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Molecular damage in aging

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Cellular metabolism and environmental interactions generate molecular damage affecting all levels of biological organization. Accumulation of this damage over time is thought to have a central role in the aging process. Insufficient attention has been paid to the role of molecular damage in aging-related phenotypes, particularly in humans, in part because of the difficulty in measuring its various forms. Recently, omics approaches have been developed that begin to address this challenge, because they can assess a sizable proportion of age-related damage at the level of small molecules, proteins, RNA, DNA, organelles and cells. This Review describes the concept of molecular damage in aging and discusses its diverse aspects from theoretical models to experimental approaches. Measurement of multiple types of damage enables studies of the role of damage in aging and lays a foundation for testing interventions that reduce the burden of molecular damage, thereby targeting aging.

Molecular damage has long been thought to be a part of the aging process[1,2] (Fig. 1). As organisms grow older, more damage accumulates as a result of internal biological processes and environmental interactions. Only some of the generated damage can be cleared or repaired[3]. The role of cumulative damage in aging, particularly human aging, has not been thoroughly assessed, in part because of limitations in and unfamiliarity with methods for measuring it. Understanding the role that molecular damage has in aging and aging-related outcomes requires the ability to simultaneously quantify many diverse forms of damage that accumulate throughout life in cells and tissues and assess their associations with chronological and biological age, aging-related diseases, healthspan and longevity.

To advance research on damage and aging in humans, the Longevity Consortium, supported by the National Institute on Aging conducted a workshop on molecular damage in aging, which served as a springboard for this Review. We describe the relationship between damage and aging, including the central place of damage in modern concepts of aging and highlight relevant types of damage that can be assessed in humans, mostly using omics approaches. The Review cannot cover all forms and manifestations of damage, but we note the importance of other processes such as inflammation or cellular senescence. Finally, we survey methods for measuring various types of damage and outline a vision of research priorities to advance our understanding of the role of damage in aging.

Intimate relationship between damage and aging

Place of damage in theoretical and mechanistic understanding of aging. Aging has many faces[4]. At the organismal level, it is characterized by increased mortality, functional decline and exponential increases in the incidence of degenerative diseases. At the cellular level, aging may be represented by the 'hallmarks of aging'[2], including both initiating events (such as genomic instability) and secondary manifestations (such as cell senescence). There are also other manifestations associated with various aspects of age-related changes. What drives all these processes, however, is the accrual of molecular damage that changes functionally relevant biomolecules in ways that deleteriously affect cellular and organismal processes. Damage comes in myriad forms such as by-products of metabolism, mutations, epimutations, errors in transcription and translation, organelle damage and other known and undiscovered forms of damage. Also, it may be endogenous (from internal biological processes) and exogenous (from the environment). There is a term, the deleteriome, which encompasses age-related deleterious processes, whose central part is molecular damage (Fig. 2). Damage can be reasonably viewed as a cornerstone of aging, its essence and its cause. Damage accumulation begins early in life, even before birth. This is clear from the analyses of somatic and germline variants, patterns of telomere shortening, DNA methylation changes and other factors[5]. As damaging mutations are inherited from parents, some damage is already present at the onset of life and the burden of these mutations is associated with functional decline, resulting in reduced lifespan and healthspan[6]. Molecular damage then manifests as cellular aging. For example, forms of intracellular molecular damage can induce cell senescence[7]. Cell senescence itself can accelerate the accumulation of intracellular damage and propagate damage to surrounding cells[8].

Aging is influenced by the rate of accumulation of damaged molecules and remediation of accumulated damage can occur through

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Fig. 1 | Molecular damage is a central element of aging concepts. Existing aging theories (shown in circles surrounding the central circle) do not agree on the nature of aging but they all consider cumulative damage as a central factor. Molecular damage accumulates throughout the lifespan, beginning early in life (in mammals before birth) and includes diverse forms discussed in this Review as well as other known and unknown forms of molecular damage.

processes such as damage repair and dilution, which happen pervasively in nature. Mammalian embryos are shown to lower the biological age during early development, presumably due to the damage dilution[9]. Notably, some organisms such as the hydra can control damage accumulation by diluting it through constant cell division and cell renewal. By contrast, organisms such as mammals that have essential nonrenewable cells and tissues (such as neurons and the skeleton) inevitably age. Some of the age-related damage in these organisms is random, but some is not. Specific forms of damage are generated by specific metabolic processes and functions. As these processes are programmed through their respective genes, these same genes superficially seem to be programmed to generate damage. While these programmatic features are evident, they have not evolved with the purpose of aging. The dilution and accelerated generation of damage suggest that damage is not simply a reflection of chronological aging, but rather a central component of the organismal aging process.

It is noteworthy that the most widely discussed concepts (theories) of aging (Fig. 1 and Box 1) recognize either the primacy of damage or its critical importance in the aging process. Although antagonistic pleiotropy, disposable soma and other evolutionary theories relate to different aspects of aging, damage is central to them. Given its centrality, one would expect age-related molecular damage to be a major focus of aging research, but this has not been the case due, in part, to the complexity of the aging process and the lack of methods to tackle damage accumulation in toto as opposed to its individual forms.

