Inflammaging and 'Garb-aging'

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Abstract

Inflammaging refers to the chronic, low-grade inflammation that characterizes aging. Inflammaging is macrophage-centered, involves several tissues and organs including the gut microbiota, and is characterized by a complex balance between pro- and anti-inflammatory responses. Based on literature data, we argue that the major source of inflammatory stimuli is represented by endogenous/self, misplaced or altered molecules resulting from damaged/dead cells and organelles (cell debris), recognized by receptors of the innate immune system. While their production is physiological and increases with age, their disposal by proteasome, autophagy/mitophagy progressively declines. This “auto-reactive/auto-immune” process fuels the onset or progression of chronic diseases that can accelerate and propagate the aging process locally and systemically. Consequently, inflammaging can be considered a major target for anti-aging strategies.

Aging is characterized by chronic low-grade inflammation

It is well established that aging is a major risk factor for all chronic diseases and geriatric syndromes [cardiovascular diseases (CVD) and neurodegenerative diseases, cancer, type 2 diabetes (T2D), arthritis, chronic obstructive pulmonary disease, sarcopenia, frailty, among others] that negatively affect health-span and longevity. Many epidemiological and biodemographic studies show that biomarkers of inflammation are robust predictors of morbidity (chronic diseases) and mortality in the elderly [1,2]. Since 2000 we have used the term "*inflammaging*" to describe the chronic, low-grade inflammation that develops with age and predicts susceptibility to age-related pathologies [3]. In Box 1 and Figure I, the experimental and conceptual origins of this theory and the steps that marked its advancement and refinement are briefly summarized. As inflammation is essential for survival, the development of inflammaging can only be explained within the framework of the antagonistic pleiotropy theory of aging. In other words, inflammation has beneficial effects towards neutralization of dangerous or harmful agents early in life and in adulthood, while these effects would turn detrimental in old age, a phase of life that, contrary to childhood, did not undergo selection during evolution. Inflammation is strictly interconnected with antagonizing mechanisms, which control and resolve the inflammatory process, collectively indicated as anti-inflammatory. Therefore the clinical outcome of inflammation depends on the balance between pro- and anti-inflammatory mechanisms, and the same stands for inflammaging, where an inhibitory counterpart (anti-inflammaging) has been postulated, as indicated in Fig. I (Box 1). The lifelong balance between inflammaging and anti-inflammaging is the *fil rouge* we will follow all along this review.

Inflammaging and anti-inflammaging: the biological structure of inflammatory markers

In most of the studies, inflammaging has been assessed and tested by measuring a limited number of inflammatory molecules, namely cytokines, chemokines and acute phase proteins, in the blood of old subjects [4]. At variance, a recent work measured 19 inflammatory biomarkers in the "InCHIANTI" study and used a PCA (**Principal Component Analysis, see Glossary**) to uncover an unexpected relationship among such markers [5]. The first component of the PCA was driven largely by the soluble tumor necrosis factor receptors (STNF-RI and STNF-RII), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-a), high-sensitive C Reactive Protein (hsCRP), IL-18 and IL-1 receptor antagonist (IL-1RA), and was strongly correlated with age. The second component was explained largely by monocyte chemoattractant protein (MCP), IL-12 and IL-8. All these molecules are involved in inflammation and strongly predictive of mortality and multiple chronic diseases, but in opposite directions [5]. It is interesting to note that the first component "*is driven by higher levels*

of both pro- and anti-inflammatory markers, indicating a more activated (but not necessarily more inflamed) inflammatory system" [5]. Together, these components explained 29% of the total variance among the considered inflammatory markers, enough to indicate their importance, but far less than 100%. Thus, the inflammatory markers involved in inflammaging appear to work within a biological structure characterized by complex and still largely unknown molecular interactions and pathways. These data also suggest that "inflammaging players" are much more numerous and not restricted to classical cytokines (see below).

Nevertheless, cytokines represent a major component in the biology of ageing and inflammaging. A study on young and elderly subjects selected for their health state according to the SENIEUR protocol, and in centenarians, reported that STNF-RI and STNF-RII plasma levels increased with age, while chemokine ligand 5 (CCL5) levels were high exclusively in centenarians [6].

Within this context, IL-6 deserves particular attention due to its strong correlation with morbidity (frailty, sarcopenia and a variety of age-related diseases), and mortality in the elderly. However, IL-6 has both pro- and anti-inflammatory functions. In chronic inflammatory diseases such as rheumatoid arthritis, inhibition of IL-6 improves inflammatory markers and symptoms [7]. On the other hand, skeletal muscle IL-6 in response to exercise is generally considered beneficial, and, the association between IL-6 production and oxidative stress after acute exercise is present only in young persons, who recover more quickly from muscle injury [8]. In a recent study [9] designed to identify biologically informed, aggregate measures of inflammation for optimal risk assessment, 15 NF-κB-mediated pathway markers of inflammation were measured in baseline serum samples of older participants of the InCHIANTI study. The results allowed the identification of an inflammation index score that included only two markers, *i.e.* IL-6 and STNF-RI. These two cytokines were tested in another large study on elderly, the Cardiovascular Health Study, resulting as the best predictor of 10-year all-cause mortality. On the whole, IL-6 is likely the most informative single marker of inflammaging and health status in the elderly.

A still open question is the contribution of the genetics of IL-6 to inflammaging. In the largest genome wide study (GWAS) ever performed on centenarians, including more than 2000 Han Chinese centenarians [10], a single nucleotide polymorphism (SNP) mapping in the *IL-6* gene locus (rs2069837) ranked at the top among genetic variants significantly correlated with longevity, contributing to 1.0% of the variance of such a complex trait as longevity. This result is remarkable for a single SNP and supports the central role of the genetics of this cytokine for ageing and longevity, as previously reported in Italian centenarians [11]. The take-home message is that studies measuring concomitantly the levels of IL-6 protein in the blood and its genetic variants in DNA

could lead to a more powerful and predictive synthetic index for morbidity and mortality in the elderly.

