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# The epigenetics of inflammaging: The contribution of age-related heterochromatin loss and locus-specific remodelling and the modulation by environmental stimuli

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## ABSTRACT

A growing amount of evidences indicates that inflammaging – the chronic, low grade inflammation state characteristic of the elderly – is the result of genetic as well as environmental or stochastic factors. Some of these, such as the accumulation of senescent cells that are persistent during aging or accompany its progression, seem to be sufficient to initiate the aging process and to fuel it. Others, like exposure to environmental compounds or infections, are temporary and resolve within a (relatively) short time. In both cases, however, a cellular memory of the event can be established by means of epigenetic modulation of the genome.

In this review we will specifically discuss the relationship between epigenetics and inflammaging. In particular, we will show how age-associated epigenetic modifications concerned with heterochromatin loss and gene-specific remodelling, can promote inflammaging. Furthermore, we will recall how the exposure to specific nutritional, environmental and microbial stimuli can affect the rate of inflammaging through epigenetic mechanisms, touching also on the recent insight given by the concept of trained immunity.

## 1. Introduction

Inflammaging is a major and ubiquitous characteristic of human aging [1] conserved across a remarkable phenotypic variability [2,3]. Increased basal levels of circulating pro-inflammatory factors, such as IL-6, TNF- $\alpha$ , IL-1R $\alpha$ , and C-reactive protein (CRP), hallmark of inflammaging, have been described during aging and have been associated with the development of a wide range of age-related pathologies and with higher risks of mortality [4,5]. Inter-individual variations in inflammaging and in its functional readouts rely on the intrinsic plasticity of the immune system and are the result of an entangled network

of factors that operate during the life course on the genetic background of each individual.

Some inflammaging sources [6] are intimately linked to time erosion: for example, cellular divisions unavoidably occur during development and maintenance of tissue homeostasis and lead to telomeres shortening, which in turns induces cellular senescence [7]. The Senescence-Associated Secretory Phenotype (SASP) includes a number of pro-inflammatory cytokines that sustain a pro-inflammatory micro-environment, potentially contributing to inflammaging as shown *in vitro* [4] and recently *in vivo* [8]. Other sources of inflammaging are not mechanistically related to the passing of time. These include lifestyle,

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exposure to environmental factors and to infections [9,10]. The accumulation of the individual response during lifespan (starting from fecundation and possibly earlier in parental gametes) can shape the pro- and anti-inflammatory phenotype of each individual, finally determining the extent of inflammaging in adulthood and old age.

The intra- and extra-cellular environments experienced by each cell during replications and quiescence, possibly contributing to inflammaging, can be recorded by an important molecular writer: epigenetic modifications. Epigenetics comprises the study of mitotically (and sometimes meiotically) heritable alterations in gene expression that do not imply changes in DNA sequence, but that rely on chemical modifications of DNA and histone proteins. Epigenetic modifications are essential mechanisms of developmental processes and are involved in the establishment of tissue-specific cells' identity. Such modifications are stable across cell divisions, but also reversible, and hence guarantee phenotypic plasticity and ability to respond relatively quickly to stimuli. Owing to their heritability, epigenetic changes are tied to cells' memory, a metaphor to indicate the ability to somehow record events that occurred in earlier cells' generations.

It is therefore not surprising that epigenetic patterns undergo during aging profound rearrangements [11–14] that have been associated with the onset of age-related diseases [15]. The technological advances for the analysis of epigenetic molecular markers achieved in the last two decades showed that, in humans, all tissues, including immune cells [16] undergo profound epigenetic changes during aging. However, our understanding of how these changes are mechanistically related to aging and age-related phenotypes – including inflammaging – is far to be complete.

Epigenetics and inflammaging are two active research areas in aging and the existence of a reciprocal relationship is almost given for granted. Nevertheless, only a relatively limited number of studies has specifically addressed this issue, investigating how age-associated epigenetic remodelling sustains inflammaging and, vice versa, how inflammaging can modulate epigenetic patterns. The aim of this review is to recapitulate these evidences. We will briefly recall changes in DNA methylation and histone modifications that occur during aging. Then, we will review and discuss the available studies on the epigenetics of inflammaging, focusing on those that describe age-dependent changes in genes relevant for inflammaging and/or associations of the epigenetic patterns with the levels of pro- and anti-inflammatory markers [17,18]. Finally, the effect of environmental factors on the epigenetics of inflammaging will be discussed.

## 2. Epigenetic modifications and their remodelling during aging

### 2.1. Epigenetic modifications

In this review we will focus on DNA methylation and histone modifications. RNA-mediated transcriptional and post-transcriptional gene silencing is often referred to as an epigenetic mechanism [19]. However, its role in inflammaging has been extensively reviewed elsewhere [20,21] and therefore will not be discussed here.

DNA methylation consists in the covalent addition of a methyl group to a cytosine, mainly within a CpG dinucleotide. In mammals, CpG dinucleotides tend to be globally under-represented but enriched in regions, known as CpG islands, typically located close to gene promoters [22,23]. CpG sites out of CpG islands tend to be methylated, while methylation of CpG islands is dynamically regulated during development [24]. DNA methylation is catalysed by DNA methyltransferase enzymes (DNMTs), while demethylation can occur passively during cell divisions or actively through the combination of oxidative/demethylation reactions and DNA repair mechanisms [25–27].