How can global damage be measured? Increased chemical diversity with age, as measured by various omics methods, may be a marker of cumulative damage. Indeed, the reactivity of cellular metabolites far exceeds their metabolic functions[10–12] and is very common in biology, with some unwanted reactions having proper names (such as Michael addition, Amadori rearrangement and Pictet–Spengler reaction). Methods are currently available that detect specific subsets of chemical and, more generally, molecular damage. It is clear that omics approaches, described in this Review, and in particular their combination may cover more damage forms than methods that measure individual forms of damage and, therefore, may better reflect the aging process in the whole organism. Omics methods have already led to important insights into the behavior of cumulative damage in model organisms. For example, metabolite profiling that quantified changes in non-targeted metabolites was used to follow age-associated trajectories of more than 10,000 metabolites in fruit flies subjected to control and nutrient-restricted diets[13]. These analyses revealed that aging is associated with increased metabolite diversity and the rise in low-abundance molecules. Further improvements in sensitivity of mass spectrometry (MS) may better characterize the aging metabolome[13]. Omics methods can also quantify and integrate the impact of age-related damage. It should be noted, however, that life is sustained by complex and dynamic systems, often making it difficult to interpret age-related changes. Such changes, as revealed by omics studies, may include responses to damage and neutral changes, in addition to the damage itself. Moreover, this balance of damaging, neutral and adaptive changes unequally applies to particular damage forms, for example mutations and epigenetic changes and transcriptomic, proteomic and metabolomic patterns. Thus, caution should be taken when inferring causal relationships between changes in molecular profiles over lifespan and aging.

Notably, damaged molecules introduced through diet may also contribute to cumulative molecular damage and influence the aging process through diet, despite the majority of biomacromolecules in the diet being digested[14]. In one study, species-specific culture media and diets were employed that incorporated molecular extracts of young and old organisms[14]. In each model organism tested (budding yeast, fruit flies and mice), the 'old' diet or medium shortened the lifespan of one or both sexes compared to the control that used the 'young' diet or medium. This finding suggests that age-associated cumulative damage is deleterious, is causally linked with aging and may affect lifespan through diet. It also suggests that age-accelerating environmental exposures might be identified through their effects on damage accumulation.

The exact contributions of particular damage forms to aging are often challenging to determine, especially in more complex organisms. In yeast, studies revealed the role of vacuolar damage[15], extrachromosomal rDNA circles (a form of DNA damage[16]), protein damage (damaged nuclear pores[17] and protein carbonyls) and other factors. One study attempted to address the topic of mutational burden by subjecting budding yeast to multiple rounds of replicative aging and assessing de novo mutations in daughters of mothers of different ages[18]. As expected, mutations increased with age, but their low numbers of <1 per lifespan excluded their causal role in aging. Conceptually, these findings should also apply to other damage types, suggesting a causal role of deleteriome (cumulative damage), as opposed to individual damage types, in organismal aging.

This view on the importance of cumulative damage in aging is also illustrated by the free-radical theory of aging[19]. This concept has been useful in defining the contribution of oxidative damage to aging, but an increasing number of studies contradicted both its centrality and sufficiency to aging mechanisms[20]. Although reactive oxygen species (ROS) are prototypical damaging by-products, their contribution to aging is governed by the metabolic organization of the cell, its protective systems and genotype. These factors are controlled by natural selection and like dietary and genetic interventions that extend lifespan, they influence aging outcomes through changes related to the composition of cumulative damage and the rates of accumulation of its various forms. Therefore, oxidative damage, like other specific damage types viewed in isolation, does