As a general comment, blood is informative of the overall inflammatory status, however blood determinations represent an average of the production of pro- and anti-inflammatory molecules from different organs and tissues, and are not informative regarding local inflammation that could play a critical role in the onset of specific chronic diseases. How to measure pro- and anti-inflammatory cytokines locally, in specific tissues and anatomical districts is a challenge for the future.

Inflammaging and anti-inflammaging: the results of metabolomics and lipidomics

A study involving 143 centenarians, 210 offspring of centenarians, 73 offspring of non long-lived parents and 21 young subjects, identified a metabolomics signature of ageing and longevity involving amino-acids (tryptophan, tyrosine, phenylalanine), lysophospatidylcholines, sphingomyelins, glycerophospholipids and an age-related complex remodeling of arachidonic acid products with a concomitant increase of both pro-inflammatory leukotrienes and anti-inflammatory compounds (HETE, EET) [12]. These changes were accompanied by unique changes in lipids biosynthesis in elderly and centenarians, with 41 lipid species (mainly phospho/sphingolipids) associated with longevity, as showed by shotgun lipidomics [13]. On the whole, these observations further support the hypothesis that increasing levels of pro-inflammatory markers with age stimulate a corresponding augmentation in anti-inflammatory markers, within the framework of a remodeling theory of ageing, that predicts that the body continuously pursues adaptive strategies to minimize the detrimental effects of accumulating damaging products [14].

Inflammaging and anti-inflammaging: the contribution of gut microbiota

The age-related differences in the gut microbiota (GM) composition among young adults, elderly, and centenarians, was recently explored [15]. It was observed that in centenarians the GM is characterized by a rearrangement in the Firmicutes population, an enrichment in facultative anaerobes, notably “pathobionts”, *i.e.* opportunistic pro-inflammatory bacteria generally present in the adult gut ecosystem in low numbers, and a marked decrease in *Faecalibacterium prauznitzii* and relatives - symbiotic species with reported anti-inflammatory properties. In such a modified GM a strong correlations between specific bacterial families and increased plasma levels of pro-inflammatory cytokines such as IL-6 and IL-8 have been found, and 9% of the total variability of the GM was related to the pattern of pro-inflammatory cytokines, suggesting that the elderly intestinal ecosystem contributes to inflammaging. A more detailed analysis of GM by **shotgun**

sequencing protocol of the fecal microbial DNA from centenarians, elderly and young people, allowed an assessment of the GM functional profile that confirmed the age-related relevant enrichment in "pathobionts" [16]. An age-related reduction of genes involved in pathways responsible for the production of Short Chain Fatty Acids (SCFA) via proteolytic fermentation, as well as an increase of bacterial genes involved in the tryptophan (Trp) metabolism pathways were observed. See Box 2 for a more mechanistic insight into the link between inflammation and a modified GM. Finally, the longest available human microbiota trajectory (age range: 22-109 years of age) was reconstructed by analyzing semi-supercentenarians (persons >105 years old, hereafter indicated as 105+), compared to adults, elderly, and centenarians [17]. People obtaining an age greater than 105 are very rare. In Italy, this population included 878 individuals out of 60,795,612 inhabitants (1:69,243 living individuals) in 2015. This study confirmed that GM in all age groups was dominated by just three families, *i.e. Bacteroidaceae*, *Lachnospiraceae*, and *Ruminococcaceae*, whose cumulative relative abundance decreased along with ageing. In particular, the abundance of *Coprococcus* and *Faecalibacterium*, was negatively associated with age, and, since a similar trend has been reported also in Chinese centenarians [17], this phenomenon can be assumed as part of the ageing process itself, regardless of lifestyle and dietary habits. Extreme longevity seems also to involve an invasion of the gut ecosystem by micro-organisms typical from other niches, such as *Mogibacteriaceae* and *Synergistaceae*, that are abundant in the periodontal environment, in accord with possible role of periodontium in inflammaging [18].

The reconstruction of human GM trajectory along ageing let clearly emerge another important phenomenon, *i.e.* the concomitant age-related enrichment of health-associated subdominant taxa such as *Akkermansia* and *Bifidobacterium* that are known to promote immunomodulation, protect against inflammation, and promote a healthy metabolic homeostasis [17], suggesting that these changes may support healthy ageing and longevity. In centenarians and 105+, also the family Christensenellaceae – the GM component with the highest heritability - increased in relative abundance and prevalence, representing a putative signature of the ecosystem of extremely long-lived people and a possible link to the heritable component of human longevity. Thus, even at the level of a complex ecosystem such as the GM, a subtle and complex balance between pro- and anti-inflammatory bacteria develops with age, likely playing a major role in the trajectory of healthy ageing (see also Box 2).

Inflammaging within the new perspective of Geroscience

In the last 10-15 years a consistent and coherent literature has accumulated suggesting that, beyond the immune system, a variety of organs, tissues and cells are capable of producing pro-

inflammatory compounds, including skeletal muscle and adipose tissue (AT) [19]. Within this perspective, inflammaging has to be considered a multifactorial, multi-organ and systemic process, characterized by complex interactions of a plethora of molecular mediators, within a larger network of basic biological mechanisms involved in ageing and age-related diseases (see also Box 3). The new field of **geroscience** identified a limited number of highly interconnected and networked molecular mechanisms involved in, and shared by ageing and the above-mentioned age-related diseases [20]. These include macromolecular damage, alteration of proteostasis, adaptation to stress, metabolism, stem cell regeneration, epigenetics, and inflammation. It is important to note that inflammation is not only one of the few major determinants of ageing but also a common outcome of other molecular pathways capable of generating inflammatory stimuli when malfunctioning. We will argue that inflammation is central in the pathophysiology of ageing, and a common biological denominator of the above-mentioned network, and thus a top target for anti-ageing strategies.

Where do the stimuli fuelling inflammaging come from?