The most studied histone modifications include acetylation, methylation and phosphorylation of histone tails [28]. Each type of modification can occur at different residues of the histone tails and is established and removed by specific classes of enzymes.

A complex and only partially characterized crosstalk governs the relationship between DNA methylation and histone modifications [29]. The ultimate consequence of this interplay is the regulation of chromatin structure and the expression of the underlying DNA sequences.

Aging is characterized by a marked remodelling of epigenetic signatures. Two types of changes have been widely characterized: 1) general loss of heterochromatin and 2) reproducible gain or loss of epigenetic markers at specific genomic locations. More recently, stochastic epigenetic modifications (that is, not shared among individuals) and increase in epigenetic variability have been described during aging [30–32], but their relevance for inflammaging has not yet been addressed and as such will not be further discussed in this review.

### 2.2. Age-associated heterochromatin loss

Heterochromatin, a tightly packaged chromatin structure, ensures transcriptional silencing and genomic stability at specific genomic domains, including telomeres, centromeres, repetitive regions and transposable elements [33]. Assembly and maintenance of heterochromatin is governed by a complex crosstalk of epigenetic modifications, that include trimethylation of lysine9 on histone H3 (H3K9me3) and DNA methylation [34].

During cell senescence a profound reorganization of chromatin structure occurs, characterized by loss of long-range chromosome interactions and gain of short-range interactions [35–37]. These rearrangements lead to a global loss of constitutive heterochromatin, a recognized hallmark of aging [38,39] occurring at the centromeric and pericentromeric regions and in retrotransposons (Alu, SVA and LINE-1) that show an increase in transcription rates and genomic copy number [40,41]. These events, observed in several eukaryotic models of cell senescence, are likely to contribute to genomic instability and to increase mutation rates by retro-transposition [42,43]. Heterochromatin loss is sustained by a decrease in histones synthesis and histones levels, as observed in cellular models of replicative senescence and in human fibroblasts from different age donors [44].

Furthermore, both histones post-translational modifications and DNA methylation undergo profound changes during aging. H3K9me3 levels are reduced with aging and replicative senescence [45,46] and in human models of age acceleration, including Hutchinson-Gilford progeria syndrome [47,48] and Werner Syndrome [49]. Concomitantly, global hypomethylation of DNA has been extensively observed in tissues from aged individuals [50] and in cellular models of senescence [51], fostered by age-associated changes in the expression and activity of enzymes involved in epigenetic modifications contributing to heterochromatin loss. For instance, expression of the histone methyltransferase SUV39H1/2 is downregulated during aging [45,46], as is the expression of DNMT1 [52].

At present it is not clear if heterochromatin loss is a side-effect of the dysregulation of the epigenetic maintenance machinery during aging or if it is - at least in part - the result of a regulated, programmed process. Indeed, although usually referred to as “global”, age-associated loss of heterochromatin is not a-specific and occurs preferentially at Alu repeats compared to LINE-1 sequences [50,53,54]. However, age-associated decrease in LINE-1 methylation has also been reported [55,56].

### 2.3. Reproducible age-associated epigenetic changes

In addition to the prominent whole genome loss of heterochromatin at repetitive sequences, several non-repetitive genomic regions, often located in genes, gene promoters and surrounding sequences also undergo epigenetic remodelling during aging. Depending on the genomic region, these changes can consist in a gain or in a loss of specific epigenetic markers (for example, acquisition or loss of DNA methylation, referred as hyper- or hypo- methylation, respectively) [11]. Epigenome-wide association studies (EWAS) have provided a consistent repertoire for the so called *normative* changes in epigenetic marks associated with

aging, that is changes at specific genomic loci that reproducibly characterize the epigenome of aged people. Most of these epigenetic markers of aging actually are DNA methylation markers, while our knowledge of locus-specific histone changes during human aging is scarcer [14,57,58]. This is mainly due to technical reasons, as purification of genomic DNA from whole blood and other human tissues is much simpler compared to chromatin recovery and histones' analysis. Furthermore, EWAS studies on DNA methylation have enormously benefited from the availability of Illumina Infinium microarrays, more cost-effective than sequencing approaches, that have been used to quantitatively measure methylation in thousands of subjects of different ages. The chip assesses CpG sites (evolving from roughly 27,000 to 480,000 and up to 850,000 with the last version of the array) representing only a minor fraction of the 28 million CpG sites in the human genome. However, the design of the microarray has proven to be highly informative in reproducibly identifying hundreds of loci that specifically undergo hypermethylation or hypomethylation with aging [59–61].

The drivers of these site-specific age-associated DNA methylation changes, as well as their functional and phenotypical consequences, are largely unknown. Plausible hypotheses include that at least a fraction of these changes is related to the accumulation of mitotic divisions [62,63] or is the result of the purposeless continuation of developmental programs [64]. On the other hand, it has also been hypothesised that stochastic events, including molecular damage, converge in stereotyped epigenetic changes at specific loci [14].

