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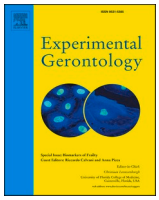

Review

Translational research into frailty from bench to bedside: Salivary biomarkers for inflammaging

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ABSTRACT

Frailty is a complex physiological syndrome associated with adverse ageing and decreased physiological reserves. Frailty leads to cognitive and physical disability and is a significant cause of morbidity, mortality and economic costs. The underlying cause of frailty is multifaceted, including immunosenescence and inflammaging, changes in microbiota and metabolic dysfunction. Currently, salivary biomarkers are used as early predictors for some clinical diseases, contributing to the effective prevention and treatment of diseases, including frailty. Sample collection for salivary analysis is non-invasive and simple, which are paramount factors for testing in the vulnerable frail population. The aim of this review is to describe the current knowledge on the association between frailty and the inflammatory process and discuss methods to identify putative biomarkers in salivary fluids to predict this syndrome. This study describes the relationship between i.-inflammatory process and frailty; ii.-infectious, chronic, skeletal, metabolic and cognitive diseases with inflammation and frailty; iii.-inflammatory biomarkers and salivary fluids. There is a limited number of previous studies focusing on the analysis of inflammatory salivary biomarkers and frailty syndrome; hence, the study of salivary fluids as a source for biomarkers is an open area of research with the potential to address the increasing demands for frailty-associated biomarkers.

1. Introduction

Advances in medical sciences, living conditions and effective public health interventions have steadily increased life expectancy over the years. The main driver for the increased average life expectancy is the decrease in child and infant mortality. However, older age mortality has also shown a decline such that life expectancy at 60 years of age has increased worldwide over the last decades (Mathers et al., 2015). In 2019, the estimated number of ageing people in the world was 1 billion and this number is expected to rise to 1.4 billion by 2030 and 2.1 billion by 2050 (WHO, 2020). Therefore, understanding ageing and ageing-related diseases has gained unprecedented importance in guiding preventive and therapeutic healthcare interventions.

Ageing is a complex phenomenon that involves metabolic and, sometimes, structural changes in each hierarchical organizational level of the body, from cells to organs, that lead to functional decline.

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Observational human studies on the older adults population suggest that physiological age does not always match chronological age and that the ageing process varies in speed and characteristics from one individual to another. Thus, it is important to be able to stratify the older adults population that has a higher risk of having an 'advanced physiological age' and ageing-related ailments as age is a risk factor for many chronic diseases and geriatric syndromes including frailty, instability, incontinence, cardiovascular disease, diabetes, and neurodegeneration (Niccoli and Partridge, 2012; Kirkland and Tchkonia, 2017).

Frailty is a condition that is associated with adverse ageing. It can be defined as an increased likelihood of unfavourable health outcomes in the face of external or internal stressors due to decreased physiological reserves (Pansarasa et al., 2019). In other words, frailty is associated with a decrease in the resiliency of the body and an increase in the likelihood of physical, cognitive disability and progressive functional loss, hospitalisation, and even death.

The higher healthcare costs associated with frailty are multifactorial, including the cost of nursing care, medicines, physiotherapy treatments, medical consultations, and emergency visits (Hajek et al., 2018). Frailty is significantly associated with other comorbidities and together they increase the use of hospital healthcare resources and related costs (García-Nogueras et al., 2017). When considering the clinical and pharmaco-economic costs of frailty, the importance of early detection, as well as risk assessment, becomes evident. That is why timely detection through the use of biomarkers is required. These biomarkers could also be used to assess the efficacy of new interventions against frailty. Such is the case of biomarkers that target clinical parameters related to the inflammatory response (Saedi et al., 2019). Currently, evidence affirms that such biomarkers in basal state measurements are associated with Fried Frailty Phenotype and Rockwood Frailty Index (Collerton et al., 2012; Mitnitski et al., 2015). Changes in the proteome of patients are thought to unify the pathophysiological process underpinning accelerated progression into frailty (Saedi et al., 2019).

Biomarkers should ideally be assessed using minimally invasive methods to decrease the risk of infection in this vulnerable population. Furthermore, the method should not be costly and labour-intensive to allow repeated testing in large numbers of people. Analyzing biomarkers in salivary fluids fits these criteria as it is a high-throughput minimally invasive process with widely used methods. The aim of this review is to briefly describe the association of frailty with inflammatory processes and diseases and to discuss potential putative biomarkers (in salivary fluids) to predict this syndrome in at-risk individuals.

2. Frailty

The ageing process is not homogenous in terms of quality of life, morbidity and survival. In this period of life, the ageing population can be affected by frailty, which is mainly defined by biomedical or psychosocial aspects. It is a physiological geriatric syndrome characterized by decreased reserve and diminished resistance to stressors as a result of cumulative decline across multiple physiological systems, causing vulnerability to adverse outcomes, including death (Walston et al., 2006). Nowadays, several potential definitions of frailty are being debated, hindering the discovery and verification of biomarkers related to the diagnosis, screening, and research of this syndrome, which includes disability, comorbidity or advanced old age.

To operationalise and standardise the definition of frailty, two complementary rather than substitutive models are commonly used. Fried et al. (2001) proposed a frailty phenotype (rule-based definition) whereby individuals are considered to be frail when they simultaneously present three or more of five listed deficits (unintentional weight loss, weakness, low levels of physical activity, slowness and poor endurance and energy). Those with no frailty-associated characteristics were considered robust, and those with one or two characteristics were considered to be in a prefrail stage (Fried et al., 2001). The frailty phenotype (Fried model) is frequently used, although it focuses more on evaluating physical function, giving little direct consideration to mental health and comorbidities (Gray et al., 2013).

Another frailty model is the one proposed by Rockwood and Mitnitski, where frailty is defined in terms of the accumulation of deficits in multiple aspects. The authors designed the Frailty Index (FI), which considers a series of health status markers (typically 30–40 items, e.g., presence or absence of a particular medical condition or poor performance on a number of functional tasks). The deficit is indicated by assigning a value 1 and the absence of by the value 0. In general, this method is considered the most reliable tool available to identify non-frailty or frailty (Rockwood et al., 1994; Rockwood and Mitnitski, 2007). Some research has found the FI reliably and accurately (O'Caoimh et al., 2019). Additionally, it has been observed that this scale is more replicable and responsive than the Frailty Phenotype (Feenstra et al., 2021). Thanks to this model, health and mortality can be predict, as well as the identification of individuals with a high risk of death and the accumulation of individuals deficits using computational modelling as observed in the investigations research by Rutenberg and colleagues. These authors generated several individual models for human health trajectories, according to age, where specific nodes of different networks appear such as frailty, physiological deficits, activities of daily living, injury and death. These nodes make new connections, creating models that facilitate the understanding of localized damage caused by frailty (Rutenberg et al., 2018; Farrell et al., 2020). This approach evaluates and identifies a large number of potential indicators (Collerton et al., 2012), combining these biomarker measures into the FI to potentially allow the detection of a systemic effect that could be masked if these variables were used in isolation (Mitnitski et al., 2015). Furthermore, this tool reflects the accumulation of organism-wide damage due to altered macroscopic (functional and cognitive) and microscopic repair processes (deficits at the levels of tissue and cells) (Howlett and Rockwood, 2013; Yin et al., 2020). When using the FI, ethnicity and societal factors need to be taken into account. For that reason, there are several frailty indexes validated around the world, to be used according to the characteristics of the population to be studied. One example of Frailty Index is Frail-VIG, which was developed to recognize the progression of frailty into four categories commonly used in clinical practice: non-frailty (Frail-VIG index score < 0.2), mild frailty (Frail-VIG index score 0.2–0.35), moderate frailty (Frail-VIG index score 0.36–0.5), and severe frailty (Frail-VIG index score > 0.5), in the older Spanish population. This index consists of 22 trigger questions that are used to assess 25 deficits from eight different dimensions, providing a final score ranging from 0 to 1 (Amblás-Novellas et al., 2017).

Some molecular changes in the frailty of older adults are reflected in biomarkers. Thus, studying these changes could be useful in frailty screening and diagnosis.

As discussed in the following sections, inflammation is one of the most relevant signs of ageing and could be a possible underlying pathogenic pathway linking frailty and ageing.