Previous research suggested that inflammatory stimuli that trigger and sustain inflammaging largely derive from long-lasting exposure to persistent viral infections and are modulated by one's own clinical history ("immune biography") [21]. A large body of evidence indicates cellular debris and misplaced/misfolded self-molecules as an important and maybe preponderant player in such a process (Fig. 1). Actually, persistent viral infections appear at least in part dispensable for the progression of inflammaging [22]. Moreover, over-nutrition is not as universal as inflammaging appears to be. At variance, millions of cells dye every day in the human body as a result of development, chemical and physical stresses, infections, and even normal homeostasis and turnover [23], and a variety of efficient mechanisms are devoted to their disposal in a way that it is not harmful for the organism. Thus, ancestral, robust, and orchestrated mechanisms have evolved and became progressively more sophisticated in order to identify, recognize, and process dying cells and their products (cell debris) [24,25]. In particular, a series of different receptors, including Scavenger Receptors, Dendritic Cell Receptors, Pattern Recognition Receptors (PRRs), among others, have the capability to recognize stressed or dying cells, and promote their degradation through engulfment in phagocytic vesicles. Recognition and clearance of dead cells is a basic biological mechanism at the core of organism biological processes and pathologies such as Alzheimer disease, autoimmune and CVD [23-26]. The main characteristic of this cleaning machinery is to avoid the production of antigenic molecules and inflammatory responses [24]. Defects in this complex machinery are associated with inflammation, auto-immune disorders, as detailed elsewhere [24]. Within this biological scenario we argue that inflammaging is largely the consequence of the imbalance

between production and disposal of cellular debris and misfolded/misplaced self-molecules, which develops with age. This process is likely at the core of the ageing process. These cell debris and self-molecules actually promote inflammation when they are recognized by PRRs, therefore a prolonged, increased exposition of these molecules leads to unwanted/unnecessary inflammatory responses. Therefore, inflammaging (and likely ageing) could be conceptualized as a peculiar type of auto-immune process where the distinction between self and not self undergoes a progressive blurring with age. As an example, centenarians have been found to have high plasma level of a variety of autoantibodies, such as Rheumatoid Factor and anti-nuclear antibodies [6], anti-beta 2 glycoprotein I and anti-cardiolipin antibodies [27], and antibodies directed to the nuclear protein poly(ADP-ribose) polymerase (PARP-1) [28]. It is interesting to note that despite the presence of antibodies comparable to those found in patients with anti-phospholipid syndrome (APS), no vascular events were reported in centenarians [27]. APS is characterized by thrombotic events, miscarriages and ischemic stroke, and these autoantibodies have likely a pathogenic role, as they are correlated with an aggressive clinical picture [29]. Antigens bound to antibodies can be eliminated through phagocytosis at the level of liver and spleen. Thus, the above-mentioned type of auto-antibodies can help long-living people in getting rid of cellular debris and misfolded self-molecules recognised as auto-antigens. In this way these auto-antibodies can contribute to anti-inflammaging by dampening the capability of auto-antigens to trigger inflammatory responses, as hypothesised by the eminent French immunologist Pierre Grabar (1898-1986).

To this scenario, nutrition, the gut microbiota and its products [15,17,30] can be added as a source of inflammatory stimuli (see Box 2 and 3). The inflammatory state sustained by over-nutrition and diets rich in high fat has been indicated as "**metaflammation**" (see Box 3)[30]. The setup of such inflammation involves different tissue ad organs at different levels of complexity and in many aspects overlaps inflammaging features. Accordingly, long-term moderate caloric restriction [31] but also time-restricted feeding, in which food consumption is restricted to certain hours of the day, or fasting-mimicking diets appear the most promising to provide pleiotropic benefits against inflammation [32]. They are likely based on increased molecular repair, such as DNA repair, and decreased production of cell garbage [33]. Caloric restriction-mimetic therapies such as metformin, acarbose, rapamycin and senolytic agents (drugs that kill senescent cells) can likely halt inflammation as they activate the disposal of unwanted/damaged cells (and cell components), therefore avoiding the activation of PRR, similarly to what calorie restriction is believed to do [34,35]. The autophagic process (and all its specialized versions, such as mitophagy and chaperone-mediated autophagy) appears of fundamental importance for the disposal of damaged organelles and proteins, and is activated by caloric restriction as well as drugs like Rapamycin and other

analogs (indicated as “rapalogs”). Actually, autophagy is reported to decrease with ageing [36,37], further supporting the idea that inflammaging is fuelled by accumulation of cellular components that are not appropriately disposed. Accordingly, the maintenance of autophagic machinery has been proven necessary to promote healthy ageing and lifespan extension, and therefore rapamycin ranks at the top of the list of possible candidate drugs for promoting healthy ageing and longevity in mammals [38].

The activity of the ubiquitine-proteasome system has also been associated with the rate of ageing [39], and human longevity is characterized by the preserved function of proteasomes [40]. A higher expression of immunoproteasome, a sign of neuroinflammation, was found in the brains of AD patients but not of healthy elderly [41]. Proteasome activation has been therefore proposed as a novel anti-ageing strategy [42]. Actually, proteasome activation through genetic manipulation or treatment with natural (*e.g.* curcumin, oleuropein) or chemical compounds extended lifespan or decelerated the progression of age-related diseases in *in vitro* systems and animal models [42].

Inflammaging and misplaced self-molecules

When cells actively proliferate, end-products of metabolism and catabolism, as well as damaged or dysfunctional proteins, get diluted into daughter cells, however in non-proliferating cells like neurons, they can accumulate *in vivo* until a crucial threshold is reached after which the cells die [43]. These products include lipofuscins, advanced glycation end-products (AGEs), Tau protein aggregates, alpha-synuclein fibrils and beta-amyloid networks. All these products can be recognised by PRRs like Toll-Like Receptors (TLR), NOD-like receptors, AGE Receptor (RAGE) and others, and can therefore induce inflammatory responses [44-46]. It is remarkable to note that many of these PRRs can also bind non-self molecular structures belonging to bacteria and viruses, collectively indicated as Pathogen-Associated Molecular Patterns, PAMPs). By analogy, the self-ligands of PRRs were indicated as Danger-Associated Molecular Patterns (DAMPs) or alarmins, because they signal the presence of cellular stressors, damage or death, rather than viral of bacterial infections. The role of DAMPs in triggering and/or sustaining inflammatory reactions is an active area of research. First of all, the accumulation of lipofuscin and AGEs increases with age, and is associated with a number of degenerative diseases where inflammation plays a role, including T2D and CVD, tumours and neurodegenerative disorders, such as Alzheimer's disease, familial amyloid polyneuropathy, diabetic neuropathy, Parkinson's disease, and Huntington's disease [47]. In particular, an increase in lipofuscin pigment is reported to be concomitant with altered inflammatory phenotype in murine ageing microglial cells [58], and AGEs are involved in the age-related macular degeneration [59]. Furthermore, transgenic mice expressing human mutant APP