#### 2.4. The epigenetic clocks

In the past years, normative changes in DNA methylation at specific CpG sites have been combined in mathematical models able to estimate chronological age [60,61,65–68]. The most popular among these models are Horvath's, based on 353 Infinium CpG probes [61] and Hannum's epigenetic clock, based on 71 Infinium CpG probes [60]. While Horvath's clock can be applied to estimate epigenetic age in a wide range of human tissues, Hannum's clock is specific to whole blood. The epigenetic age predicted according to these clocks is highly correlated with chronological age in physiological conditions, while deviations from this correlation can be informative of the presence of biological signs of accelerated or decelerated aging. This is relevant as, for example, epigenetic age acceleration – that is, an older biological than chronological age – has been found in individuals affected by age-related conditions such as Down Syndrome, Werner Syndrome, Parkinson's and Alzheimer's diseases, and it has been correlated with morbidity and mortality (reviewed in Ref. [69]); on the other hand, epigenetic age deceleration – that is, a younger biological than chronological age – has been found in models of successful aging such as centenarians and their offsprings [70,71]. Further refinements in Horvath's and Hannum's predictions have been proposed to take into account variations in the cellular population composition (*cell counts*) when measuring DNA methylation from whole blood [71]. As DNA methylation patterns differ among blood cells types [72,73], changes in their proportions can affect the estimation of epigenetic age of DNA extracted from whole blood. The intrinsic epigenetic age acceleration (IEAA) corrects Horvath's prediction for blood cell counts to control this bias. This means that, although calculated from whole blood, this estimation of epigenetic age is independent of blood cell types dynamics, also affected by aging. On the contrary, the extrinsic epigenetic age acceleration (EEAA) integrates Hannum's estimation with known age-related changes in blood cells composition and is thus informative of immune system aging (immunosenescence). Accordingly, EEAA is correlated with markers of immunosenescence, while IEAA is not [74].

### 3. Age-associated epigenetic changes and inflammaging

In the following paragraphs we discuss some examples on how age-

associated epigenetic changes are related with and can functionally contribute to inflammaging. It will be evident that current knowledge in this field is still sparse and that a comprehensive view of the role of epigenetics in the regulation of inflammaging remains an active area of research.

#### 3.1. Age-dependent heterochromatin loss and inflammaging

Evidences in the literature suggest that the quantitative loss of heterochromatin during aging can contribute to inflammaging. Agrawal et al. evaluated the activation of human monocyte-derived dendritic cells, measured by the membrane expression of the co-stimulation molecules CD80, CD86 and by the secretion of the cytokine IFN- $\alpha$ , found to be upregulated, upon intracellular delivery of genomic DNA by lipofectamine [75]. They showed that these markers of activation were expressed more strongly when the delivered DNA was extracted from blood of old (age 65–90 years) compared to young (age 20–35 years) donors. Importantly, they experimentally demonstrated that the higher immunogenicity of DNA from old donors was related to its hypomethylation compared to young donors. This experimental set-up potentially mimics what happens *in vivo* when self DNA is released in plasma and phagocytised by immune cells. Cell-free DNA (cfDNA) levels increase during aging as a result of tissue necrosis, defective clearance of apoptotic cells and inflammation itself [76–78]. It can be speculated that age-associated hypomethylation and, more in general, heterochromatin loss, increases the pro-inflammatory properties of cfDNA. Indeed, unmethylated cfDNA resembles microbial DNA and it is sensed by several types of intracellular receptors present in the cytosol or in the endosomal membranes of various cell types, including dendritic cells [79]. For instance, among the many DNA immune sensors, TLR9 -part of the Toll like receptor family expressed mainly in the endosomal compartment- recognizes DNA motifs containing the dinucleotide CG, mostly unmethylated in bacteria, and upon binding with DNA, its activation results in the transcription of IFN- $\alpha$  and IFN- $\beta$  as well as other pro-inflammatory genes [80].

Other evidences in literature support a role for age-associated DNA hypomethylation in increasing DNA immunogenicity. In nonagenarians plasma levels of total and unmethylated cfDNA were associated with the inflammaging markers IL-6 and CRP and with increased frailty [81]. Furthermore, Bellizzi et al. demonstrated that global hypomethylation of buffy coat cells was associated with frailty in 65–85 years old individuals [82]. Alu demethylation is likely to contribute to these significant associations, as in an independent study blood differential LINE-1 methylation was not associated to CRP levels [83]. It is worth noting that global genomic hypomethylation appeared to be delayed in a cohort of offspring of long-lived parents (mean age 69.8 + 1.6 years) [84]. Although these subjects did not differ specifically in the parameters related to inflammaging (CRP, IL-6) when compared to age-matched individuals, they displayed a better health status compared to controls [85].

It therefore follows that the epigenetic status of cfDNA can have an effect on the global inflammaging profile, although several questions remain unanswered. First, it is not clear to what extent cfDNA is able to promote immune response *in vivo* during aging [86,87], a well-known issue in elderly vaccination – treated in more details in the dedicated review in this issue –, nor to what extent circulating DNases, ensuring rapid cfDNA clearance, are involved [88]. In addition, the origin of cfDNA is not well characterized; it is plausible that different tissues undergo age-associated heterochromatin loss to different extent and upon different stimuli, thus differently contributing to the overall immunogenicity of cfDNA.