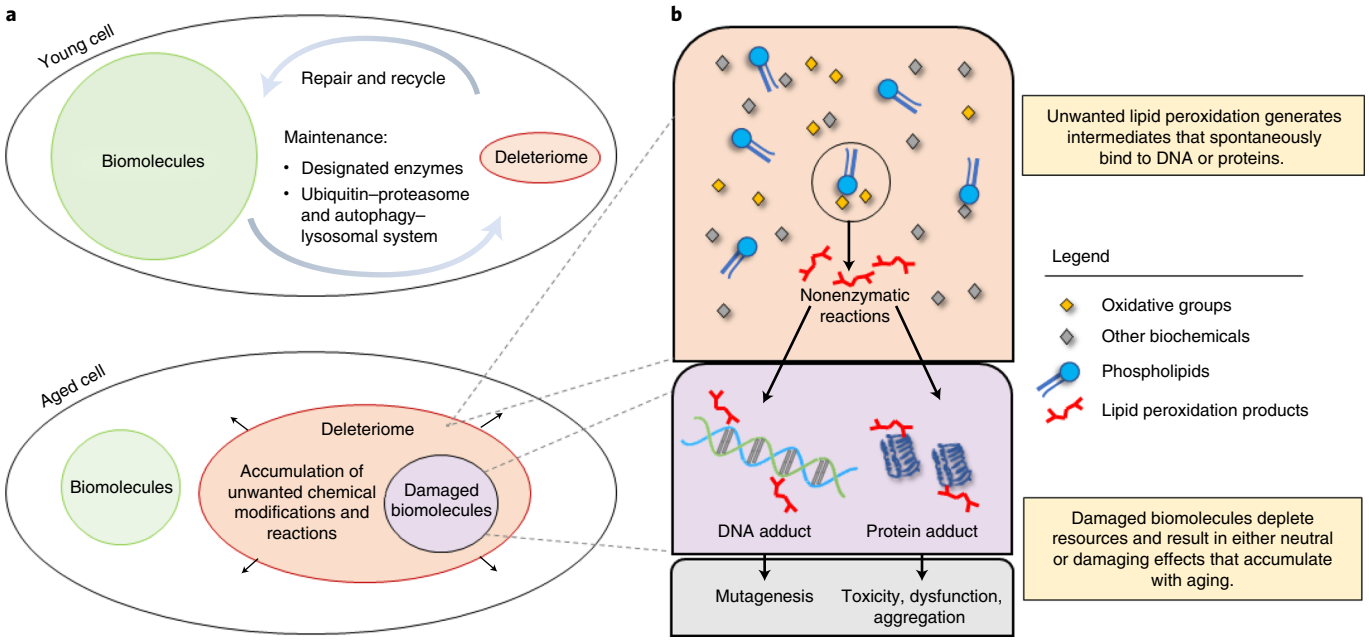

Fig. 2 | The burden of damage expands with age and affects cellular biomolecules at all levels of biological hierarchy. a, In young cells, the damage burden is low, as much of the damage, but not all, is manageable by maintenance processes such as designated enzymes that clear toxic side-products of imperfect cellular metabolism. Irreparable proteins may be cleared by the ubiquitin-proteasome or autophagy–lysosomal systems. In aged cells, in addition to the rise of irreparable damage, the repair systems may be damaged themselves and accumulation of damage to biomolecules becomes prevalent, stemming further damage. **b**, An example of how unwanted chemical modifications and reactions can lead to damaged biomolecules. In this example, lipid peroxidation products spontaneously, through nonenzymatic reactions, form DNA and protein adducts that lead to damage.

not represent a major cause of aging. Rather, inevitable accumulation of damage in the form of numerous molecular species helps to define the true root of aging through their combinatorial effect.

Below, we review many important damage types at the molecular level, which can be characterized by current omics approaches, in particular damage to the genome, epigenome, transcriptome, proteome and metabolome (Table 1). We posit that measurement of these and other types of damage would provide a picture of the role of major types of damage in human aging.

DNA damage

Somatic mutations. With age, DNA accumulates somatic mutations driven by DNA replication and transcription[21,22]. With recent advances in single-cell sequencing technologies, it is now possible to better distinguish mutations from sequencing errors and other noise. An analysis of single neurons from people aged 4 months to 82 years revealed that mutation abundance is increased in older individuals in all brain regions[23]. Notably, the same types of mutations (point mutations) are increased in the brains of patients with DNA-damage-repair-deficient progeroid diseases such as xeroderma pigmentosum and Cockayne syndrome, diseases caused by a defect in nucleotide excision repair and characterized by premature neurodegeneration[24]. Mutations accumulate most rapidly during early and late life[25]. Most mutations in the nuclear and mitochondrial genomes are either neutral or, less frequently, detrimental to cell function.

Mutation accumulation varies across cell types. A single-cell multiple displacement amplification analysis of liver cells from humans aged 5 months and 77 years revealed that mutations are significantly increased with age in hepatocytes, whereas in adult liver stem cells the mutation rate is much lower[26]. Age-related mutations accumulate in specific patterns across the genome, as revealed by cancer genomics[27–29]. In addition to driving mutagenesis, DNA damage that cannot be replicated drives other outcomes, such as cell death or senescence[30]. DNA strand breaks and stalled replication forks are potent drivers of these cell fates if not rapidly repaired.

Somatic DNA damage. Evidence supporting a role of DNA damage in aging includes: (1) all organisms have multiple conserved DNA repair mechanisms that have evolved to protect the nuclear genome; (2) genetic defects that interfere with the ability to repair DNA damage cause accelerated aging; (3) the vast majority of diseases of accelerated aging have a component of genome instability or intolerance of genotoxic stress; (4) skin in areas of the body exposed to the sun and UV radiation ages more rapidly than nonexposed skin; (5) cancer therapy with genotoxic agents accelerates aging and the onset of age-related diseases[31] and (6) DNA damage is a potent driver of cellular senescence, which has an important role in aging and age-related diseases[32]. Despite this compelling case for endogenous and exogenous DNA damage driving aging, formal proof of how, when and where somatic DNA damage does so is lacking. The reason for these gaps in knowledge is that it is challenging to measure endogenous DNA lesions, metabolites that damage nucleic acids and DNA repair capacity in tissues.

As in the case of many other forms of damage, cross-sectional studies show increased DNA damage in old organisms compared to young. This includes increased abundance of oxidative DNA lesions (such as 8-oxodG[33] or cyclopurine adducts[34]) and single-strand breaks[35]. There is also increased expression of surrogate markers of genotoxic stress, such as modified histones (γH2AX) and DNA damage response (DDR) proteins (such as p53, p21 or GADD45), as well as evidence for activation of the DDR (such as increased phospho-p53, phospho-ATM and 53BP1 nuclear foci). It is also well established that in aged mammals, expression and activity of antioxidants are reduced[36], with a concomitant increase in reactive oxygen species (ROS)[37], providing increased opportunity for DNA damage to occur. Yet, DNA repair is so efficient that a cross-sectional measure of DNA damage is thought to reflect only

Box 1 | How damage is viewed by different aging concepts

The programmed theory of aging envisions a genetically defined, evolutionarily conserved program whose purpose is to force organisms toward the aging path over time and ultimately eliminate older organisms from the population to preserve resources for the younger individuals[121]. Damage is viewed as a mediator of such an aging program.

Antagonistic pleiotropy and mutation accumulation theories[122–125] consider aging as nonprogrammed and posit that it occurs due to the accumulation of germline mutations that are either neutral (in mutation accumulation) or beneficial (in antagonistic pleiotropy) in pre- and early reproductive life, but are deleterious in late life. As these mutations have no deleterious effects on fitness during the period of most active reproduction (they are deleterious later in life), they cannot be eliminated by natural selection from the population and are passed to the next generation. These evolutionary theories typically consider damage accumulation as a key factor in progressing through age, although the molecular basis of these theories is not well defined[126].