3. Older adults and the immune system

Significant changes in the immune system occur in advanced age. These alterations in immune function and effectiveness that are linked to older age are known as immunosenescence (Wilson et al., 2017). Adverse effects of age-associated immune decline are thought to manifest in an increased risk of infections and cancer, a decreased response to vaccines and an increase in autoinflammatory diseases (Wilson et al., 2017; Fulop et al., 2018). If an infection cannot be effectively controlled by the immune system, it may become chronic and eventually lead to the development of chronic inflammatory diseases and/or inflammation-related diseases (neurodegenerative diseases, cardiovascular disease, diabetes, frailty and some cancers). Hence, the declined strength of the immune response with age is considered to be one of the underlying causes of age-related morbidity and mortality.

In the age associated immune decline, marked quantitative and qualitative changes occur in the lymphocyte compartment. The number of naïve T cells is significantly reduced, probably due to the long-term effects of thymic involution and the depletion of reserves due to antigen contacts during life (Fulop et al., 2018). This makes it harder for the older adults to fight off new pathogenic challenges or mount effective immune responses to new vaccination. Conversely, memory T cells increase, especially those specific to CMV, leading to persistent infection and immune stimulation (Pawelec, 2018). Moreover, this chronic stimulation can lead to T cell exhaustion, which impairs T cell effector function (Fulop et al., 2018). Quantitative changes in the T cell compartment include an inversion of the CD4/CD8 ratio (Wikby et al., 2005). The T cell receptor repertoire is also reduced in immunosenescence (Egorov et al., 2018; Thomas et al., 2020), as shown in Fig. 1.

Another effect of thymic involution is the decrease in the efficiency of the thymic education process, so that more self-reactive T cells escape to the periphery, increasing the risk of autoimmune responses and promoting an inflammatory milieu in the body (Egorov et al., 2018; Thomas et al., 2020). In terms of B lymphocytes, the number of plasma cells in the bone marrow of older people is reduced, and in some cases, there is less class switching and somatic hypermutation. These changes can hamper vaccine response and long-term immunity due to a compromised antibody response (Ventura et al., 2017; Frasca et al., 2020).

However, as more human studies are carried out, it has become increasingly difficult to trace the health consequences of immune deterioration due to ageing (López-Otín et al., 2013; Fulop et al., 2018).

In frailty, limited evidence supports alterations in the T cell compartment of the adaptive immune system. The first set of evidence, that comes from the results of the post hoc analysis of the data nested-case control study which showed that frail older women had significantly higher counts of $CD8^+$ and $CD8^+CD28^-$ T cells, while $CD4^+$ T cell frequencies were significantly lower in the frail than in the non-frail (Semba et al., 2005).

Another piece of evidence comes from the Multi-Centre Acquired Immune Deficiency Syndrome (AIDS) Cohort Study (MACS), where the patients with (HIV) infection had an increased vulnerability to the frailty phenotype, which was determined by $CD4^+$ T cells counts, regardless of plasma HIV viral load or antiretroviral therapies (Desquilbet et al., 2007; Desquilbet et al., 2009).

Finally, evidence from a pilot study, showed that frail people had higher T-cell counts, which were expressed at the CC chemokine receptor 5 (CCR5), causing a pro-inflammatory type 1 phenotype (Loetscher et al., 1998) and, contributing significantly to several inflammatory conditions compared to non-frail people. The increased $CCR5^+$ T cell frequency in fragile older adults was not attributed to $CD8^+$ T cell-associated fragility, and a gradual increase in $CCR5^+$ T cell counts has been observed (De FU, et al., 2008).

Another phenomenon related to age-associated immune decline and with the innate arm of the immune system is inflammaging. As will be discussed in the next section, inflammaging refers to a chronic, subclinical, and sterile inflammatory response in the body that arises at old age (Pansarasa et al., 2019). This inflammatory response is correlated with many geriatric syndromes, including frailty.

4. Inflammation and frailty

Inflammation is a complex immune response to harmful stimuli, such as pathogens, irritants, or damaged cells. Once a pathogen breaks through the physiological, physical and chemical barriers of the body, the inflammatory response of the innate immune system is the first line of defence to eliminate the pathogen (Prasad et al., 2016). This response is pivotal for limiting the spread of pathogens or the accumulation of harmful cells in the body. However, the concomitant destruction of the healthy surrounding tissue resulting from the bystander effect requires strict control of this process. Therefore, anti-inflammatory mechanisms such as IL-10 secretion is required to limit the damage caused by the inflammatory response. The immune-privileged status of the delicate tissues of the body, such as the eye and the brain, further underscores the destructive potential of the inflammatory response (Lopez Angel et al., 2021). The harmful effects of unchecked or misguided inflammatory responses also manifest themselves in autoimmune diseases. In older people, inflammation is associated with morbidity, a decline in functional autonomy and frailty (Pansarasa et al., 2019). The relationship of frailty with some lipoproteins could also be indirectly explained by inflammation: a decrease in high-density lipoprotein (HDL) is related to increased inflammatory status (Abbatecola et al., 2004). Hence, an increase in HDL- cholesterol is also related to disability, mortality and decreased longevity and, as such, is a determinant of frailty syndrome (Fulop et al., 2018).

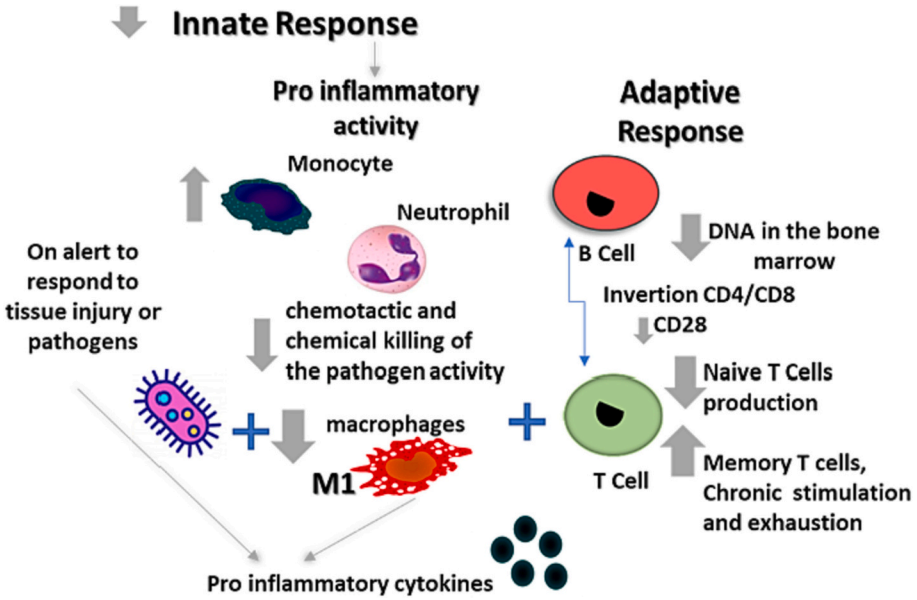

Fig. 1. The immunosenescence in older people interferes in a number of innate and adaptive immune cells aspects like thymic involution, altered T and B cell responses, altered naïve/memory ratio, increased serum levels of IgG and IgA, a chronic low grade inflammation.

The delicate balance between pro-inflammatory and anti-inflammatory mechanisms tips towards the pro-inflammatory side with ageing. This phenomenon is called inflammaging and is triggered by non-specific innate immunity. Inflammaging is characterized by a chronic, systemic, and low-grade, inflammatory status in the absence of infections, which can mediate tissue damage such as endothelial damage (Franceschi et al., 1995). It manifests through elevated serum levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α), C-reactive protein (CRP) and decreased anti-inflammatory cytokines such as IL-10 (Baylis et al., 2013; Juárez-Cedillo et al., 2019).

The underlying causes of inflammaging have not been clearly elucidated, however it is deemed to be multifactorial. It is related to infectious agents encountered throughout life, including latent or persistent viral infections (Haq and McElhaney, 2014), changes in microbiota and metabolic and cellular dysfunction (absorption of bacterial toxin such as LPS) (Li et al., 2011; Lee et al., 2016; Cardoso et al., 2018). More specifically, inflammaging is thought to be the result of the cumulative effects of stimulation and assault from defective autophagy, loss of protein homeostasis and increased endoplasmic stress that increase cellular 'garbage' such as misfolded proteins, mitochondrial dysfunction and oxidative stress, senescent T cells, secretory profile of senescent cells (senescence-associated secretory phenotype, SASP), gut microbiota dysbiosis and 'leaky gut' (Fulop et al., 2018; Fülöp et al., 2019). These events culminate in the assembly of the NLRP3 inflammasome and associated secretion of pro-inflammatory cytokines (López-Otín et al., 2013).