#### 3.2. Gene-specific age-dependent epigenetic changes related to inflammaging status

One of the most remarkable data linking age-dependent epigenetic

remodelling and inflammaging is that the promoter of tumor necrosis factor alpha (*TNF- $\alpha$* ) gene shows age-dependent DNA demethylation in peripheral blood leucocytes and monocytes-derived macrophages [89]. Using a reporter gene construct, Gowers et al. demonstrated that the methylation status of *TNF- $\alpha$*  promoter regulates its transcription, with hypomethylation associated to upregulation of the gene. Remarkably, this result was confirmed in an independent study [90] where DNA methylation and gene expression were assessed in peripheral blood mononuclear cells (PBMCs) from 5 young and 5 old donors.

The C-X-C motif chemokine 10 (*CXCL10*, also known as interferon gamma-induced protein 10, IP-10), whose secretion is induced by interferon gamma (IFN- $\gamma$ ) in monocytes, endothelial cells or fibroblasts, is a chemoattractant and regulator of immune cell adhesion, for example on endothelial cells. It was shown that methylation of *CXCL10* has an age-associated pattern similar to *TNF- $\alpha$*  gene [91]. In a cohort of 361 healthy young and old adults, DNA methylation of *CXCL10* promoter decreased with age in whole blood and was negatively associated with plasma concentrations of the chemokine; a gene reporter construct confirmed the inverse correlation between DNA methylation and expression of the gene. Interestingly, *CXCL10* plasma levels were negatively associated with working memory performance in the same cohort.

Age-specific epigenetic regulation has been demonstrated also for IL-23. The behaviour of this major regulator of T-cell differentiation towards the proinflammatory Th17 axis has been poorly investigated in the frame of aging physiology [92] although it may seem paradoxical. IL-23 induces the differentiation of naive CD4 + T cells into pathogenic helper T cells (Th17/ThIL-17) and its inactivation in animal models leads to the suppression of the development of autoimmune diseases [93,94]. In humans, production of IL-23 by whole blood cells upon TLR stimulation was found to be decreased in frail elderly subjects [95]. On the contrary, IL-23 levels are higher in plasma from 12- and 24-month-old compared to young mice belonging to the senescence accelerated mouse resistant 1 (SAMR1) strain [96], as well as in stimulated dendritic cells from old C57BL/6 mice compared to young ones [97]. In this latter study, upon TLR ligation, dendritic cells from old mice showed an increase in dimethylation of lysine 4 on histone H3 (H3K4me2) levels at the promoter of the subunit p19 of *IL23*. This chromatin remodelling was not observed in dendritic cells from young mice, indicating that selective changes in H3K4 methylation occur with aging and contribute to the increase in IL-23 protein production observed in aged mice.

The Krüppel-like factor 14 gene (*KLF14*), involved in adipose tissue regulation, has a role in modulating macrophages inflammatory response and in the pathogenesis of atherosclerosis [98]. Interestingly, several independent reports described age-dependent hypermethylation of the CpG island of *KLF14* promoter in several tissues, including whole blood, PBMC, liver and pancreatic islets [90,99–102], although no correlation with gene expression in PBMC or islets was observed. Hypermethylation with aging was observed also in mouse spleen, adipose tissue, kidney, lung, colon and whole blood [103], and in adipose tissue its hypermethylation is associated with decreased *KLF14* expression and increased *TNF- $\alpha$*  and *IL12* expression (Th1 pathway of T-cell differentiation).

Decreased autophagy in macrophages with aging substantially contributes to chronic inflammation [104]. Transcription of two autophagy genes, *ATG5* and *LC3B*, was found to be downregulated in macrophages from aged versus young mice, and this reduced expression is associated with DNA hypermethylation of the promoters of the two genes [105].

At the current stage of knowledge, the genomic region whose DNA methylation level is mostly associated with aging is the one mapping in the promoter of *ELOVL2* gene, encoding for an endoplasmic reticulum-bound enzyme that catalyses the elongation reaction necessary to the synthesis of the  $\omega$ -3 fatty acid docosahexaenoic acid (DHA). DHA has anti-inflammatory effects and is the precursor of pro-resolving lipid mediators (resolvins, protectins and maresins). Progressive

hypermethylation of *ELOVL2* occurs in almost all human tissues [62,106] and has been observed also in mouse [107]. So far, *ELOVL2* hypermethylation has not been associated to transcriptional down-regulation of the gene and the functional consequences, if any, of this normative epigenetic mark are not known. However, it is worth noting that *ELOVL2*-deficient mice exhibit hyperactivation of M1 macrophages and a pro-inflammatory phenotype in adipose tissues. Future studies should evaluate if *ELOVL2* hypermethylation with age can contribute to inflammaging by impairing DHA-mediated anti-inflammatory processes.