(mAPP) in neurons, and RAGE in microglia, displayed enhanced IL-1β and TNF-α production, increased infiltration of microglia and astrocytes, accumulation of Amyloid beta, reduced acetylcholine esterase activity, and accelerated deterioration of spatial learning/memory [48].

Beside molecules like AGEs or misfolded proteins, PRRs can also bind many other "normal" cell components [19,24,49]. A brief list of these molecules is given in Table 1. They can be considered part of the cellular "self", and usually they do not stimulate immune/inflammatory reactions. However, they can be sensed by PRRs when they are misplaced, and outside their normal, physiological location. This misplacement can be accompanied by modifications such as peroxidation, and is the result of membrane rupture or active extrusion in the cytoplasm or in the extracellular space. This "misplaced self" is rapidly disposed of by phagocytes. As mentioned, PRRs act within the framework of the innate immune response and induce an inflammatory response. It is interesting to note that the misplacement of self-molecules (that can engage these receptors) appears to increase with age, further supporting the hypothesis that inflammaging is fuelled by an increased exposure of cell components. Among the DAMPs whose level increases with age, we can include cell-free nuclear DNA [50]; HGMB-1, a chromatin-associated nuclear protein that can be bound by RAGE, TLR4 and possibly other PRRs [51]; and S100a9 (calgranulin B) that can reinforce inflammation together with S100a8 by serving, among other, as ligand for RAGE [52]. A special case of DAMPs source is the mitochondrion. As mitochondria are reminiscent of their ancestral bacterial origin, their components [circular, under-methylated mitochondrial DNA (mtDNA), specific lipids such as cardiolipin, and formylated peptides] share with bacteria the capability to bind to PRRs. Thus mitochondria can be considered a favourite source of DAMPs. Circulating mtDNA can bind TLR9 [53], and its concentration is reported to increase with age [54]. It has been observed that the *in vivo* levels of TNF-α, IL-6, RANTES and IL-1Ra are positively associated with the concentration of mtDNA, and that the *in vitro* stimulation of monocytes with mtDNA concentrations similar to the highest levels observed *in vivo* resulted in an increased production of TNF-α [54]. mtDNA, when oxidized and released into the cytosol, can also be recognized by NLRP3 inflammasomes, leading to Il-1β maturation and release [55]. Inflammasomes are very important intracellular molecular platforms for the ignition of inflammation upon binding with either foreign (non-self) antigens or misplaced/damaged self molecules, including cardiolipin, ATP, urate crystals among others [45,56-58]. Inflammasome activates caspase 1 and in turn this leads to maturation of some cytokines, the main one being IL-1β (see also Box 3).

Cell-free nuclear DNA (cf-nDNA) circulates in the blood [59], and increased amount of cf-nDNA is found during pregnancy, sepsis and cancer [59]. The entire genome is represented in cf-

DNA but, notably, parts of it such as non-coding sequences, seem to be overrepresented, suggesting an active and selective vesicle compartmentalization of DNA molecules [60-62]. Several examples of cellular DNA fragment uptake and integration have been reported [63,64]. Recently, cf-nDNA levels have been associated with inflammatory markers and frailty signs, while unmethylated cf-nDNA, RNase P-coding cf-nDNA, and Alu repeat cf-nDNA were significantly elevated in the nonagenarians compared with the young controls [65].

Propagation of inflammaging: the communicomes

Local and systemic inflammaging favours the onset of chronic diseases when additional determinants (genetic risk variants, unhealthy lifestyle, early events favouring inflammation with late effects in adult and old age) are involved. It is likely that chronic diseases accelerate organismal ageing in a vicious cycle that is difficult to stop [26]. In favour of this assumption, several data suggest that cell senescence can propagate from cell to cell by a bystander effect ("senescence-induced senescence") [66] and transmitted in a paracrine fashion by activation of inflammasome and IL-1 signalling [67]. Local propagation can be particularly important in pathologies such as cancer in the elderly, where a systemic inflammaging is present [19,20,68]. Moreover, systemic inflammation can accelerate ageing via ROS-mediated exacerbation of telomere dysfunction and cell senescence, in the absence of any other genetic or environmental factor [69]. Inflammaging is likely also propagated by the secretion of damaged cellular components produced by compromised, stressed or senescent cells and organelles where a pathological event is occurring. The inflammatory phenotype originates inside the cells with the contribution of different organelles and the nucleus, and various mechanisms, such as telomere attrition, DNA Damage Response (DDR), mitochondrial dysfunction, proteasome/lysosomes alteration, inflammasome activation and ER-stress (Fig. 1). Most of these mechanisms can generate misplaced self-DAMPs that in many cases are not confined within the cell, and actively or passively leave the cells, being taken up by other distal cells. These molecules can trigger a feed-forward propagation/amplification cycle of inflammation/inflammaging, where different shuttles actively secreted by cells such as exosomes and ectosomes - generally defined as extracellular vesicles (EVs) [70] - are involved. They influence not only nearby acceptor cells but also distant cells by travelling via circulatory and lymphatic systems (see Box 4).