The disposal soma theory of aging[127] offers an evolutionary basis for the buildup of global age-related damage, suggesting that, as resources are limited, organisms have to invest them in both reproduction and maintenance; the maintenance part is imperfect and therefore damage accumulates.

The hyperfunction theory of aging[128–130] suggests that aging is caused by the persistence of the overactivity of genes resulting in excessive functions that lead to damage, rather than the other way around. While this model gives the damage a secondary role, the overactivity of genes and damage may be said to work hand-in-hand to bring about aging phenotypes.

The free-radical theory of aging[19,131,132] suggests that molecular damage results from the reactivity of partially reduced molecular oxygen produced by metabolic processes. However, free radicals are only one potential damage source, so this theory has somewhat grown out of favor in recent years. There are also other theories involving particular damage types, which are conceptually related to the free-radical theory of aging.

A more recent deleteriome[56] concept suggests that endogenous damage is a by-product of imperfect metabolic processes and results from most if not all biological functions. In this model, damage is the consequence of life and exists in the myriad of forms[20,133]. As the number of these damage forms is greater than the means of protection against it, damage accumulates. The deleteriome encompasses molecular damage as well as other age-related deleterious changes at all levels of biological organization.

the preceding few weeks of exposure to genotoxins, whether endogenous or exogenous[38].

Perhaps the biggest challenge to directly quantify somatic DNA damage is that cells often respond robustly to genotoxic stress. Acute activation of the DDR is advantageous and is a potent tumor suppressor mechanism; however, chronic activation of the DDR, either due to repeated episodes of genotoxic stress or complex DNA lesions that are irreparable, leads to activation of cell fate decisions[39]. While there are a variety of cell fates, genotoxic stress clearly drives apoptosis[40] and cellular senescence[41,42]. This exacerbates the challenges of measuring DNA damage because if DNA damage triggers apoptosis, the cells with the damage are dead and eliminated. By contrast, if DNA damage drives senescence, the cell will persist, DNA damage is measurable and surrogate markers of genotoxic stress become amplified (γH2AX, 53BP1 foci, p53 and p21 levels) along with cellular senescence biomarkers (p16, p21, β-galactosidase and senescence-associated secretory phenotype factors). In addition, senescence rewires cellular metabolism[36] and senescent cells may release damaged DNA into the circulation in extracellular vesicles[43]. This DNA makes up part of circulating cell-free DNA, which increases with age and is associated with adverse age-related outcomes[44,45].

Liquid chromatography (LC)–MS/tandem MS is the most sensitive and specific method to measure endogenous DNA damage but it is laborious and expensive[46]. It also requires previous knowledge about what to measure, where and when. Thus, a tiered approach is recommended, starting with evidence for genotoxic stress based on the cellular response to DNA damage and then measures of endpoints that inform about whether cells are in a state of increased oxidative stress implying increased DNA damage. With these criteria met, DNA lesion quantification can proceed to create a correlation between DNA damage levels and aging or age-related diseases (Supplementary Table 1).

Routine measurement of DNA damage levels will require the identification of stable DNA lesions that can be quantitated by LC–MS/tandem MS or with antibodies. Currently, the only antibodies to detect endogenous DNA adducts commercially available are against 8-oxodG[47] and M_1G (pyrimido[1,2-*a*]purin-10(3*H*)-one). Measuring the adductome is a promising emerging technology[48]. This allows the quantification of the total burden of DNA damage in tissues or cells without identifying the adducts themselves. Ultimately, longitudinal studies are needed to determine whether various DNA adducts accumulate over time in vivo or increase toward the end of life in association with disease. It will also be important to determine whether the DNA damage measured in cells or bodily fluids that are readily collected from humans and model organisms reflect the DNA damage burden of the entire soma.

Mitochondrial DNA damage. Mitochondrial oxidative phosphorylation (OXPHOS) supplies 90% of molecular energy and is dependent upon the coordinated expression and interaction of genes encoded in the nuclear and mitochondrial genomes. Human mitochondrial DNA (mtDNA), a maternally inherited 16,569-bp genome containing genes critical to OXPHOS, commonly exhibits a mixture of standard and mutated states known as heteroplasmy[49]. Unlike somatic DNA, there are many copies of mitochondrial DNA in a cell. Heteroplasmic mutations and rearrangements of mtDNA are not repaired and therefore, accumulate with aging in various tissues[50]. In general, organs with the highest ATP requirements and the lowest regenerative capacities, such as the brain, heart, retina, auditory neuroepithelia and skeletal muscle, are the most sensitive to the effects of mtDNA mutations.

Because mitochondria perform diverse functions in different tissues, specific mutations in mtDNA lead to mitochondrial diseases resulting in a broad spectrum of abnormalities. Many of these diseases result from high heteroplasmy loads (>80% burden of pathologic mtDNA mutation) and cause neurologic, sensory, movement, metabolic and cardiopulmonary impairments. For example, one study found that mtDNA heteroplasmy sequenced at 20 loci known to be linked to inherited mitochondrial diseases was associated with reduced cognition, vision, hearing and mobility[51]. Another study found that increasing heteroplasmy in a single locus, m.3243 A>G was associated with multiple features of aging including poorer cognitive performance, decreased grip strength and increased risk of mortality[52]. The potential importance mtDNA mutations is further illustrated by mutator mice that accumulate mtDNA mutations in an approximately linear manner over their lifetime and develop profound respiratory chain deficiency and premature aging phenotypes[53]. Thus, the accumulation of mutations in mtDNA with age at specific sites in the mitochondrial genome, where mutations can be quantified, provides a measurement of the proportion of mitochondria having a mutation at that locus.