The main immune players in inflammaging are monocytes and macrophages, especially M1 subtypes that produce pro-inflammatory cytokines and chemokines (Li et al., 2011; Munawara et al., 2021). Immune cells also change their surface marker expression and are less efficient in the production of reactive oxygen species. These cells are in a state of immune paralysis and favour the production of pro-inflammatory over anti-inflammatory cytokines. The immune paralysis could be due to increased cortisol levels. This cortisol increase is a compensatory mechanism for the decreased dehydroepiandrosterone (DHEA) levels that comes with physiological ageing. After a stress response in an ageing context, there is not a complete recovery of all innate cell functions (Namioka et al., 2017) (Fig. 2).

SASP is one of the links that tie inflammation, adverse ageing, and frailty together. Cellular senescence is associated with cell cycle arrest, resistance to apoptosis and shortened telomeres (Thomas et al., 2020). Senescent cells accumulate in multiple tissues during ageing due to compromised clearance by leukocytes. Senescent cells have a unique SASP that includes many proinflammatory cytokines, interleukins, interstitial collagenase (MMP1) or collagenase 3 (MMP13) (Coppé et al., 2008) that play an important role in many diseases, such as atherosclerotic complications (Namioka et al., 2017).

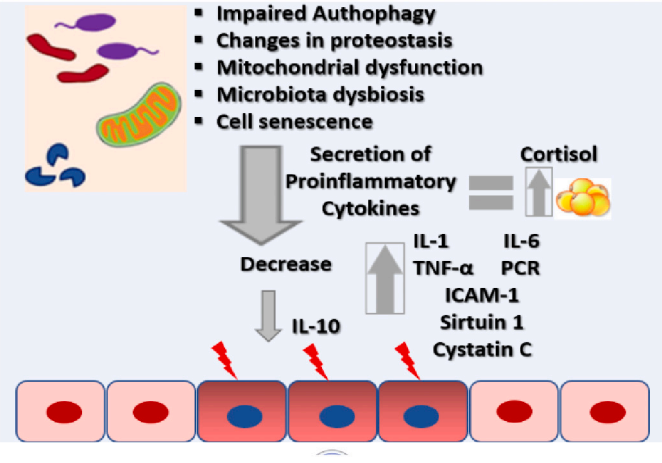

Fig. 2. In Inflammaging several factors can dysregulate intracellular homeostasis during ageing, intensifying the secretion of inflammatory cytokines and chemokines.

Inflammaging is one of the contributing factors to age- and frailty-related ailments including neurological and cardiovascular diseases (Thomas et al., 2020).

5. Guidelines for selecting frailty potential inflammatory biomarker

Considering the aim of this review, which is to decipher putative biomarkers for predicting frailty, guidelines are required for the proper selection of potential biomarkers whose presence or alteration could be related to frailty. However, this is not an easy task, as the molecular changes associated with ageing are still not well understood or systematically characterized as it would be required to define a specific panel of biomarkers useful for diagnosis and/or prognosis.

Recently, clinical endpoint-focused studies have been set up, examining different biomarkers candidates, to identify known and new markers associated with ageing and disease and their relation to ageing process. Among these studies, two will be briefly described as they have served as examples in the design and implementation of research. The first one, Targeting Ageing with Metformin "TAME" (Justice et al., 2018), is based on a geroscience-guided intervention. This model was proposed by NIH Biomarker Definitions Working Groups and FDA guidance markers. This research involved a double-blind randomized clinical trial (6-year duration) using metformin to target the molecular ageing pathways. The aim was to extend human life by preventing and reducing the incidence of multimorbidity and functional impairment caused by the biomarkers present in this population. In the absence of a set of specific ageing biomarkers for such studies, a panel of experts was convened to select the biomarkers according to the following criteria: (a) reliability and feasibility of biomarker measurement in a clinical setting; (b) relevance of the biomarker in ageing, (to ensure that changes were constant); (c) consistent data from the biomarker (to predict all-cause mortality, clinical and functional outcomes); and (d) responsiveness to intervention in a short period of time. Biochemical markers, clinical and physiological measures (handgrip speed, grip strength, cognitive assessment, spirometry, and blood pressure, among others) were included in this trial and had to be appropriate for the population and the size, duration, cost, and logistics on the trail. The sample consisted of a non-diabetic male and female population aged 65 to 80 years. Initially, 258 biomarkers related to ageing or age-related diseases were selected:229 out of 258 were based on a comprehensive literature review, while 29 were clinically selected (cancer, cognitive, cardiovascular, etc.) during the project. Following the biomarker selection criteria, 77 biomarkers were omitted as they were not blood-based. Another, 39 were excluded due to low reliability and viability in large trials or because required an immunoplex assay, which can be reliable, but not on a large scale, as well as showing inconsistency in the detectability of several cytokines that were part of these biomarkers. As a result, the use of 86 biomarkers was evaluated, based on: 1) frequency of use; 2) usefulness for diagnosis or follow-up of clinical disease; and 3) expert opinion (relevance and impact on the literature via PubMed and data published in Diabetes Prevention), on at the magnitude the effect of the biomarker across publications, face validity of the biomarker and sensitivity to change within a minimum time frame of 6 years. It should be noted that each biomarker was separately screened for each clinical disease and functionality, excluding acute or severe diseases due to the difference in the results obtained. Furthermore, the estimated effect size was calculated with every cause of death and a screening of the interventions and percentage changes was performed. If these were available, the biomarker was considered suitable to be evaluated using the metformin treatment and comparing it with the reference or control group. Based on this systematic process, only 8 biomarkers were suitable for study in this trial at the following levels: Inflammatory (IL-6,

TFNRIII, CRP), Stress Response and Mitochondria (GDF15), Nutrient Signalling (IGF-1), Renal Ageing (cystatin C), Cardiovascular (NT-proBNP) and Metabolic Ageing (haemoglobin A1c). The combination of biomarkers is able to predict morbidity, disability, and mortality more accurately. The epigenetic clock of an ageing biomarker is strongly associated with chronological age and predicts mortality risk and clinical outcomes, although it has not yet been epidemiologically tested.

The second study (Ahadi et al., 2020) was a cohort of 106 healthy individuals from 29 to 75 years of age that were examined by different types of omic measurements (transcripts, proteins, metabolites on blood mononuclear cells and cytokines assays using serum, clinical assays). Nasal and gut microbiomes were analysed using 16S rRNA sequencing in a quarterly visit for up to 4 years with additional samples acquired during a period of physiological stress. A new age-related marker was identified by comparing insulin-resistant (IR) to insulin-sensitive (IS) individuals. Peripheral blood mononuclear cells were analysed and 51 laboratory tests were obtained from each participant per visit, generating 18 million data points. Ageing was identified through changes in marker levels over a short period of time (2–3 years) and defined types of molecular pathways that also changed over time in specific individuals, known as ageotypes, which provide a molecular assessment of personal ageing as reflected in each subject's lifestyle, which was useful in this study for monitoring and intervening in the ageing process. To understand patterns within and across individuals and to perform omics analyses, three types of identification analyses were performed 1) identification of markers and pathways that were associated with the age of the individuals. 2) identification of molecular ageing, differentiating between participants who were insulin resistant and those who were insulin sensitive and 3) identification of personal markers and pathways that differed between individuals as they age.

A systematic analysis was carried out to find markers (median gene expression, plasma proteins, metabolites, cytokines, microbes and clinical markers) that showed a strong correlation (using Spearman's test) with the median age of each study participant. After analysis of body mass index (BMI) and sex, the results identified 184 molecules with different tendencies and levels of positive and negative association.

Concerning age-related biological pathways, there was a significant elevation in the acute phase movement response, Toll-like receptor, inflammatory and coagulation pathways of the different types of molecules. Ten of the 184 molecules found in this research were identified in the insulin-resistant group, while none were found in the insulin-sensitive group.

This study has shown how over time individuals are capable of change. Analysis of analytes collected from 43 subjects at 5 medical visits over 700 days showed significant changes. No significant correlation between dietary intake and time was observed in 28 subjects with dietary habits (assessed by standardised feeding tests). It was noted that the dietary intakes were generally stable and dietary alteration was not responsible for longitudinal changes in clinical markers.

In addition, physical activity, medication and body mass index were assessed at each visit. Using the phenotypic age formula, which reports metric changes over time at an individual level, results showed that people age at different rates in different biological mechanisms known as ageotypes, which were present in four major mechanisms: immunological (where an age-related increase in molecules was observed), metabolic, hepatic, and renal regulation and dysregulation. Moreover, increased inflammation was observed earlier in insulin-sensitive (IS) subjects than in insulin-resistant (IR) subjects.