In animal models, strong evidence in favor of the propagation hypothesis of the ageing phenotype emerges from the heterochronic parabiosis experiments in rodents, where an aged phenotype was induced in young mice by connecting their circulatory system to that of old animals [71,72]. This suggests that soluble molecules can lead the ageing process, while others can reverse it. The soluble, circulating, informative molecules (including those that accelerate or delay ageing)

are collectively indicated as "**communicome**" (see Box 4) [73]. Communicomes may act both at local, intercellular and systemic levels [66] as depicted in Figure 1, but at present it is not clear if and how much inflammatory molecules and cellular debris contribute to the heterochronic parabiosis-mediated ageing.

Among the main actors of propagation, an increasing importance is attributed to short nucleic acids, such as microRNAs (miRs). MiRs are well known 17-25 nt long epigenetic regulators that circulate in the blood and other body fluids at different level, transported by various shuttles. Some miRs appear to be modulated with age. As an example, it has been shown that blood circulating miRs (c-miRs) -21-5p;-126-3p increased during ageing [74-76]. Noteworthy, miR-21-5p plasma level decreased in centenarians, while c-miR-21 dramatically increased in patients affected by CVD [74] and c-miR-126-3p - highly expressed in endothelial lineages and hematopoietic progenitor cells - was found to decrease in diabetic patients and in particular in those with unstable control of glycemia [75]. On the whole, these data suggest that inflammatory endothelia-associated circulating miRs could be involved in inflammaging and eventually in the propagation of ageing and metabolic diseases (CVD and T2D). Further, c-miR-146a is an important regulator of the interplay among DDR, cell senescence and inflammaging, as recently reviewed [77]. miR-21; -126a and -146 are inflammation-associated miRs [78], but other c-miRs with possible pro-inflammatory roles have been identified [76,79,80]. At present, the study of circulating miRs in ageing and major age-related diseases, as well as their role in EVs- and communicome-mediated propagation of ageing, is a topic of intense research which can greatly benefit from the use of **Next Generation Sequencing** techniques.

Concluding Remarks and Future Perspectives

The search for the final determinants of ageing is an endless quest. The link between chronic stress, microbiota-gut-brain axis, and increased inflammatory state is emerging as a unified body-brain-mind framework to understand ageing and age-related diseases [81]. We propose here a unifying perspective which has the potential to put together in a coherent framework fragmented pieces of evidence published in different field ranging from apoptosis, cell death, cancer and obesity. In the present form, inflammaging remains macrophage- and innate immunity-centred, but becomes more systemic and dynamic, capable of propagating and fully immersed in the basic biological processes of ageing identified in the last twenty years. One of the main novelties here is the hypothesis that "the enemy comes from within". In other words we suggest that the dominant stimuli fuelling inflammaging are self-endogenous molecules and that, from this point of view, inflammaging can be largely considered an auto-immune, auto-inflammatory process. This conceptualization is far

reaching from an immunological perspective, and offers a comprehensive umbrella for and a link with a variety of inflammatory and auto-immune diseases that are usually treated separately from ageing and inflammaging. A number of crucial questions remain unanswered (see Outstanding Questions), including the quantitative contribution of cellular garbage to inflammaging (which has not been measured yet). As discussed above, the accumulation of garbage (cell debris, misplaced/misfolded proteins, but also senescent or apoptotic cells) seems to be a physiological and inescapable process. Therefore, the use of drugs like rapamycin and its analogs, proteasome activators and other drugs, that preserve garbage disposal, but also lifestyle interventions such as caloric restriction or its mimetics, represent promising candidates to counteract inflammaging and its detrimental effects of health in the elderly.

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BOX 1: Inflammaging: evolution and immunosenescence

As we suggested in a 2000 article entitled "*Inflamm-aging: An Evolutionary Perspective on Immunosenescence*", [3], this inflammatory theory of ageing stemmed from the confluence of two concomitant investigations; the evolution of immune responses and the ageing of the immune system. Comparative and phylogenetic studies from invertebrates to mammals led to a unifying hypothesis whereby immune response, inflammation and stress response, phenomena in which the macrophage plays a key role, are part of an integrated and evolutionary highly conserved set of functions crucial for survival, and aimed to counteract all kinds of potentially harmful stressors impacting on the body [82]. The study on immunosenescence allowed the identification of some of its basic characteristics, such as the expansion of memory cells, the decrease and even the exhaustion of naive cells, the shrinkage of the T-cell repertoire, and the global reduction of the "immunological space" [83]. However, not all immune responses undergo an age-related decline, but instead the innate immune system is largely spared, and in particular the capability to mount inflammatory responses is up-regulated in the elderly. In the 2000 seminal paper [3], by merging these two perspectives we proposed an oversimplified but heuristically powerful framework; both innate immunity and adaptive immunity appear to be hyper-stimulated in old people; the inflammatory status becomes pervasive over time owing to the exposure to a variety of stressors (including the continuous exposure to antigens conceptualized as particular types of stressors) occurring beyond the age of reproduction, largely unpredicted by evolution. We soon faced the paradox of centenarians, i.e. the difficulty of reconciling inflammaging with the results emerging from studies on centenarians, showing that such exceptional individuals who largely avoided or postponed chronic pathologies, were highly inflamed and characterized by increased plasma levels of inflammatory cytokines, acute phase proteins, and coagulation factors (IL-6, IL-18, CRP, Serum-amiloid A, fibrinogen, Von Willebrand factor, resistin) [4]. We noticed that, concomitantly with the abundance of inflammatory compounds, other powerful anti-inflammatory

molecules such as adiponectin, TGFbeta1, cortisol, were also present at high level in the blood of centenarians. Accordingly, we hypothesized that what really matters for a healthy and long life is not simply to have low levels of pro-inflammatory compounds but to reach a balance between inflammatory and anti-inflammatory responses [4]. A timeline of the milestone progresses in the inflammaging theory is reported in Figure I; for references, see [3,4,15,19,20,82,84,85].