Epigenetic damage

The epigenome is another target for omics approaches[54]. Functional studies revealed that age-related changes in the epigenome have a strong impact on the aging process. Many chemical changes in the epigenome with aging, similar to changes in the genome, can be broadly conceived as 'damage'[55,56]. Epimutations also resemble chemical modifications of other molecules, for example metabolites and proteins. Examples of epigenetic damage are unwanted changes in cytosine methylation as well as in diverse post-translational modifications of histones. The latter are particularly prone to assessment by omics approaches (proteomics). Other examples of epigenetic changes are decreased levels of the core histones and their replacement with histone variants, as well as altered noncoding RNA expression. Such epigenetic damage alters local DNA accessibility, which in turn results in abnormal gene expression, genomic instability and activation of transposable elements.

Some studies assessed cumulative epigenetic damage in the form of Shannon entropy, where the degree of predictability and levels of randomness are assessed. An increase in entropy at a CpG site with advancing age would mean its methylation status becomes harder to predict. The fact that aging is accompanied by an accelerated increase in Shannon entropy is consistent with damage accumulation[57]. Notably, these entropy effects differ for the CpG sites that increase, decrease or remain unchanged in methylation status with age. Thus, DNA methylome remodeling may have a role in aging and provides a framework to better understand the molecular changes in aging.

An advance in the field associated with epigenetic changes has been the analysis of DNA methylation at hundreds of sites, which may serve as a 'clock' of organismal aging[58–60]. The best-known human clocks include the Horvath pan-tissue clock based on chronological age[58], the Hannum blood clock[61], the PhenoAge clock based on blood biochemistry parameters associated with disease[62] and the GrimAge clock, which accounts for future mortality risk[63,64]. Numerous other human clocks have also been developed. Epigenetic clocks have been widely used in recent years to address various questions in the aging field, such as the difference in aging rates among tissues and species, association with factors that affect the aging process and association with various age-related diseases[60,65]. The difference between chronological and biological (epigenetic) age assessed by these clocks, is termed 'age acceleration' and it has been consistently associated with mortality in human cohort studies[62,63].

The epigenetic clock studies have also been extended to other organisms, most notably mice, where both single tissue and multi-tissue clocks are available that are based on chronological age[66–70]. In addition, universal epigenetic clocks have been developed to assess biological age across species[71]. Mouse clocks were applied to test the effect of longevity interventions (such as calorie restriction, rapamycin and growth hormone receptor deficiency) on aging, as well as conversion of somatic cells to induced pluripotent stem cells, which showed an age close to zero[58,67,72,73]. Some interventions showed the potential of lowering the epigenetic age[74,75].

Thus far, age-related changes in the epigenome have been quantified primarily using human Illumina DNA methylation chips and by subjecting mouse samples to reduced representation bisulfite sequencing. As with other omics studies, DNA methylation may be used to assess age-related damage, but the contribution of damage to the observed epigenetic changes is currently unknown. Indeed, even though it is expected that many methylation changes are damaging, some of them may be adaptive.

RNA damage

Like other biomolecules in the cell, various RNA species, such as messenger RNA, transfer RNA, ribosomal RNA and small RNAs, get progressively damaged with age, which includes chemical modifications, transcriptional errors, cross-links, unwanted RNA breaks and other forms of damage. A recent work also described changes in splicing patterns as a function of age across model organisms[76]. By analyzing more than 1,000 human samples, another study found ~50,000 splicing events that correlated with age in a tissue-specific manner[77]. There is evidence that age-related changes in splicing are functionally related to processes that have been implicated in lifespan control. In nematodes, splicing factor *Sfa-1* mediates the lifespan-extending effect of caloric restriction and its overexpression was shown to extend lifespan[78]. Other forms of damage to RNA include variance in gene expression, expression of repetitive elements and erroneously expressed genes in tissues. Additionally, altered noncoding RNA expression is intimately linked with epigenomic damage. RNA damage may be partially counteracted by RNA quality control mechanisms, such as nonsense-medicated decay, which eliminates coding mRNA molecules with premature stop codons[79]. Colliding ribosomes were also suggested to detect damaged transcripts[80].

In contrast to DNA damage, there has been less focus on damage to RNA; however, it may be directly assessed by RNA-sequencing (RNA-seq), which is currently among the most common omics methods, and in the future this approach to RNA damage may extend even to single cells. For example, one study identified differentially expressed 'dark matter' transcripts that do not map to exons[81]. RNA-seq, as well as microarray profiling, is commonly used to assess changes in the expression of genes with age and some of these changes also reflect damage. Several comparative studies have searched for conserved molecular signatures of aging as defined by sets of genes whose expression changes with age across tissues and organisms. For example, a study comparing mice, rats and humans found common genes that were differentially expressed with age in certain tissues[82,83]. However, such studies do not distinguish between changes that are damaging and those that represent compensatory, adaptive responses to damage. With the release of large-scale RNA-seq datasets, such as human GTEx[84] and mouse Tabula Muris[85], it may be possible to examine patterns of RNA damage across cell types and tissues.