As not all organs or tissues age at the same rate, the tissue specificity of age-associated epigenetic changes should be taken into account when considering their contribution to inflammaging. A recent study demonstrated that age-associated DNA methylation changes are largely tissue-specific [106]; however, the situation is highly multifaceted, as some loci concordantly change in several (if not all) tissues [62], while others show opposite behaviours (hyper- and hypo-methylation) in different tissues [99]. Epigenetic changes that occur in cells of the adaptive or innate immune system can directly modulate their pro- and anti-inflammatory activity. At the same time, however, the epigenetic remodelling of other tissues can also have an impact on inflammaging, as it can affect the inflammatory phenotype of the microenvironment.

### 3.3. Epigenetic clocks and inflammaging

Despite the large number of studies that have used Horvath's clock to investigate age-related conditions, few have specifically considered its association with inflammaging. A weak but statistically significant positive correlation between EEAA and CRP levels (and, to a lesser extent, between IEAA and CRP) was found in a cohort of 4490 individuals aged from 50 to 79 [108]. However, in the same study the authors failed to validate this correlation in an independent, smaller cohort of 407 subjects including males and females aged 30 years or older. Irvin and colleagues provided a deeper analysis of the association between IEAA/EEAA and inflammaging-related circulating markers in a cohort including 830 subjects [109]. They confirmed the positive association of EEAA with CRP and they showed positive associations of EEAA with the level of circulating IL-6 and *TNF- $\alpha$*  that were dependent on T cell type (naïve, regulatory, memory) percentages [110]. Association between EEAA and CRP levels was confirmed also in a small cohort including 23 nursing home elderly aged 82–98 [111]. Taken together, these results suggest that the association between age acceleration estimations and inflammaging is mediated by the age-associated remodelling of the immune system compartments in blood [112], rather than being an intrinsic characteristic of the aging process.

## 4. Regulation of epigenetic patterns by inflammaging

As it often occurs in such complex interactions, it is to be expected that chronic age-related inflammation also impinges on the remodelling of epigenetic patterns, despite very limited work existing in this direction.

In particular, if on one side heterochromatin loss can drive inflammaging, some evidences suggest that the reverse mechanism may also operate, i.e. inflammaging can sharpen heterochromatin loss, thus establishing a vicious circle. An *in vitro* model of inflammatory stress show that treatment with IL-6 induces global hypomethylation of LINE-1 repeats [113]. In addition, nuclear DNA damage, that can be triggered by chronic inflammation [114], can induce global hypomethylation by promoting a re-localization of DNMTs to damaged chromatin [115].

*KDM6B*, also known as *JMJD3*, is an inducible demethylase which removes the repressive epigenetic marks H3K27me2 and H3K27me3 [116]. The expression of *KDM6B* is induced by various inflammatory mediators, including IL-4 and TGF- $\beta$ , via the NF- $\kappa$ B pathway [117], and in turn the enzyme regulates inflammatory and immune responses. *KDM6B* expression triggers cell senescence, possibly by controlling the



nuclear localization of p53 [118,119] and by regulating the expression of INK4 box genes [120]. Overexpression of *KDM6B* in glioma promotes the expression of several cytokines associated with the SASP [121]. At the same time, however, it has been reported in *C. elegans* that overexpression of *KDM6B* increases lifespan [122].

Although these studies provide some mechanistic hints on how inflammatory mediators can modulate the activity of the epigenetic enzymes, future research will have to determine to which extent and with which specificity the inflammaging milieu can modulate epigenetic modifications.

## 5. Epigenetic signatures of inflammaging

A limited number of studies exists on the direct relation between epigenetic signatures and inflammation, and it is indeed among the aims of this review to recollect and harmonize existing work in this view. Rather than considering the association between age-associated epigenetic changes and inflammaging, whose data are currently missing, these studies have specifically evaluated the association between DNA methylation and pro- and anti-inflammatory cytokines in cohorts of elderly subjects.

One of the first studies of this kind evaluated the association between circulating CRP and genome-wide blood DNA methylation in a cohort of 966 African American participants (mean age: 66 years old) enriched for hypertensive subjects [123]. They identified 257 differentially methylated CpG sites mapping in 240 genes enriched for immune response and inflammatory functions. In the study from Nevalainen et al. the authors evaluated the association between circulating IL-6 and transcriptomic/epigenomic profiles in PBMC from 156 nonagenarians [124]: significant associations were found in men but not in women and further mapped on genes involved in inflammatory responses, such as *IL1RN*, *CREB5* and *FAIM3*.