Pathway analyses of personal ageing molecules in different individuals revealed distinct ageing pathways for each subject. In addition, different subjects experienced different gut microbial changes over time. There are two parameters in this study on an individual and a population level. Thus, molecules can show a significant trend among a community on a personal level, but not on a population level.

The availability of time-dependent markers of personal ageing potentially allows action on ageing at the individual level.

The aim of identifying, selecting and studying age-related biomarkers in both investigations was met, but the systematisation to achieve it is rather different and can sometimes become complex. The observational and directed strategies of Justice et al. go from the general to the specific, selecting large numbers of biomarkers for study and gradually discarding them down to only eight biomarkers that met the previously established criteria (which were more focused on the presence of the biomarker in the literature and the opinion of experts). The biomarkers studied were plasmatic, discarding other types of fluids, focusing on the clinical level (diseases as well as functionality) and those changes observed over time. Meanwhile, the study by Ahadi et al. uses omics analysis, which at present is still a largely unexplored area in the study of biomarkers of any fluid (proximal and peripheral fluids). This is particularly true in an ageing population, where the strategy of biomarker analysis can go from the specific to the general since this study focuses on the individual participants. Among the 106 subjects of the study, molecular patterns of Insulin Resistance or Insulin Sensitivity were identified in each participant, studying the relationships of these molecules with nutrition, physical activity and medication. Both studies highlight the importance that the immune system has in ageing due to the changes observed over time.

However, for the purposes of this review we opted for the study of Ahadi et al. as it covers the study of salivary biomarkers related to physical and functional factors of frailty. These will be as follows:

There are twenty neuroinflammatory markers associated with cognitive impairment and physical frailty. These include elevated levels of IL6 (Wikby et al., 2005), C-reactive protein (CRP), tumour necrosis factor (TNF-alpha and beta), uric acid, IL1-beta, IL1-alpha, erythrocyte sedimentation rate (ESR), cortisol/dehydroepiandrosterone ratio, IL1RA, IL-13, CD4, CD8, IL6R, IL-18, TNF-a receptor I (TNFR1), TGFbeta, IFNalpha, IFNbeta, cortisol, homocysteine, fibrinogen, Intercellular Adhesive Molecule-1 (ICAM-1), circulating osteogenic progenitor (COP) cells, and beta 2-microglobulin (B2M) (O'Bryant et al., 2010; Lee et al., 2016; Ferrucci and Fabbri, 2018). It is important to mention that some studies have linked elevation of these pro-inflammatory cytokines in cognitive fragility to hippocampal and thalamic atrophy, which is associated with low levels of anti-inflammatory cytokines, causing cognitive impairment and a reduction in neuronal plasticity, which will be reflected in executive functions, processing speed and selective care (Mooijaart et al., 2013; Namioka et al., 2017).

White blood cells (WBC) in peripheral blood (PB) are circulating immune cells. White blood cell counts, including neutrophil counts, are increased in frail individuals. High WBC counts were predictive of future frailty in healthy people aged 60 years (Wilson et al., 2017). It is also associated with cardiovascular or cerebrovascular events and cancer mortality (De Martinis et al., 2006; Li et al., 2011). Neutrophils are of prime importance for the inflammatory response since they are the first leukocytes to arrive at the site of inflammation and initiate the cascade of events leading to inflammation. Higher neutrophil counts are associated with low physical activity and frailty (Wilson et al., 2017). In ageing, neutrophils lose some of their chemotactic and chemical activity to kill pathogens and hence can contribute to systemic inflammation (Dewan et al., 2012). In the lymphocyte compartment, there are fewer CD28 expressing T cells and CD4/CD8 counts are reversed in frail individuals according to the IRP (Ventura et al., 2017).

Interleukin 6 (IL-6) is a pleiotropic proinflammatory cytokine involved in the regulation of immune responses. It is the most potent and broadly effective stimulant for acute-phase protein production by human hepatocytes (Tilg et al., 1994). IL-6 also promotes the expansion and activation of T cells and the differentiation of B cells. IL-6 levels have been found to be important markers for many diseases. High serum levels (for example 2.5 pg/ml) predict incident disability and frailty, in particular, slower walking speed (Ferrucci et al., 1999; Ferrucci et al., 2005). Also, higher levels of IL-6 are correlated with a higher number of chronic diseases (Fabbri et al., 2015) including cardiovascular, neurological (dementia, Parkinson's disease), musculoskeletal, cancer,

chronic fatigue syndrome and clinical depression (Hiles et al., 2012).

In the frailty context, increased IL-6 is associated with cognitive disability, sarcopenia and mortality (Wilson et al., 2017). Conversely, single nucleotide polymorphism in IL-6 gene that is associated with higher plasma levels of this cytokine is less frequent in centenarians, underscoring the potential association of IL-6 with mortality and unhealthy ageing (Wilson et al., 2017).

CRP, IL-6, TFN-α and neutrophil count showed positive associations with Fried model, Rockwood model and inverse associations with albumin (Aziz et al., 2015). Increased levels of CRP, IL-6 and TFN-α are associated with insulin resistance in older adults nondiabetic subjects (Abbatecola et al., 2004), coronary heart disease and heart failure events (Fulop et al., 2006).

Increased IL-6 levels are also associated with a limitation in the instrumental activities of daily living (Li et al., 2011).

C Reactive Protein (CRP) is a protein secreted by the liver in response to IL-6 and is a prominent member of the acute-phase inflammatory proteins. It promotes the production of pro-inflammatory cytokines while circulating in blood plasma as a stable homopentamer. CRP binds to phosphocholine, a common constituent of polysaccharide coatings of bacterial pathogens and mammalian cell membranes. It then acts as an opsonin, facilitating the phagocytosis of pathogens and dead or dying cells (Mortensen and Zhong, 2000; Volanakis, 2001). CRP is considered a broad biomarker of systemic inflammation of clinical significance (Stoner et al., 2013). Elevated levels in plasma are associated with progressive vascular disease (Wang et al., 2011), age, depression (Duggal et al., 2014), sarcopenia, metabolic syndrome (Fulop et al., 2012), disability and cognitive decline in individuals over 65 years of age, but it is not always a significant mortality predictor. There is a tight correlation between serum levels of CRP and IL-6. Significant correlations were found between CRP levels and handgrip. Interestingly, CRP has emerged as a sex-specific frailty marker where an association between frailty and women has only been made for women aged between 60 and 90 (Gale et al., 2013).

Salivary CRP levels and blood CRP are particularly likely to be correlated because CRP is synthesized primarily in the liver. There is no local production of CRP in the mouth and its most likely to reach saliva via the blood, which is why there is a higher dilution in salivary CRP levels (Ouellet-Morin et al., 2011). Out et al. (2012) suggests that salivary CRP at low levels is a better indicator of systemic CRP, whereas, at higher levels, it is less likely to correlate with blood levels (Out et al., 2012), as it can indicate an abnormally high infiltration from blood due to local inflammation, tissue damage or poor oral health (Engeland et al., 2019).

Tumour Necrosis Factor-alpha (TNF-α) is a pro-inflammatory cytokine and endogenous pyrogen produced by natural killer cells (NK), macrophages, microglia and other cell types (Slavish et al., 2015). It also plays a role in the stimulation of acute-phase response, and its increased levels are associated with leukocyte telomere length (Pavanello et al., 2017). TNF-α activates prostaglandins and other substances related to pain perception, and numerous other processes throughout the body, including remodelling of tissues and apoptosis of tumour (Fülöp et al., 2019) cells and dysregulation (Fülöp et al., 2019; Goodsell, 2005). TNF-α is a potential biomarker for frailty (Hubbard et al., 2009). In older adults people, TNF-α is associated with cardiovascular disease (Stoner et al., 2013), osteoporosis, Alzheimer's disease (AD) (Franceschi et al., 2001), Diabetes Type II (Recasens et al., 2005) and mortality (Giacomini et al., 2018).

Pentraxin 3 (PTX3) is secreted in response to TNF-α by macrophages and blood vessel components. It has been associated with weight loss or leanness in the older adults hypertensive population and its high levels are associated with cognitive impairment and potentially with frailty in this population (Yano et al., 2010).

Interleukin 1 (IL-1β) is the proinflammatory cytokine that mediates the host response to microbial invasion, inflammation, tissue injury and immunologic reactions (Garlanda et al., 2013). IL-1 β is synonymous with leukocytic endogenous mediator, lymphocyte activating factor, as well as mononuclear cell factor, catabolin, osteoclast activating factor and hemopintin-1. IL-1β is produced as a result of the proteolytic cleavage by the inflammasome, mainly by macrophages and epithelial cells. In addition to attracting leukocytes into inflamed tissues, IL-1 β also causes degranulation of basophils and eosinophils, stimulates thromboxane synthesis in macrophages and neutrophils and potentiates the activation of neutrophils by chemoattractant peptides. IL-1 β is mitogenic for mesangial cells in the kidney, glial cells in the brain and keratinocytes (Ganter et al., 1989).