Protein damage

The chemistry of protein damage. Like other biomolecules, the proteome is under constant attack by reactive molecules such as oxygen, glucose and water[86]. Spontaneous oxidation of methionine, cysteine, histidine and tyrosine by nonenzymatic pathways leads to dysfunctional protein products, with protein carbonyls that historically have been the most studied[87]. There are also pathways where aspartic acid and asparagine residues racemize, isomerize and deamidate into dysfunctional products (Fig. 3). These serve as prototypes for how spontaneous, nonenzymatic damage to biomolecules contributes to aging[12]. Many forms of protein damage can be recognized by enzymes that repair that damage, such as reductase-mediated repair of oxidation damage of methionine sulfoxide residues[88–91]. But, there are also many damage forms for which there is no protection.

Post-translational modifications of proteins. Post-translational modifications (PTMs) are an understudied area of protein damage with aging. They include, but are not limited to, oxidation, glycation, formylation, methylation, phosphorylation, SUMOylation, ubiquitination and crosslinking. Protein carbamylation has also been studied as a hallmark of aging[92]. Many PTMs have been observed and quantified but many more, 'putative' PTMs have been predicted to exist or are yet to be discovered entirely[93]. Some well-known PTMs have important roles in normal biological functions, but these same modifications, however, may also represent damage when occurring at incorrect positions in proteins, where instead of supporting a function, they disrupt it. For example, oxidation of a

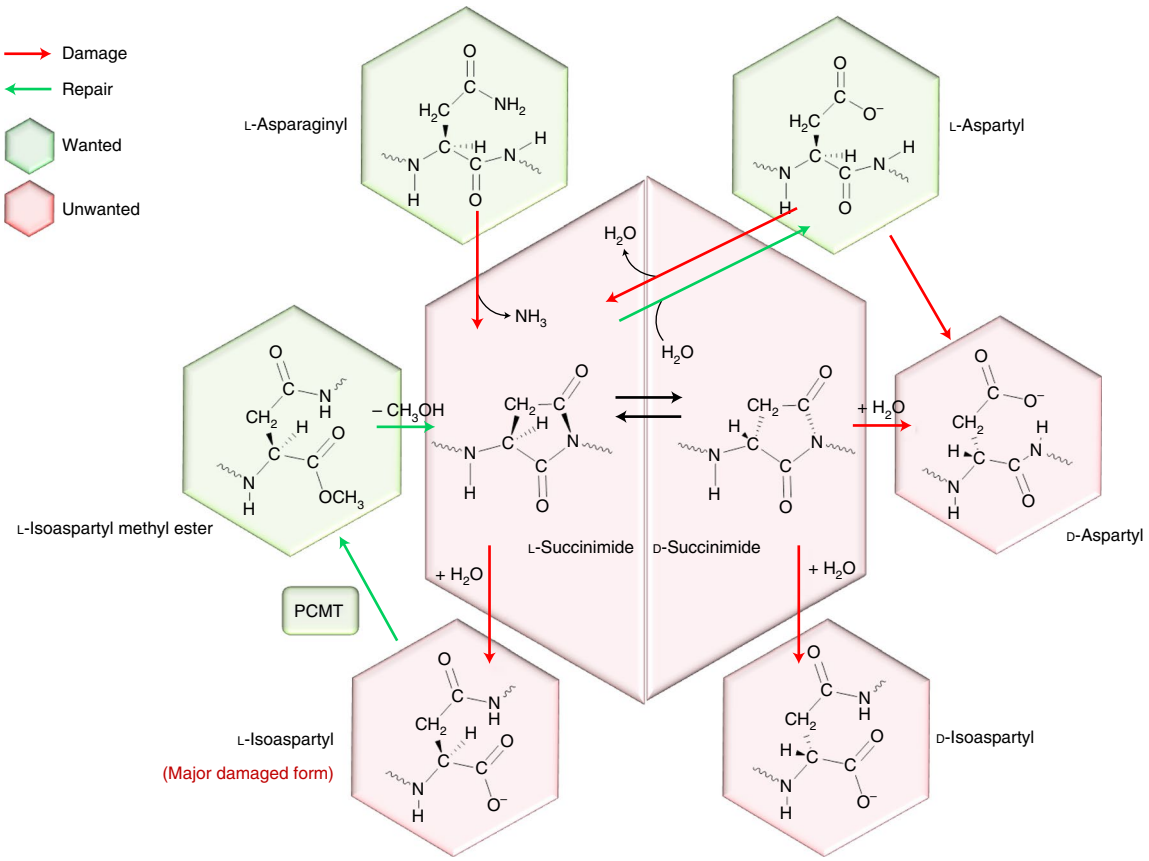

Fig. 3 | A prototypic example of damage in the form of amino acid residues. Asparagine and aspartate residues in proteins may be damaged (red) and repaired (green). Some forms of damage to these amino acid residues are generated spontaneously, and some may be repaired by designated enzymes, such as PCMT.

particular methionine residue in actin supports depolymerization of the protein[94], whereas methionine sulfoxidation at other residues would represent damage[95]. An even more common situation is represented by nonregulatory PTMs, which are modifications that have no biological function. For example, formation of methionine sulfone residues has no function but is damaging as this modification cannot be repaired. Like other forms of damage, post-translational age-related damage to proteins contributes to cellular and tissue dysfunction that characterize the aging process.