In a subsequent study, whole blood genome-wide DNA methylation and gene expression were associated with CRP levels in adult individuals of European (8863 participants) and African-American (4111 participants) ancestry [125]. Fifty-eight CpG sites were found to be reproducibly associated with CRP in both cohorts, explaining 6% of the variation in CRP plasma levels, and 4 of them mapped on genes whose expression correlates both with DNA methylation and CRP levels. Pathway enrichment analysis on this methylation signature of inflammaging included IL-6 and IL-9 signalling and several of the identified CpG sites mapped on genes involved in the regulation of inflammation, such as *AIM2* and *SOCS3*. *AIM2* encodes for an IFN- $\gamma$  inducible protein that recognizes altered or mislocalized DNA and initiates the assembly of the inflammasome by promoting caspase-1 activation and pro-IL1 pro-IL18 cleavage [126]. Methylation of *AIM2* was negatively associated with the expression of the gene and with CRP levels; interestingly, we found that in publicly available datasets *AIM2* methylation decreases with aging (data not shown) in agreement with age-associated increase in CRP levels. Finally, *SOCS3* is induced by various cytokines, including IL-6, IL-10, and IFN- $\gamma$  and it encodes for a negative regulator of *STAT3* signalling. *SOCS3* methylation was negatively associated with CRP levels in an independent study [127] where whole blood and adipose tissue DNA methylation was evaluated. Importantly, of the 58 CpG sites identified by Ligthart et al., 25 were demonstrated to be associated with circulating levels of TNF, IL-6, IL-8 or IL-10 in an independent study performed on a small cohort including 14 community-dwelling adults from 48 to 78 years old [128].

The efficacy and conceptual appeal of epigenetic clocks has prompted the search for predictors to better characterize life- and health-span. As a result, recent efforts have produced a new clock, termed DNAm PhenoAge [129]. The model was built to include CpGs whose methylation is associated with a set of 10 parameters informative of phenotypic age (chronological age, albumin, creatinine, glucose, lymphocyte percent, mean cell volume, red cell distribution width, alkaline phosphatase, white blood cell counts and CRP). The

inclusion of CRP among the selected parameters is noteworthy, along with the presence of a CpG site mapping on *AIM2* in the list of 513 probes included in the model. DNAm PhenoAge was shown to outperform the previous version of the clocks in terms of prediction for all-cause mortality and aging-related morbidity and to positively correlate with the transcription of genes involved in several pro-inflammatory signalling pathways.

## 6. Modulation of the epigenetics of inflammaging by environmental stimuli

In the past years a wealth of studies has provided robust evidence that environments that have been experienced not only during adulthood, but also in utero, infancy and childhood shape the immune phenotype and contribute to explain its functional variations during aging [2,130,131]. Epigenetic remodelling has often been brought into play to explain the effect of early events on the development of cardiovascular, metabolic, neurodegenerative and psychiatric diseases in adulthood [132–134]. Dietary components, environmental agents, stressful exposures and drugs have been shown to affect gene-specific and whole genome methylation [135,136] and some of them are currently under investigation to assess their ability to reverse disease-associated epigenetic profiles [137–141]. Despite this large amount of evidences, little is known about how these epigenetic modifiers combine and interact during the life course, and even less is known on their effect of the epigenetic patterns that sustain or dampen the inflammatory phenotype in the elders. In the following paragraphs, we will specifically discuss current knowledge on the role of environmental agents in mediating the interaction between epigenetics and inflammaging. As it will be evident, most of these studies are descriptive and mechanistic insights are still missing. As a consequence, it is hard to conclude whether the observed epigenetic changes are cause or consequence of the chronic inflammatory phenotype. What seems more likely is that a reciprocal interaction between the levels of pro- and anti-inflammatory cytokines and epigenetic marks exists.

### 6.1. Nutritional compounds

Nutritional habits can affect inflammaging and dietary interventions are promising strategies to modulate it and treat age-related diseases [1,5,137,142–145].

Caloric restriction and caloric restriction mimetics (for example, resveratrol and quercetin) have anti-inflammatory properties [144,146]. They act by orchestrating a complex network of molecular pathways [147] that include the activation of the histone deacetylase Sirtuin 1 (*SIRT1*). *SIRT1*-dependent deacetylation of histones and transcription factors (NF- $\kappa$ B, AP-1) negatively regulates the expression of inflammation-related genes [96,148–150]. *SIRT1* expression decreases with aging [151] and it is conceivable that interventions prompting its activation could counteract age-associated chronic inflammation.

Mediterranean diet (MedDiet), universally known for its beneficial anti-inflammatory effects, is particularly enriched in compounds able to modify the activity of epigenetic enzymes [137]. In the “Prevenición con Dieta Mediterránea” (PREDIMED) study the effect of MedDiet on genome-wide DNA methylation was evaluated in a cohort of 36 subjects (age range: 55–80 years) at high risk to develop cardiovascular disease [152]. The eight genes (*EEF2*, *COL18A1*, *IL4I1*, *LEPR*, *PLAGL1*, *IFRD1*, *MAPKAPK2*, *PPARGC1B*) identified as differentially methylated with respect to baseline are all involved in the regulation of inflammation. Notably, in the same study methylation of *EEF2*, which controls TNF- $\alpha$  translation [153], correlates with plasma levels of TNF- $\alpha$  and CRP, whose concentrations decrease after MedDiet intervention.