Together with the other proinflammatory cytokines TFN- α and IL-6, IL-1β, plays an important role in pain sensitivity (Mika et al., 2013) and fatigue that can restrict physical activity and contribute to frailty (Thoma et al., 2011). An analysis performed on geriatric patients admitted to the trauma centre of a hospital showed that frail individuals had higher IL-1β serum levels as compared to non-frail individuals (Palmer et al., 2019).

Interleukin 4 (IL-4) is a Th2 interleukin that is produced by T lymphocytes, granulocytes and mast cells (Landau et al., 2019). It has an anti-inflammatory effect and has been defined as a "prototypic immunoregulatory cytokine" (Mika et al., 2013). Given the association of frailty with cardiovascular diseases, this cytokine can be an indirect biomarker for frailty.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that inhibits the synthesis of numerous cytokines (TFN- α, IFN-γ, IL-2 and TFN-β) and suppresses Th1 proinflammatory responses. IL-10 secretion is low in nonstimulated tissues and it regulates its own expression in a negative feedback loop. IL-10 also enhances B lymphocyte survival and proliferation (Fulop et al., 2006) as well as the production of IgA, which is the main immunoglobulin in salivary immunity (Stoner et al., 2013). IL-10 is a myokine, as exercise fosters an environment of anti-inflammatory cytokines. The frequency of *IL-10* single nucleotide polymorphism, which increases serum levels of this cytokine, is increased in centenarians and is speculated to be preventive against sarcopenia (Wilson et al., 2017). In frailty syndrome, the direct role of IL-10 is not clear yet.

Intercellular Adhesive Molecule-1 (ICAM-1) had higher levels for both cognitive impairment and physical frailty (Lee et al., 2016). This biomarker is associated with increased production of IL-6, and TNF-α, suggesting that it is related to the pro-inflammatory cascade (McCabe et al., 1993).

Albumin is an abundant protein in body fluids, such as plasma, synovial fluid and cerebrospinal fluid that functions as a carrier protein. It is involved in the body's inflammatory response to acute or chronic infections or conditions that are associated with a persistent low-grade inflammatory state, such as obesity. Low serum albumin levels are prospectively associated prospectively with an increased total mortality (Phillips et al., 1989). Low plasma albumin levels in the older adults with type II diabetes is associated with frailty (Yanagita et al., 2020). This hypoalbuminemia has been proposed to be an indicator of malnutrition.

Therefore, albium could have a potential frailty biomarker status as a result of the complex interaction of the nervous system, endocrine system, immune system and diet, all of which are major contributing factors to frailty.

6. Infectious diseases associated with frailty

Altered immune responses related to ageing make the older adults more susceptible to new infections, such as influenza. The frail population, in particular, is more susceptible to infectious diseases than younger persons (Matheï et al., 2011).

In older adults the respiratory infections may also negatively affect frailty status during and after recovery from infection. The information currently available on the association of frailty with the severity of infectious diseases is limited, however, it is known that frailty has been associated with a lower probability of recovery in older people

hospitalised for influenza and acute respiratory illnesses (Lees et al., 2020). Hughes and cols. found that more severe respiratory episodes may have had a detrimental impact on frailty compared to less severe episodes. Sometimes chronic respiratory illnesses are exacerbations of underlying diseases (Hughes et al., 2019).

Regarding influenza, if this infection leaves older adults with a lasting increase in frailty, the true burden of influenza could last beyond the period of illness and lead to long-lasting impacts on a person's health. Investigations reported that the frailty of hospitalised older adults with influenza and acute respiratory illness was associated with lower odds over recovery (Lees et al., 2020).

Recent research in Asia has observed that the prefrailty is only associated with the occurrence of pneumonia, deterioration in physical strength, nutritional status deficit, impaired oral function, depression, and home boundness. It was also noted that frailty in older adults was significant in association with hospitalisation due to pneumonia compared to non-frailty (Iwai-Saito et al., 2021). The other study performed in China has shown that frailty increases the 1-year mortality associated with community-acquired pneumonia and, thus, it is a risk factor for severe pneumonia (Luo et al., 2020).

Another respiratory viral infection is coronavirus disease (COVID-19) caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (Goyal and Goyal, 2020). The development of COVID-19 in people with frailty is associated with poor prognosis and high fatalities. One study has shown a high disease burden increased the likelihood of hospitalisation, with men being more likely to be admitted than women. In the same study in women, age was a protective factor for COVID, as it was shown that young women had a higher incidence of infection, while men did not, but were at risk. The crude incidence of this pathology increases with age, as well as certain pathologies caused by immunosenescence (Feijóo et al., 2022).

7. Neurodegeneration and frailty

There is evidence for the association between frailty and cognitive impairment based on the shared pathophysiological mechanisms such as vascular diseases and inflammation (Panza et al., 2017). Evidence suggests that mental health and cognition may be affected even from the early stages of frailty (Han et al., 2014). This combination leads to increased levels of disability and a lower quality of life compared to frailty or cognitive impairment alone (Feng et al., 2017).

In 2013, the International Consensus Group organized by the International Academy on Nutrition and Aging (I.A.N.A) and the International Association of Gerontology and Geriatrics (I.A.G.G) defined the characteristics of cognitive frailty: 1) existence of physical frailty and cognitive impairment and 2) exclusion of a clinical diagnosis of Alzheimer's Disease or any other form of dementia (Kelaiditi et al., 2013). Several pathological changes associated with cognitive decline and the pathological processes of dementia, such as insulin resistance, chronic inflammation, and mitochondrial dysfunction, are associated with obesity (De Felice and Ferreira, 2014; Seo et al., 2021). In addition, cognitive impairment is related to frailty, sharing pathophysiological bases and some outcomes such as disability, gait disturbances, falls, fractures, hospitalisation and even mortality (Robertson et al., 2014; Casas-Herrero et al., 2019). Cognitive frailty can be considered as the initial step of neurodegeneration, which has brought together physical and cognitive frailty.

Two markers measured in serum/plasma were associated with cognitive impairment and frailty: Sirtuin 1 and cystatin C. Sirtuin 1 has been reported to be involved in the pathway that controls the expression of amyloid-beta peptides, which form the plaques associated with Alzheimer's disease (AD), through ADAM10 (Donmez et al., 2014). Levels of Sirtuin 1 decrease with age but this decline is more pronounced in individuals with cognitive impairment and frailty compared to age-matched healthy individuals (Kumar et al., 2014). On the other hand, decreased serum cystatin C is associated with cortical and subcortical atrophy, contributing to a higher risk of cognitive impairment (Liu et al., 2014), and, consequently, to falls, unstable and slow gait speed, poor balance and slow reaction times (Casas-Herrero et al., 2019; Robertson et al., 2014). These are the main motor manifestations of cognitive frailty (Montero-Odasso et al., 2016). Therefore, people who suffer cognitive frailty with an inflammatory process will be more susceptible to neurodegenerative diseases, such as AD, as pro-inflammatory cytokines might be responsible for the inflammatory state in the brain (Petrus and Lee, 2014). Infections can induce amyloid-β peptides, which have true antimicrobial activity while contributing to the maintenance of inflammatory state via the innate immune system (Fulop et al., 2016). Hence, bacterial amyloid proteins could influence β-amyloid deposition in the brain via two different pathways: induction of neuroinflammation or molecular mimicry, i.e., misfolding of neuronal proteins through cross-seeding (Friedland, 2015). As some authors hypothesized, gut microbiota might play a role in regulating amyloid deposition in the brain through the modulation of inflammation (Cattaneo et al., 2016). Altered nutrition, immune system and the structural changes associated with ageing can promote proinflammatory gut dysbiosis. Many microbial taxa are underrepresented in frail older people, including those that produce metabolic mediators such as short-chain fatty acids (SCFAs) that modulate inflammation and improve insulin sensitivity and anabolic responses (Di Sabatino et al., 2018). Moreover, facultative anaerobes, which are associated with increased inflammatory cytokines, become dominant in the intestines of older people (Biagi et al., 2010). Several authors underscored the fact that many gut bacterial taxonomies can produce amyloid proteins. These proteins could be phagocytized by the enteric immune system cells and then delivered to the central nervous system or directly enter the systemic circulation as a consequence of a "leaky gut", that is, altered mucosal permeability induced by microbiota dysbiosis (Friedland, 2015). This has been corroborated by a study that detected Gram-negative bacteria-derived lipopolysaccharide (LPS) in the hippocampus and temporal cortex of AD patients in a post-mortem analysis (Zhao et al., 2017). The importance of the microbiota in modulating systemic inflammation and neuroinflammation has also been underlined by the association between oral microbiota overgrowth in periodontitis and AD (Harding et al., 2017; Shoemark et al., 2015). Other authors have also emphasized the potential role of the vagal nerve in transducing gut microbiota signals to the brain (Leung and Thuret, 2015). Overall, the increased local and systemic inflammation that occurs during inflammaging can contribute to neurodegenerative processes, as exemplified by AD.