BOX 2. Inflammaging and changes of the gut microbiota: implications and perspectives

The changes occurring in the GM of the elderly can have far reaching biological consequences owing to the important physiological anti-inflammatory role of SCFA and the complex role of tryptophan (Trp) in brain physiology, within the gut-brain axis [81]. SCFA (acetate, n-propionate and n-butyrate) are produced in high amounts by commensals bacteria (e.g. clusters IV and XIV of Clostridia), and, other than an important energy source, they are strong anti-inflammatory molecules that regulate host metabolism and immunity [86]. Butyrate contributes to intestinal homeostasis because it acts as energy source for colonic epithelial cells, facilitates the differentiation of CD4+ T cells into Treg cells, induces TGF-beta secretion by epithelial cells, and triggers the production of IL-10 and retinoic acid by dendritic cells and macrophages [86]. These actions promote the resolution of intestinal inflammation, thus avoiding the leakage of bacteria and bacterial-derived inflammatory compounds into the blood [86]. Reduced plasma levels of Trp are related to increased immune activation and can contribute to inflammaging [16]. Actually it has been observed that the GM of old people and centenarians is enriched in bacteria that consume Trp, affecting its bioavailability; accordingly, a reduction of Trp in the plasma of centenarians has been observed [12]. As the GM heavily impacts on the health of the host and is involved in inflammaging [87], the possibility to modify and adapt it towards a personalized pro-health ecological system is attractive. Indeed, both physical exercise and diets such as the Mediterranean diet, can modify GM, and physical exercise can attenuate the dramatic

effect of high fat diet thus reducing inflammation [85,88,89]. Moreover, the administration of pre- and pro-biotics can help in modulating GM composition also in the elderly.

BOX 3: Chronic low-grade inflammation and obesity

It is well established that high nutrient intake and obesity are linked to higher chronic inflammation, whose conceptualization and underpinning mechanisms are described in the metaflammation theory [30]. This theory is based on the observation that daily nutrient intake can stimulate low-grade inflammatory response, indicating that nutrition is pro-inflammatory *per se* [90]. High nutrient intake represents a stress for several tissues resulting in the chronic activation of specific inflammatory paths. This contributes to increase the overall inflammatory tone locally, in particular in those organs that play a major role in the metabolic homeostasis such as AT and liver. One of the most paradigmatic examples of nutrient-fuelled inflammatory response is the NLRP3 inflammasome and its modulation by dietary fatty acids. In human monocytes, palmitic acid promotes the heterodimerization of TLR1 and TLR2, inducing NF-κB-mediated expression and activation of NLRP3 inflammasome, resulting in IL1β secretion [91]. On the contrary, in macrophages, omega-3 fatty acid inhibits TLR1-TLR2 heterodimerization and abolishes NLRP3 inflammasome activation [92]. It is worth noting that NLRP3 is one of the most important molecules of inflammaging, because its silencing is able to stop the process at the organismal level [93].

Chronic nutrients excess, and in particular high fat diet, leads to an increase of the inflammatory response, that in turn and over time activates immune cells in metabolic tissues such as AT and in liver, promoting local chronic inflammatory foci. This nutrient-triggered inflammation is higher in specialized metabolic cells such as adipocytes and hepatocytes, which are more sensitive to high calorie diet. AT is at the heart of metaflammation. In obesity adipocytes increase in volume, being less vascularized and at their mechanical limit they frequently undergo cellular death, releasing cytokines, debris and excess fatty acids, all fuelling the inflammatory cascade in AT [30]. A pivotal

event in metaflammation is endoplasmic reticulum (ER) stress. In obesity, AT shows increased levels of ER stress [94], which leads to the activation of the unfolded protein response (UPR). Three main ER transmembrane enzymes responsible for UPR (PERK, IRE-1 and ATF-6) can activate several inflammation-related molecules, including JNK and IKK, leading to increased expression of inflammatory cytokines [95,96], NF-κB and PKR. In addition, it has been recently demonstrated that ER-stress can activate NLRP3 inflammasome, resulting in IL-1β activation through its cleavage and secretion [97]. On the whole, many of the metaflammation mechanisms fit the garb-aging framework, suggesting that metaflammation can contribute to inflammaging.

BOX 4: Extracellular vesicles (EVs), communicome/secretome and the propagation of inflammaging

EVs are spherical bilayered proteolipidic vesicles with an average diameter of 20-1,000 nm, and are enriched with various bioactive materials, including proteins, DNA/RNA (microRNAs or miRs), and lipids (http://evpedia.info) which are released and can be taken up by other cells ("communicome/secretome"). Emerging evidence suggests that cells can use EVs to dispose of cellular debris in case of lysosome malfunction [98]. EVs may mediate the extracellular spread of misfolded/pathogenic as well as viral or bacterial proteins. EVs-shuttled molecular communication undergoes age-related modifications [99] and mediates the propagation of senescent cell phenotype [99,100]. A recent work identified 998 molecules in the *in vitro* secretome of normal human dermal fibroblasts from donors of different ages. Seventy of these proteins, involved in matrix degradation and pro-inflammatory processes, exhibited an age-dependent secretion pattern similar to that observed in senescent cells [101]. Indeed, it is known that senescent cells acquire a specific pro-inflammatory secretory phenotype termed SASP (senescence-associated secretory phenotype), which might be an important contributor to chronic inflammation.

Fibroblasts also exhibit a unique age-related pattern of proteins, distinct from the canonical SASP, likely reflecting a specific process of skin ageing. Similarly, studies were performed to identify *in*

vitro the communicome/secretome from human preadipocytes [102], human peripheral blood mononuclear cells (PBMCs) [103], and human umbilical vein endothelial cells (HUVECs) [77,104]. Results obtained using the last three cell models suggest three different characteristics of the communicome/secretome as follows: i. Cell type specificity; ii. Sensitivity to exogenous/endogenous stimuli; iii. Capability to modulate molecular mediators of senescence or inflammation based on different stimuli. In fact, preadipocytes secretomes of omental and subcutaneous preadipocytes were demonstrated to be distinct, with omental preadipocytes able to induce more macrophage/monocyte chemoattraction, in part through IL-6/JAK-mediated signaling. PBMCs apoptosis, induced by irradiation, stimulated the expression of pro-angiogenic factors, the shedding of EVs including exosomes, and the production and release of oxidized phospholipids, in solution or incorporated into EVs. Importantly, the HUVEC senescent phenotype was characterized by an increase in several pro-inflammatory cytokines as well as the expression of miR-146a, which was also found increased in *ex vivo* circulating angiogenic cells (CACs) of Chronic Heart Failure patients, compared with healthy controls. In particular, miR-146a was observed to decrease its target interleukin-1 receptor-associated kinase 1 (IRAK1) which is partially responsible for regulating the inflammatory response [77]. Recently, telomeric repeats-containing RNA (TERRA) - telomere-derived non-coding RNAs that contribute to telomere function in protecting chromosome ends- were identified as cell-free form (cfTERRA) and enriched in extracellular exosomes. These cfTERRA-containing exosomes stimulate inflammatory cytokines when incubated with immune responsive cells, thus suggesting a possible role in inflammaging propagation [105].