Among the nutritional compounds, zinc has attracted particular attention owing to its anti-oxidant and anti-inflammatory properties [154] and zinc deficiency being associated with higher susceptibility to

infections and systemic inflammation [155,156]. Plasma zinc concentrations decline with age [157–159] possibly due to lower zinc dietary intake in the elderly, also as a consequence of age-related alterations in sensitivity for salty taste [160]. Evidences from mouse models suggest that age-associated zinc deficiency can also have epigenetic basis. Thymocytes, splenocytes and bone marrow cells from aged mice displayed intracellular decrease in zinc concentration with respect to young animals and concomitantly show hypermethylation of the CpG island at the promoter of *SLC39A6* gene, encoding for a zinc transporter [161]. This did not affect baseline expression of the gene, which on the contrary was upregulated in splenocytes after lipopolysaccharide (LPS) stimulation in young but not in old mice. Not only zinc deficiency can be epigenetically mediated during aging, but it can also exert its pro-inflammatory action by epigenetic mechanisms [162,163]. Indeed, zinc deficiency was associated with hypomethylation and overexpression of *IL6* in cell culture models and in cells from aged mice and humans [163]. At present, however, it is not clear if and to what extent dietary zinc supplementation could improve the inflammatory landscape of the elderly [164].

### 6.2. Pollutants

Exposure to environmental pollutants can contribute to aging and age-related diseases by sustaining a chronic pro-inflammatory status [165]. Accordingly, air pollutants (particulate matter, black carbon, ozone) and persistent organic pollutants (organic compounds deriving from inappropriate disposal of chemical and electronic products) have been associated to increase in pro-inflammatory cytokines in multiple independent studies [166–168]. DNA methylation changes in genes relevant to inflammaging have been reported in humans and animal models exposed to environmental pollutants. For example, in the Normative Aging Study, exposure to black carbon and particulate matter was associated with changes in methylation of IL-6 and IFN- $\gamma$  [169] and hypomethylation of LINE-1 in blood cells [170]. The latter was observed also in a cohort including adults living near an electronic waste area, together with an increase in plasma levels of IL-6 and IL-10 with respect to controls [168]. If on one side these studies suggest an important role of epigenetics in mediating the pro-inflammatory effects of environmental pollutants, on the other side it is still not clear if and how long these changes can persist after acute exposures [171,172] and, more in general, how the extent, the frequency and the duration of exposures can ultimately impinge on epigenetic patterns and contribute to chronic inflammation in the elderly.

### 6.3. Socioeconomic environment

Lower socioeconomic status (SES) is associated with increased chronic inflammation [173,174], earlier onset of age-related disease and increased mortality [175]. The fact that individuals with lower SES have unhealthy lifestyle habits and higher rate of risk factors for chronic diseases explain only in part these associations, suggesting that psychosocial stressors and socioeconomic adversity can directly impinge on the regulation of chronic inflammation. Early life seems to be a sensitive window in which the experienced SES is particularly important in determining the inflammatory phenotype in adulthood [176].

Some recent reports suggest that epigenetic modifications can mediate the impact of SES on inflammaging. In the pSoBid cohort, recruited in the NHS Greater Glasgow Health Board area and including subjects from 35 to 64 years of age, the total content of methyl groups in blood cells (global DNA methylation) was decreased in most socioeconomically deprived group of participants, independently from the diet, and inversely correlated with IL-6 levels [177]. A study performed in macaques showed that dominance rank, a proxy for social status, was associated with PBMC altered methylation in inflammatory genes (*NFATC1*, which regulates the expression of cytokines in T cell; *CXCR2*,

which encodes for a IL-8 receptor; and *PTGS2*, which encodes for a key enzyme in prostaglandin biosynthesis) [178]. These observations were confirmed and expanded in blood from a cohort of 857 adult participants from the EPIC study, where different indicators of SES were taken into account [179]. In monocytes from more than 1200 participants from the Multi-Ethnic Study of Atherosclerosis (MESA; mean age: 70 years), low childhood SES, low adulthood SES social mobility and living in disadvantaged neighborhood were associated with altered methylation of several inflammation-related genes (including *CCL1*, *CD1D*, *F8*, *KLRG1*, *NLRP12*), and expression of a subset of these genes was associated to methylation levels [180,181].

### 6.4. Microbial exposures

Interesting insights in this field come from the work of the biological anthropologist Thomas McDade, who adopted an ecological framework to study chronic low-grade inflammation in adulthood. McDade showed that adults from low-income countries, such as Philippines or Ecuador, have lower circulating levels of CRP with respect to age-matched subjects from industrialized countries like the United States [10]. This phenotype was not explained by differences in genetic background or adulthood BMI, but was ascribed to the marked differences in nutritional and microbial exposures experienced early in life and in infancy. According to this view, the higher burden of infectious diseases in low- and middle-income countries actively engages the immune system during critical stages of its development, resulting in the ability to effectively turn on or off the inflammation as appropriate in adulthood and, possibly, in old age. On the contrary, the immune system of individuals born in more sanitized environments is less trained and more prone to a pro-inflammatory phenotype. Interestingly, McDade and colleagues found that in a cohort of 494 young adults (age 20–22 years) DNA methylation of genes involved in inflammatory processes (*CD8A*, *APBA2*, *IL1A* and *PIK3C2B*) was associated with exposure to animal feces and season of birth, two proxies of the intensity and diversity of microbial exposures in early life [182]. Some of the identified CpG sites correlated with the values of an inflammation index built up from the plasma levels of CRP, IL-6, TNF $\alpha$ , IL-10, IFN $\gamma$ , IL-1 $\beta$  and IL-8. Although these results are of potential interest for the study of the epigenetics of inflammaging, it is difficult to extrapolate what happens in the elderly, given the young age of the participants.