8. Chronic diseases and frailty

Ageing is the greatest risk factor for a majority of chronic diseases driving both morbidity and mortality. New findings suggest this might be reciprocally true: diseases and/or their treatments may accelerate ageing pathologies (Kennedy et al., 2014). The link between ageing and chronic diseases is multifactorial, including changes in the gut microbiome, metabolic dysregulation and the immune system.

In terms of metabolism, Type II diabetes, like other chronic diseases or comorbidities, can exacerbate the metabolic dysregulation associated with ageing. One of the most important questions to be addressed is how overnutrition and obesity can affect the metabolome of ageing. These issues are relevant to inflammation as well, as adipose tissue is a major source of inflammatory cytokines (Kennedy et al., 2014). An increased inflammatory state can lead to frailty and dysregulations in the body systems. As mentioned earlier, inflammation can be a common underlying cause of adverse ageing and chronic disease (Salvioli et al., 2013).

Chronic diseases confer a high healthcare cost, and some diseases are associated with greater hospitalisation or overall healthcare costs than others. The association between frailty and chronic diseases is, thus, one of the factors contributing to frailty-associated healthcare costs.

8.1. Cardiovascular diseases

Hypertension, diabetes, obesity (especially abdominal obesity), and sedentary lifestyle appear to be shared factors of frailty and cardiovascular disease (CVD). Low-grade inflammation is an important factor in the development of CVD. Veronese et al. (2017a, 2017b) showed that pre-frailty was associated with a 23 % greater risk of CVD, whereas frailty was associated with a 70 % greater risk of CVD (Veronese et al., 2017a, 2017b). Frailty can be associated with an increased risk of CVD due to inflammaging as well as compromised muscle structure and function.

8.1.1. Atherosclerosis is one of the inflammatory diseases that can lead to coronary artery disease, peripheral arterial disease or cerebrovascular disease (Fulop et al., 2018). Frailty has been shown to increase the risk of atherosclerosis (Korada et al., 2017). In this disease, endothelial cells are damaged and foam cells accumulate. Foam cells increase as a result of the deposition of cholesterol-containing low-density lipoprotein (LDL) particles on the endothelium. This process induces an inflammatory reaction, including interaction among innate cells, adaptive immune cells and endothelial cells (Libby et al., 2011). Foam cells are formed when macrophages engulf lipid-containing particles. In atherosclerosis, there is the hardening of the arterial wall as well as a narrowing of the lumen that can hinder the perfusion of the organs. Senescent T cells contribute to atherosclerosis by secreting macrophage stimulating IFN-ɣ (interferon-gamma) (Thomas et al., 2020). Studies show that high CRP and IL6 levels are associated with an increased risk of cardiovascular diseases in older people (Ferrucci and Fabbri, 2018).

8.1.2. Hypertension is one of the most prevalent diseases of the cardiovascular system (Liu et al., 2020). Systolic hypertension is the most common form of primary hypertension in the ageing population and is partly associated with higher pulse pressure and pulse wave velocity (Staessen et al., 2000). Hypertension is more common among frail individuals as compared to pre-frail and non-frail individuals (Liu et al., 2014). Hypertension and frailty can share a common pathophysiology involving arteriosclerosis, inflammation or sustained oxidative stress (Liu et al., 2020).

8.2. Sarcopenia

Sarcopenia is the muscle atrophy associated with ageing. Sarcopenia is associated with hypoplasia (the decline of fibre number) as well as skeletal muscle atrophy (the reduction of fibre size) (Narici et al., 2003). The concomitant decrease in muscle strength is exacerbated by the decrease in type II muscle fibres and mitochondria, and the increase in fat deposition in the parenchyma (Wilson et al., 2017). Sarcopenia can lead to changes in gait and physical performance in older adults. Hence, it is associated with loss of mobility, reduced independence, and increased mortality.

Sarcopenia can be the result of hormonal changes, such as increased cortisol/(DHEA), malnutrition, reduced physical activity, and immunosenescence and inflammaging (Wilson et al., 2017). A significant amount of metabolic energy is spent on cytokine production in inflammaging, thereby reducing the amount of energy available for other essential functions such as the maintenance of muscle mass (Fulop et al., 2016). Neuroinflammatory markers IL-6, TNF-alpha, and CRP were found to be elevated in older adults with sarcopenia (O'Bryant et al., 2010). In terms of energy, skeletal muscle mitochondrial dysfunction is also prevalent in advanced age, decreasing in volume and number, as well as reduced biogenesis (Petersen et al., 2012).

There is a "gut–muscle axis" involving microbial mediators that influence skeletal muscle anabolism and physical exercise, favouring gut microbiota biodiversity (Grosicki et al., 2018). High-fat diets may promote gut microbiota dysbiosis, and thus support systemic inflammation. Fabbri et al. (2017) showed that a higher total fat mass is accompanied by a simultaneous loss in lean body mass, predicting a faster rate of decline in muscle quality in the older adults (Fabbri et al., 2017). Recent studies have also shown that gut microbiota is involved in the physiopathology of frailty by promoting chronic inflammation and anabolic resistance. Therefore, gut microbiota could be a mediator between frailty and sarcopenia.

One of the biggest challenges nowadays is to determine which comes first, sarcopenia and then frailty or frailty, then sarcopenia. However, there are limitations to the information available. It should be considered that these pathologies represent a permanent interruption to the homoeostasis of organisms with a limited probability of change (Cesari et al., 2014). Sarcopenia and frailty are distinct but related conditions. Sarcopenia is not a biomarker of frailty; however, the absence of sarcopenia may be important to exclude a diagnosis of frailty. It will also have to be considered that the prevalence of sarcopenia or frailty also varies depending on the techniques used to evaluate the different parameters of these diseases (Davies et al., 2018).

A Japanese cross-sectional study has shown that participants with sarcopenia and frailty (measured by the frailty phenotype criteria) had poor quality of life, high incidence of falls, and low levels of vitamin D, protein, and energy. The study also shows that frail people havea higher percentage of lower limb, muscle strength weakness and require more long-term care, due to the intensity of falls (Mori and Tokuda, 2019).

8.3. Skeletal pathologies

Physical frailty is related to skeletal pathologies and cognitive impairment that share common pathophysiological bases and outcomes, such as gait disturbances, falls, fractures, disability, and even mortality (Robertson et al., 2014; Casas-Herrero et al., 2019). Bone fractures are related to significant complications, such as increased fear of falling, loss of autonomy, risk of disability, decreased quality of life, and anticipated mortality in old people (Bailly et al., 2014; Bonafede et al., 2016; Amouzougan et al., 2017). Age-related low bone mass weakens the skeleton and increases the likelihood of bone fractures (Amouzougan et al., 2017; Bonafede et al., 2016). The incidence of hip fracture (HF) is an increasing global medical problem that mainly affects aged populations (Dhanwal et al., 2011), as well as vertebral fractures, albeit to a lesser degree (Walters et al., 2017).

HF has severe clinical consequences because of the associated morbidity, patient's susceptibility to severe depression and loss of autonomy. These factors contribute to reduced health, poor or partial recovery and frailty (Duggal et al., 2014). The incidence of bacterial infections in HF patients before surgery is 43–48 %, while 20–30 % of patients die due to HF (Edwards et al., 2008). HF triggers a state of acute stress that shares similarities with severe burns, sepsis, and stroke, resulting in progressive immunosuppression (Xiu et al., 2013) and an increase in infection risk.