Glossary:

Communicome: this term was dubbed by Dr. Tony Wyss-Coray [73] to indicate the proteins that carry information from a cell to another. In this review, the word "communicomes" is used to indicate the whole of the molecules (not exclusively proteins) actively secreted or passively released in the blood stream. We intend that every organ/tissue has its own "communicome", which can be (totally or partially) different from that of other organs/tissues.

Inflammaging: this term was dubbed by Dr. Claudio Franceschi [3] and is the contraction of "inflammation + aging" to indicate the low grade chronic inflammation characterizing the ageing process. See also Box 1.

"Garbage": in this review this term is used to indicate all the cellular and molecular products usually disposed by specific enzymes or enzymatic complexes. These products can also be recognized by cleaning receptors and phagocyted by other cells, activating the inflammatory response. In this review the new term "garb-aging" is proposed (contraction of "garbage + aging"). This term indicates the production and eventually accumulation of "garbage", *i.e.* misfolded/misplaced self molecules, as a cause of inflammation and inflammaging.

Geroscience: interdisciplinary field aimed at understanding the relationship between ageing and age-related diseases. Since ageing is the major risk factor for most non-genetic chronic diseases, understanding the role of ageing in the onset of disease will open up new avenues for disease prevention and cure.

Metaflammation: this term was dubbed by Dr. Gökhan S. Hotamisligil [30] and indicates metabolically triggered inflammation. It is mainly triggered by nutrients and metabolic surplus, while exploiting the same signaling pathways involved in classical inflammation.

Next generation sequencing: this term is used to indicate the most advanced technique of DNA/RNA sequencing based cDNA fragments, library preparation and massive parallel sequencing with instruments of third generation.

Principal Component Analysis (PCA): unsupervised statistical analysis applied to reduce sample variance when a great number of data are analyzed simultaneously. The use of orthogonal components and data transformation into vectors allows the identification of groups of data with similar variance or co-variance.

Secretome: the whole of secreted molecules. The term is more often referred to as the secreted proteins of a proteome in a given species. The communicomes can be considered partially overlapping with the secretome.

Shotgun sequencing: approach used to decode a genome by shredding ("shotgunning") it into smaller fragments of DNA which can then be individually sequenced. The sequences of these fragments are then ordered, based on overlaps in the genetic code, and finally reassembled into the complete sequence.

Table 1. A list of normal cell components that when misplaced are recognised as DAMPs, with their site of origin and the main receptors engaged. Legend: TLR, Toll-Like Receptor; NLRP, Nodd-like Receptor P; RAGE, Receptor of Advanced Glycation End-products; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; MBR, Mannose Binding Protein.

DAMPS	**Origin**	**Engaged receptors**	**REF**
mtDNA	Mitochondria	TLR9, NLRPs	[53,54,58]
N-formyl peptides	Mitochondria	Formyl peptide receptor-1, NLRPs	[106]
Cardiolipin	Mitochondria	NLRPs	[56]
Histones	Nucleus	TLRs	[107]
High Mobility Group Box 1 protein (HMGB1)	Nucleus	RAGE, NLRPs, TLR4	[57,74]
Nuclear DNA (CpGs)	Nucleus	TLR9	[108]
Heat Shock Proteins (e.g. HSPA1A, HSP90AA1); ER chaperons (CRT, ERp57, GP96)	Cytoplasm, mitochondria, Endoplasmic reticulum	TLR2, TLR4, NLRPs	[45,109,110]
Cathepsin B	Lysosomes	NLRPs	[58]
Triphosphate nucleotides (ATP, UTP)	Cytoplasm	NLRPs	[45]
S100 proteins (including S100a8, a9 and a12)	Cytoplasm – granules (neutrophils)	RAGE, TLR4, TLR9	[57,75]
Lipids (fatty acids, ceramides)	Cytoplasm, membranes	TLR4, NLRPs	[58,111]
Crystals (e.g. monosodium urate, cholesterol crystals, calcium pyrophosphate dihydrate)	Cytoplasm	NLRPs, TLR2, TLR4, CD14	[45,57]
Hyaluronans	Extracellular matrix	NLRPs	[45]
Altered N-glycans	Serum proteins	DC-SIGN, MBR	[112]

Figure Legend

Figure 1 (Key Figure): Propagation of inflammaging.

The production of pro-inflammatory compounds can start inside the cell as a consequence of: 1. a response to dysfunctional organelles (mainly mitochondria); 2. defective disposal of dysfunctional organelles through autophagy/mitophagy; 3. stress of the endoplasmic reticulum; 4. the activation of the inflammasome platform by misplaced self molecules, 5. defective disposal of misfolded/oxidized proteins by the ubiquitin/proteasome system; 6. Activation of the DNA Damage Response or the induction of cell senescence. Inflammation can then spread out of the cell to proximal or distal tissues and organs directly (cytokines) or via signaling molecules that engage receptors such as PRRs or are engulfed within target cells. These include self molecules either normal (but misplaced) or modified (indigestible end products) such as nucleic acids (cf-nDNA, mtDNA, etc.), proteins and products of lipid metabolism, small molecules, etc., collectively indicated as self garbage (see text). These molecules can be passively released by cell breakage (spillover of self garbage) or secreted via extracellular vesicles (EVs) that can contain also signaling molecules such as miRNAs.