Measuring specific post-translational modifications of proteins. MS is the method of choice to study and quantify PTMs. The identification of protein oxidation requires only a peptide database search to include oxidation in the variable modification list to identify these PTMs. Although computationally expensive, a vast landscape of protein oxidation can be seen because oxidation may occur during sample handling. Nε-(carboxymethyl)lysine (CML) is an advanced glycation end-product formed on proteins by combined nonenzymatic glycation and oxidation (glycoxidation) reactions and CML and pentosidine, which are the only chemically characterized advanced glycation end-products known to accumulate in proteins with age[96,97]. Like phosphorylation, carboxymethyl lysine is measured by MS following specific enrichment methods[98]. Lysine methylation is a physiological PTM of tau protein that changes qualitatively with aging and disease[99]. For most other low-abundance PTMs, enrichment steps to purify peptides with specific PTMs is standard. For example, phosphorylation quantification on serine threonine and tyrosine amino acids supports the presence and contribution of decreased mitochondrial oxidative phosphorylation during aging to impaired cellular metabolism[100].

For most other low-abundance PTMs, enrichment steps to affinity purify peptides with specific PTMs is standard. For example, phosphorylation, measured primarily on serine threonine and tyrosine amino acids, are affinity purified by using metal catalyzed or antibody-bound supports to bind phosphorylated peptides specifically. Other antibody-based approaches are useful for identification of ubiquitinated peptides[101]. Finally, as proteins age, their propensity to develop cross-links increases and a number of proteins can be measured by MS and quantified computationally[102].

Repair and removal of damaged proteins and other forms of intracellular damage (autophagy). The importance of repair mechanisms in aging can be illustrated by the case of asparagine and aspartic acid residues in proteins. L-Asparaginyl and L-aspartyl residues are crucial to the structures and functions of many proteins, but these desirable residues can nonenzymatically transform to forms that represent the molecular damage. The prevalent L-isoaspartyl form may be particularly toxic to cells and therefore requires a strategy for its removal. Indeed, the protein L-isoaspartyl methyltransferase (PCMT) converts L-isoaspartyl residues back to L-aspartyl residues via the L-isoaspartyl methyl ester (Fig. 3). In *Caenorhabditis elegans*, overexpression of PCMT extends lifespan, whereas in mouse models, PCMT knockout caused seizures and dramatically shortened lifespan[103]. In this model, damaged aspartyl/asparaginyl residues accumulated in the brain to a greater amount than in other tissues. But the response was also unexpected; blocking repair increased brain size and improved performance in spatial learning (hippocampus) and rotarod (cerebellar) tests. This is an example of how blocking a repair pathway leads to toxic effects, but also induces

Table 1 | Types of age-related molecular damage and approaches to its assessment.

Class of molecule or process	Type of damage	Measurement
RNA	Transcriptional errors and RNA editing errors	RNA-seq and whole-genome or whole-exome sequencing
	Splicing errors	RNA-seq
Metabolites	By-products of metabolism	Targeted and untargeted metabolite profiling
	Exogenous molecular species	Targeted and untargeted metabolite profiling
Proteins	PTMs	MS
Genome (DNA)	Structural damage	Genome sequencing
	Somatic mutations	Single-cell or clonal genome sequencing
	Clonal expansions	Clonality assays
	mtDNA heteroplasmy	Mitochondrial genome sequencing
Epigenome	Hypomethylation, hypermethylation of DNA	Bisulfite sequencing and DNA methylation arrays
	Histone modifications and chromatin remodeling	Proteomics
		ChIP-chip arrays
Autophagy failure	Decreased clearance of damaged molecules and organelles	Quantification of lipofuscin granules
		Proteomics

compensatory changes that may be beneficial. It also illustrates the complexity and surprising results of translating a single chemical change to phenotypic changes that tends to occur, particularly in mammals.

It should be noted that generation of spontaneous and inflicted molecular damage is the inherent property of biological systems. The most dangerous molecules produced by these reactions may be repaired by designated enzymatic and other cellular systems, but during aging, these and other forms still accumulate. Moreover, the pathways leading to the synthesis of these enzymes are themselves susceptible to damage with time resulting in an exponential rate of accumulation of damage which may contribute to explaining the exponential rise of phenotypic damage with age.

Proteins that cannot be repaired or do not have designated repair enzymes may be cleared by two proteolytic processes—the ubiquitin–proteasome and autophagic–lysosomal systems[104]. Decline in cellular proteostasis is a hallmark of aging[2] and the consequent failure to clear damaged proteins contributes to the accumulation of intracellular damage with aging.

Chaperones identify misfolded proteins and promote their refolding or triage them for degradation through the ubiquitin–proteasome or lysosomes. Protein intermediates or partially folded states can undergo hydrophobic collapse into globular conformations in the crowded cell environment. Some aggregates contain exposed, reactive side chains, which leads to nucleation of further aggregation or development into amyloid fibrils. If an antibody has been developed for an identified, aggregating protein, then fluorescence microscopy may be used to measure aggregation. An approach to quantifying general cellular aggregates may be immunostaining with ubiquitin, as proteins in the aggregate are often polyubiquitinated.

Maintenance of cellular quality requires degradation of intracellular components, including damaged biomolecules and structures, by the proteasome and lysosomes. There are several types of autophagy that coexist in all mammalian cells: macroautophagy, chaperone-mediated autophagy and endosomal microautophagy[104]. Macroautophagy includes cellular sequestration and delivery to lysosomes of cytosolic components, including mitochondria (mitophagy), lipids (lipophagy), aggregates of proteins (aggregophagy) and pathogens (xenophagy), among others. In chaperone-mediated autophagy and endosomal microautophagy, selective proteins are targeted one by one to lysosomes or late endosomes, respectively, for their degradation. The lysosome contains enzymes that digest these products into constituents, including amino acids and fatty acids, which can be recycled back to the cytosol and reused in synthesis of other proteins or used as energy substrates. The consequences of decline in autophagy with aging, in the face of continued generation of damage, include loss of cellular quality control and accumulation of damaged biological structures, as well as an energetic compromise and deregulation of important cellular functions such, as adaptation to stress or defense against pathogens.