8.4. Metabolic pathologies

8.4.1. Diabetes mellitus (DM)

Recent studies have shown that DM, insulin resistance and lipotoxicity increase neutrophil infiltration, macrophage proliferation and the secretion of inflammatory mediators, contributing to a pro-inflammatory state in the body (Ferrucci and Fabbri, 2018). DM is also related to frailty and pre frailty, as it is characterized by decreased reserves in multiple physiologic systems. In 2019, the European Society of Cardiology (ESC) identified diabetes as one of the main cause of CVD (Jiménez Navarro et al., 2020). Also, DM plays an important role in the development of atherosclerosis due to the excessive production of angiotensin II that can cause mitochondrial damage by increasing chronic inflammation (Eguchi et al., 2018). The prevalence of this pathology varies around the world (Soysal et al., 2016), as it can occur as a part of the metabolic syndrome or as a single disease. There is a possibility that DM-associated episodes of hypoglycaemia play a role in the increased risk of frailty (Niccoli and Partridge, 2012).

Metabolic Syndrome refers to a collection of disorders linked to

abdominal obesity, dyslipidaemia, systemic hypertension, and insulin resistance (Andersen et al., 2016)Metabolic Syndrome is characterized by higher levels of leptin that are associated with the prevalence of frailty (Hubbard et al., 2010). In cognitive functions, this Metabolic Syndrome is related to a deterioration in executive functions (Lin et al., 2015). As the name suggests, it is associated with a systemic metabolic disruption, involving the endocrine and immune system. The immune system plays a role in its pathophysiology and, at the same time, is affected by this pathology. Thus, a proinflammatory state and chronic low-grade inflammation are observed in this syndrome.

8.4.2. Obesity

Obesity is indicated by a higher than normal body mass index (BMI) and changes in body fat/muscle composition. It is associated with many conditions, including insulin resistance. Insulin resistance increases inflammation via central obesity, adipocytes release C-C chemokine 2 (CCL2) and tissue accumulation of M1 macrophages (Ferrucci and Fabbri, 2018), monocytes and T lymphocytes (Vandanmagsar et al., 2011). In turn, the accumulation of B lymphocytes and macrophages causes an increase in body mass (Weisberg et al., 2003). Many studies have indicated that an increase in BMI, waist circumference, intramuscular and pericardial adipose tissue is associated with higher frailty scores and probably with sarcopenia (Hubbard et al., 2010; Crow et al., 2019; Karczewski and Snyder, 2018).

8.4.3. Dyslipidemia

Numerous studies have examined this pathology; however, the results are inconsistent. One set of results showed that HDL concentration was higher in people in a state of non-frailty (61.7 m g/dl) vs. pre-frailty (58.1 mg/dl $p = 0.04$). However, another study showed that pre-frailty and frailty are associated with a higher risk of low HDL and High LDL concentration (Sergi et al., 2015). These outcomes are similar to the ones in Reykjavik, which showed that HDL and LDL levels were lower and triglyceride levels were higher in frail versus non-frail people, reaching even higher levels in those subjects with metabolic syndrome (Veronese et al., 2017a, 2017b). Cholesterol may induces NLRP3 inflammasome which stimulates the production of IL-1β, producing atherosclerotic complications and perfusion problems (Ordovas-Montanes and Ordovas, 2012).

9. The use of proteomics for salivary biomarkers of frailty

The aetiology of frailty has been associated with changes in several physiological situations including inflammation (Walston et al., 2002). Many of the observed alterations in frailty syndrome occur at the protein level (such as defects in protein synthesis and metabolism) abnormalities of mitochondrial function, redox imbalance and dysregulation of myocyte apoptosis (Marzetti et al., 2012; Joseph et al., 2012). Thus, the discovery of protein biomarkers could be a step forward for the diagnosis and prognosis of this multifactorial pathology.

Currently, there is no single biomarker that achieve sufficient predictive, diagnostic and prognostic accuracy to be routinely introduce used in clinical practice, either alone or in combination with clinical and demographic data (Lippi et al., 2015).

Understanding the molecular mechanisms and the complexity of frailty syndrome is one of the present challenges for the scientific community. Although the knowledge in this area has notably increased in the last years thanks to the development of new approaches in genomics and the proteomics (and proteomics applications in translational biomedical research).

Currently, proteomics studies focused on frailty biomarkers are limited, as most of them have used small a cohort for evolution and aim to confirm the role of inflammatory mechanisms in frailty (Pan et al., 2020). Such is the case of the FRAILOMIC initiative (www.frailomic.org), which is based on proteomic approaches that aim to identify a set of biomarkers, that will represent the basis for developing “ready to use kits”. This strategy could be used in clinical practice to improve prediction capacity, early diagnosis and the monitorization of frailty (Erusalimsky et al., 2016).

The assessment of a large panel of biomolecules (bioactive proteins, including proinflammatory cytokines and metalloprotease, referred to as the SASP involved in the pathogenesis and progression of frailty, is a strategy of a model multivariate/multidimensional) (Calvani et al., 2015). This approach strongly relies on the use of proteomics techniques, that enable the identification of previously untargeted proteins, as well as the detection of proteomic signatures reflecting specific timeframes. As a result characterizing people at greater risk of developing frailty or pre-frailty, as well as frail patients at enhanced risk of progressing towards a worse cognitive and functional decline (Frantzi et al., 2014) are identified.

Hence, to understand the aetiology and consequences of frailty, new methods should be designed to allow patients stratification, prevention, early diagnosis, and treatment monitoring of frailty in the older population. Currently, invasive tests are the gold standard identifying biomarkers of inflammation processes by obtaining fluids like plasma and/or serum. These tests are uncomfortable for individuals and require highly trained personnel. By contrast, other more accessible proximal fluids, such as saliva, are being studied to identify relevant biomarkers. This approach has several advantages over other conventional ones, such as reduced health costs as a result of non-invasive and stress-free specimen collection that does not require specialized healthcare professionals and settings. Furthermore, sample collection is safer and simpler thanks to the possibility of self-collection. Therefore, repeated specimen collection is not challenging either more straightforward (Slade et al., 2003) comparisons between plasma and saliva proteomes have indicated that 20–30 % of proteins found in saliva are present in blood (Yan et al., 2009). Scientists have long recognised that saliva serves as a mirror of health, containing the full repertoire of proteins, hormones, antibodies and other analytes that are frequently measured in the blood tests (Slowey, 2015). Also, the analysis of salivary is being carried out for a wide range of conditions including oral cancers and CVD (Gohel et al., 2018; Khurshid et al., 2018). Despite these advantages, some biobanking features also need to be developed to highlight the need for high-quality clinical salivary samples, collected under standardised conditions, to provide an excellent biomarker validation pipeline; the aim to finding new projects is to create new research to discover diagnoses and treatments for frailty. This approach will have to consider that the relationship between saliva and ageing is not a static one and also that oral cavity harbours a variety of microbes, influencing local and systemic health. Additionally, it is necessary to take into account that the strength and nature of the associations between saliva and blood immune markers may depend on the oral and/or physical health of the population under study. Acute illness or inflammation of the oral or systemic compartment may affect the degree of correlation between salivary and serum immune markers. In addition to oral, physical health, age, smoking, blood contamination, test collection time and sleep quality can have acute and chronic effects on the oral immune environment, all these factors will have to be controlled (Riis et al., 2014). Establishing measurement reliability is a key component of validating new bio measures (de Almeida et al., 2008). It is worth mentioning that findings on inflammatory markers in saliva are inconsistent and the studies employ many differing single-plex or mono-plex approaches, making it hard to compare their results (Slavish et al., 2015). In terms of sample collection, most researchers collected saliva using the passive drool method (salivette). Several studies have reported poor correlations between saliva and blood for inflammatory markers (Minetto et al., 2007; Sjögren et al., 2006; Riis et al., 2014), but others show a significant correlation in those same biomarkers (Nam et al., 2019). This correlation was identified in IL-1β, CRP and IL-6 (Nam et al., 2019). One of the reasons for the inconsistencies across studies could be the time of sampling (Slavish et al., 2015). Furthermore, although there is some infiltration of protein from blood into saliva, as noted above, most

proteins cannot easily cross the multiple barriers between these two compartments. So, the question is whether these inflammatory markers reflect a local or a systemic response. For example, IL-6 may come from local tissue macrophages and the acinar cells of the salivary glands, and consequently salivary IL-6 may reflect a local inflammatory response rather than a systemic metabolic response. In terms of salivary inflammatory biomarkers for frailty, evidence suggests that the serum of frail older people has increased levels of myeloid-derived suppressor cell subset, which can suppress T cell responses, as well as the pro-inflammatory cytokines required for the differentiation and resulting immune dysfunction. For this reason, several studies have found higher levels of inflammatory markers in saliva than in blood (Miller et al., 2014).