Franceschi et al. “Inflammaging and Garb-aging”

Outstanding questions

- How much of the inflammaging process is accounted for by the production/accumulation of cellular “garbage”? Can this process be prevented by favouring the correct disposal of garbage through the ubiquitin-proteasome system and/or the autophagy/mitophagy machinery? Can compounds capable of proteasome or autophagy activation represent a novel anti-inflammaging strategy?

- When does inflammaging starts? As early as in utero (or immediately after birth) or later on in adult life? In other words, can this phenomenon be considered physiological and useful for development and survival until adulthood and detrimental later on in the post-reproductive period largely not foreseen by evolution?

- Can we dissect the inflammaging seen in healthy aging (compatible with the attainment of longevity, as exemplified by centenarians), from the disease related/specific inflammaging that has serious pathological consequences?

- How inflammaging and anti-inflammaging are coordinated? Which are the mechanisms co-regulating the two concomitant phenomena?

- Is there a role for circulating miRNAs in the propagation of inflammaging in healthy and pathological aging?

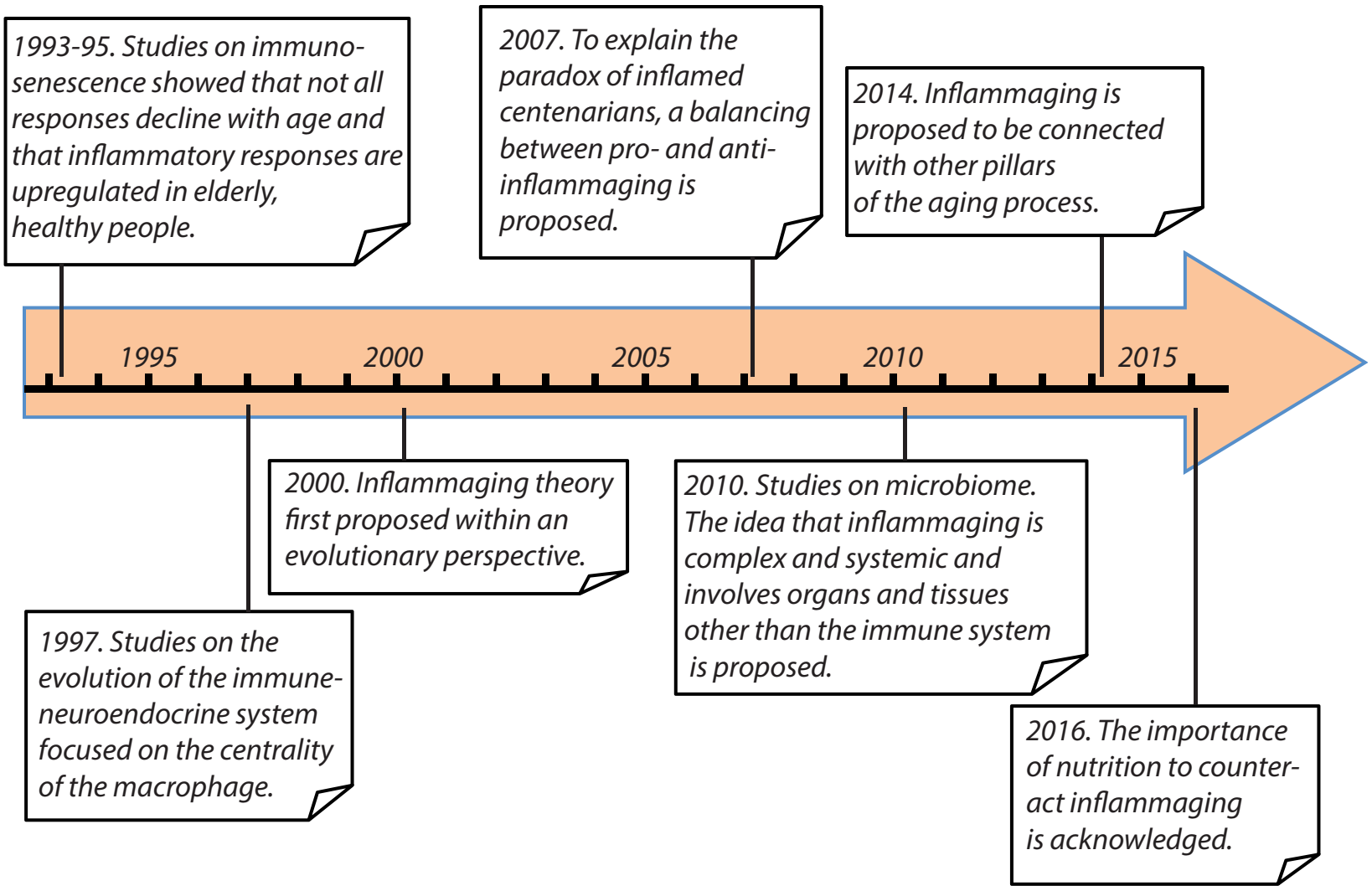
- To what extent the different microbiota of our body, other than that present in the gut, impinge upon inflammaging? How much do these microbiota contribute to inflammaging? Can we counteract inflammaging and its deleterious effects by targeting the gut microbiome using *ad hoc* pro- and pre-biotics?

- Can we counteract inflammaging and its deleterious effects by an integrated comprehensive strategy including all the above-mentioned approaches plus diet (e.g. Mediterranean diet), age-appropriate exercise and less stressful healthy lifestyle?

- To what extent anti-aging drugs such as metformin, acarbose, rapalogs, and NSAIDs modulate inflammaging? Studies in this regard are urgently needed.

- Is it possible to set up a personalized anti-inflammaging strategy, taking into account age, gender, gut microbiota composition, blood levels of pro- and anti-inflammatory cytokines, agalattosylated N-glycans, inflamma-miRs as well as cellular debris?

Franceschi et al., Figure I



Franceschi et al.Figure 1