Further contribution to disentangle the complexity of the role of infections in the crosstalk between epigenetics and inflammaging may come by a phenomenon that has been recently framed under the name of *trained immunity* [183]. The term has been relatively recently coined [184] to distinguish this mechanism from what is usually intended by “memory” in the adaptive immune system (i.e. *trained* versus *adaptive* immunity) and describes previously overlooked means by which the host innate immune system copes with infections.

Infection encompasses a wide range of situations where blood circulating monocytes are often recruited in tissues to differentiate in resident macrophages. By doing so, macrophages are key contributors for the control of bacteria or fungi but also for orchestrating repair mechanisms. In recent years, it has been uncovered that pathways of differentiation may be diverse based on the genetic programs they unfold, accompanied by very diverse consequences. For example, in severe sepsis macrophages undergo long term functional palsy making them tolerant to further infection insults. Conversely, in the course of a productive response, they reach a state of permanent modifications that make them more prone to react to a second challenge with the same microbial stimulus by an increased production of pro-inflammatory cytokines [185]. This long-term activated (*trained*) phenotype relies on epigenetic changes in monocytes chromatin, consisting in an increase in mono- and trimethylation of histone H3 at lysine 4 that has been associated with an up-regulation of the expression of pro-inflammatory cytokines [186].

It is likely that many types of trained immunity do exist depending

on context and inducers. Interestingly, trained immunity can also be severely down-modulated after encounter with fungi which may represent a protection mechanism against septic shock, extending the complexity and entanglement among diverse types of infections and their outcome [187].

In aging, where the innate compartment of the immune system is hyperfunctional, leading to the state of inflammaging, the question would be to know if the past history of immune reactions of an individual can imprint to the immune system, and more specifically to its innate compartment, a behavior bias susceptible to explain the wide range of aging phenotypes found to exist among individuals as well as their association with co-morbidities [2,188]. Trained circulating monocytes are present several months after stimulation, but studies at longer time points are still missing. Recently, the concept of trained immunity has been found to be also applicable to myeloid precursor cells in the bone marrow [189,190]. Although the role of epigenetic changes has not been yet addressed in this setting, these observations extend the potential consequences on inflammaging of microbial exposure, vaccination or other environmental factors like diet. For example, a Western-type diet for 4 weeks followed by a chow diet for 4 weeks induced profound transcriptional and epigenetic changes in circulating monocytes and bone marrow progenitors in the atherosclerosis-prone *Ldlr*<sup>-/-</sup> mice [191]. These partial answers seem to indicate that trained immunity can contribute to inflammaging and age-related diseases, but further studies are required in this sense.

## 7. Conclusions

We presented a focused review of the available experimental evidences linking inflammaging with epigenetic mechanisms and in particular with age related epigenetic remodelling. Altogether such evidences support the idea that these phenomena can be causally linked. Some clues indicate that inflammaging and epigenetic remodelling can sustain each other, likely feeding vicious circles and representing a significant contributor to age-related physiological decline and diseases. However, our knowledge in this field is still limited and further research is needed that explore this topic, in particular to overcome a number of the study designs' shortcomings.

In particular, most of the studies that have analysed the association between inflammaging markers and epigenetic changes were performed in whole blood. Although whole blood is one of the most accessible sources of biospecimens in human, it is difficult to infer mechanistic insights from these measurement, given the high variability in blood cell types that can differently contribute to the epigenetic signal and to the inflammaging phenotype. The epigenetic characterization of selected populations of cells is thus needed, together with mechanistic studies in cellular and animal models. Furthermore, the relationship between systemic and local inflammaging [192,193] should be addressed also from an epigenetic point of view. Recent results from our group demonstrated that fibroblasts from centenarians, a well established model of healthy aging, show reduced production of IL-6 compared to cells from old-age donors, possibly due to hypomethylation of the *RNAseH2C* gene which degrades RNA:DNA hybrids (Storci et al., submitted). Conversely, the same gene was hypermethylated in atherosclerotic plaques and cancer tissues. Thus, future studies will have to consider the contribution to inflammaging of epigenetic changes in non-circulating cells, including resident macrophages.

Another issue is related to the effects on inflammaging of (early) environmental exposures. Although this topic needs long lasting longitudinal studies, the combination of results from animal models with the targeted analysis of existing and newly generated data will provide insights on how the turn of the events experienced during the life course ultimately results in a lower of higher grade of chronic inflammation in the elderly.

In conclusion, there is the urgent need to further elucidate the connection between epigenetic remodelling and inflammaging. Besides

shedding light on the molecular basis of chronic inflammation in the elderly, these studies will result in a new category of markers of inflammaging. Indeed, epigenetic modifications have proven to be extraordinary sources of robust and reproducible biomarkers of aging and age-related phenotypic traits, more stable than RNA or protein based biomarkers. Lastly, the current thrive of epigenetic drugs and interventions will soon provide new strategy to combat inflammaging.

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