Recently a study on IL-6 using saliva showed that has a significant relationship with the levels of frailty and physical activity. In this study, significant differences were detected between robust and fragile individuals in saliva concentrations (Gómez-Rubio et al., 2022).

Currently, the discovery phase of biomarker in human salivary fluids relies on untargeted approaches that could resulting in the identification of a vast number of potential biomarkers. Untargeted approaches are also referred to as unbiased since the discovery of new putative biomarkers is performed without any prior assumptions as to which ones could be potentially promising. However, the methods for carrying out an untargeted analysis tend to be relatively intensive and cannot handle the immense complexity of the salivary fluid proteome. From a practical point of view, this limits the analysis to the most abundant proteins, which represent only a narrow or small fraction of the proteome. Accordingly, an untargeted analysis may be more relevant when using human salivary fluids rather than blood, such as fluids close to the affected tissues. This approach can be used to identify an initial set of protein targets that may be further evaluated using more sensitive methods.

10. High-throughput molecular technologies for salivary biomarker discovery in older adults

Rapid and simple molecular testing is important for the clinical application of salivary inflammatory biomarkers. The methods used range from more conventional immunoassays to the most advanced techniques. Progress in several high throughput techniques in sample analysis has allowed screening many individuals for many parameters.

Immunoassays are tests used to quantify specific biomarker levels within a sample, using biomarker concentrations to generate a standard curve. Its values are then interpolated onto the standard curve to define biomarker levels (O'Brien et al., 2006; Haran et al., 2012). Standard microplate readers used for immunoassays such as enzyme-linked immune-absorbent assay (ELISA) include software that supports absorbance detection for bioassays and calculations of the corresponding concentrations based on interpolation of the optical density onto the standard curve (Xiao and Wong, 2012).

Different proteomics approaches have been used for the characterization of the proteome in proximal fluids. Mass spectrometry (MS) has become one of the most powerful technologies in the last years in proteomics characterization. The use of surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS), which allows rapid, high-throughput detection of proteins in minute sample volumes without pre-processing, can be used in the diagnosis of many diseases. The use of mass spectrometry requires smaller amounts of material and is higher throughput than the conventional sequencing methods (Amado et al., 2005). Analysis of protein biomarker employing two-dimensional difference gel electrophoresis (2D-DIGE) coupled with MS (identification of 300 proteins approximately) and liquid chromatography-MS (LC-MS) can identify >1050 proteins in a sample (Hu et al., 2005; Xiao and Wong, 2012). It has been shown that only 1929 proteins can be analysed in human saliva, compared to the 3020 found in the plasma proteome, of which 597 can be found in common between two sample types (Ohshiro et al., 2007).

Another proteomic technique is the Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MALDI- TOF) mass spectrometry (MS/MS) analysis (Schwartz et al., 1995). MALDI-TOF, SELDI-TOF and SDS-PAGE (sodium dodecyl sulfate [SDS], polyacrylamide gel electrophoresis [PAGE] and isoelectric focusing [IEF]) are the most widely used techniques. The first and the second ones have been employed to study the protein composition of whole saliva and specific gland saliva, with or without stimulation, which allowed the separation of >30 bands (Filaire et al., 2004). A weakness of IEF using carrier ampholytes is the need for prior desalting of samples, which is time-consuming and could lead to protein loss.

A screening advantage of SELDI-TOF-MS over MALDI-TOF-MS is its ability to perform a miniaturized on-chip prefractionation of saliva samples. Optimization of buffer systems can potentially increase resolution and the results help in the design of purification schemes to isolate specific salivary proteins with classical chromatographic methods (Schipper et al., 2007). Salivary protein gels are not different from the protocols used for staining other kinds of samples, including Coomassie blue (higher detection limits), silver staining and fluorescent dyes (high dynamic range). Problems associated with 2DE include poor recovery of hydrophobic proteins and limited resolving power for proteins with low/high relative molecular mass (Ms) or basic pIs (Peng et al., 2003). A disadvantage of the use of polyacrylamide gels is their inability to resolve and detect the low-molecular-weight proteome (<10 kDa).

Then, Multiple Reaction Monitoring (MRM) or Selected Ion Monitoring (SRM) has emerged as the method of choice for performing validation with a large number of candidate biomarkers for validation/verification in saliva and other fluids.

MRM is a tandem mass spectrometric technique (MS/MS) that is performed on triple-quadrupole mass spectrometers. This technique involves the selection of a precursor ion, a peptide that acts as a surrogate for the protein of interest, which is selected by the first quadrupole. The precursor ion (protonated intact peptide) is then fragmented in the second quadrupole, and one of the fragments is selected by the third quadrupole, reaching the detector. This precursor/product ion pair is referred to as a transition and the quantitation of the protein is based on the signal that reaches the detector. This fragmentation occurs in an LC/MRM-MS experiment on a millisecond time scale; different transitions can be selected as a function of the retention time. This type of analysis allows the quantification of hundreds to thousands of peptides/proteins, and as a consequence, the proteins present in the sample (Cheng et al., 2020).

Finally, it can be important to highlight that multiplex-MRM assays can be employed as biomarker discovery platforms due to their feasibility in the quantitative identification of up to several hundreds of proteins in a single LC/MRM-MS analysis; hence, this platform could be compared with protein microarrays. Several biomarker studies have been performed using this approach.

In general, the performance of MRM was at least as successful as ELISA in several other pathologies Optimal performance is expected to be achieved in frailty, as described in SASP-Atlas (Proteomics atlas of senescence-associated-secretome for ageing biomarker development) recently developed by Birgit Schilling and collaborators (Basisty et al., 2019). This is the first database containing the contents of exosome and soluble secretomes, that can be used to identify SASP components or biomarkers candidates for senescence burden and identify SASP components, ageing and related diseases (Nelson et al., 2012).

One aspect to keep in mind is that there is a difference between biomarkers that fail during the verification process and those that cannot even be evaluated, mostly due to a lack of suitable antibodies or affinity reagents. In fact, in both situations, the biomarkers are commonly considered unverifiable.

Another important aspect is the cost of a complete discovery-to-verification study. Anderson et al. discussed the challenges and costs of performing a complete discovery-to-verification study (Anderson

et al., 2019).

11. Discussion

Frailty is associated with adverse ageing and is a growing public health problem worldwide, leading to severe impairment in people who suffer from it. This chronic condition is associated with comorbidities and high healthcare costs. Given both its health and economic impact, biomarkers for early intervention and prevention are crucial. Salivary fluids offer an advantage over blood since they are easily accessible and safe to collect. Inflammatory response and hence inflammatory proteins have been shown to play a role in many clinically relevant diseases. Further research is needed to better determine the clinical relevance of salivary inflammatory biomarkers.

Currently, there is limited literature that focuses on the study of salivary inflammatory biomarkers and even less so regarding frailty syndrome. Studies on the viability and validity of salivary inflammatory markers in frailty would be highly valuable for research and clinical purposes. Thus, clinical studies with large sample cohorts need to be designed and performed to enable the examination of multifactorial aspects (such as age, gender personality factors or socio-economic status that may potentially alter stress levels, stress responses and immune function) involved in frailty, which could establish a direct correlation that might increase knowledge on this pathology. Recent advances in high throughput protein analysis techniques make this type of high scale screening feasible. This opens up an opportunity to exploit the potential of salivary inflammatory biomarkers in frailty screening and management.

12. Conclusions and futures perspectives

Considering the biological and clinical findings obtained thus far concerning the complexity of frailty syndrome, its incidence has grown in the last years. It has been observed that many of the clinical alterations in this syndrome are related to immune dysfunction that initially occurs at the protein level. For this reason, it is crucial to study and identify proteins, inflammatory biomarkers in this case, using saliva as a proximal fluid, which has been shown to have a high protein level. Hence, non-invasive collection of salivary fluids, as well as the use of sensitive methods and molecular techniques for proteomic analysis in translational biomedical research, allow for diagnosis, prognosis and prediction of the best treatment for frailty.

CRediT authorship contribution statement

Alfonssina Niebla-Cárdenas performed the literature search and wrote the manuscript. Halin Bareke wrote the manuscript. Manuel Fuentes García carried out the design and conceptualization of the manuscript. Halin Bareke, Ana Silvia Puente-González, Roberto Méndez Sánchez and Manuel Fuentes Garcia reviewed the manuscript. Eva Arroyo-Anlló, Pablo Juanes-Velasco, Alicia Landeira-Viñuela, Angela-Patricia Hernández, Enrique Montalvillo, Rafael Góngora discussed the manuscript. All authors edited the manuscript and approved the submitted version.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

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

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