Lysosomes are where all autophagy degradation takes place. Therefore, measuring changes in lysosomal number and properties (acidification, enzymatic activity and lysosomal-associated membrane protein expression) inform on autophagic function. An indirect assessment of the robustness/activity of the lysosomal system is the detection of accumulation of lipofuscin, an auto-fluorescent pigment, which reflects a general failure of lysosomal digestion of damaged and undigestible residues of macromolecules and organelles[105].

The steady-state of autophagy can be determined in frozen or fixed samples by measurement of changes in both gene expression[106] and proteins involved in the autophagic process and image-based procedures, such as immunohistochemistry (IHC) and electron microscopy (EM). There are also activity-based proteasome probes that, when paired with fluorescence imaging, can determine 20S and 26S proteasome activity in cell lysates[107]. The failure of macroautophagy leads to morphological and functional changes in organelles such as swollen and deformed mitochondria, which can be recognized by EM. Analysis of lysosomal degradation of cargos and receptors that deliver cargo to autophagosomes are required to fully characterize the status of a cell's autophagy[108]. The accumulation of modified proteins (damaged or bearing unwanted PTMs) and the metabolic disbalance and subsequent alterations in metabolite levels also provide indirect information on proteasome and/or autophagy efficiency. The appropriate degree of activity of the autophagy system depends on the state of the cell: high levels of autophagy may be an appropriate response to increased rate of damage or an inappropriate activation that may itself damage components of the cell.

There have been few applications of measurements of autophagic activity to human longevity and aging. It was found that offspring of centenarians have better proteostasis and preserved macroautophagy in peripheral T cells than their noncentenarian spouses and that this correlates with their immune function[109]. Appreciation of the value of using multiple approaches to quantifying autophagy and information on the dynamics of this process offers the opportunity to directly investigate the role of its decline or failure in aging.

Metabolite damage

Forms of metabolite damage. Metabolites are chemicals. Anything that can happen to metabolites in the laboratory can also happen to them in cells[110], for example the various types of oxidation, but also hydration, hydrolysis, nitrosylation, isomerization and other chemical reactions. In a test tube, important metabolic intermediates such as tetrahydrofolate polyglutamate[111] or NADPH[112] are chemically unstable. Indeed, many essential metabolites, from bis- and diphosphates to aldehydes, gain their critical functions in metabolism from their inherent chemical reactivity. In addition, classic metabolic

enzymes are not perfect for example, glyceraldehyde-3-phosphate dehydrogenase can inadvertently hydrate NADH into its useless form NADHX[113]. Other enzymes show reaction promiscuity in addition to substrate ambiguity[114], building up unwanted metabolite pools over time. To keep metabolites in their native chemical states serving their metabolic functions in cells, a plethora of helper enzymes called damage control enzymes are required. During aging, the defense system exhibited by such damage repair enzymes may become less effective. Hence, the extent of dysfunctional and damaged metabolites will change over time and become a readout as well as contributor to the accelerated effects of aging.

Even the best-studied genomes (those of humans, mice, nematodes, fruit flies and budding yeast), have numerous genes of unknown function. Notably, many of more recently functionally characterized genes code for repair proteins[115]. Virtually all enzymes exhibit side activities that often represent <0.1 % of the classical activity and even sometimes $<10^{-6}$ for the most specific enzymes. The products generated by side activities have been neglected until recently when it was realized that a new category of enzymes, metabolite repair and clearance enzymes serve to destroy the most important side products and avoid their accumulation, which might otherwise be toxic, causing disease.

An example of a side activity is the production of L-2-hydroxyglutarate by L-malate dehydrogenase and lactate dehydrogenase, two abundant enzymes. Their apparently tiny side activity ($<10^{-6}$ compared to the regular activity) leads to the daily production of grams of L-2-hydroxyglutarate in humans. An FAD-linked mitochondrial enzyme reconverts L-2-hydroxyglutarate to α-ketoglutarate, avoiding its accumulation, which is toxic particularly to the brain. The metabolic disease L-2-hydroxyglutaric aciduria, which is due to inactivating mutations in the repair enzyme, leads to progressive neurodegeneration and increased incidence of brain tumors.

In glycolysis, there seems to be at least as many distinct repair reactions as the 11 classical reactions of glycolysis[116]. This huge diversity of side products related to glycolysis suggests that hundreds and probably thousands of different side products may be formed in cells. It is likely that only some of them are eliminated by repair and clearance enzymes. But at least for those that are eliminated, it is possible to evaluate their potential toxicity in cell-based experiments. Such experiments have shown that some of the side products are indeed extremely toxic.

Clearly, many more defects of metabolite repair exist that contribute to aging. Some recognized metabolite repair diseases manifest early in life, as the consequences of protein-truncating variants in these genes are usually quite severe at that time. Other metabolite repair diseases, such as L-2-hydroxyglutaric aciduria, may be diagnosed later in life (probably due to mutations that do not completely inactivate L-2-hydroxyglutarate dehydrogenase). It is clear that many types of metabolite damage are not fully dealt with by enzymes of metabolite repair. This is particularly true for the damage that occurs at an extremely low rate and gives rise to products that only very slowly accumulate in cells and organs.

Measuring metabolic damage and repair. Nontargeted metabolomics is the best tool to study the extent of buildup of damaged metabolites over time. Here, a combination of assays using LC with high-resolution MS (HRMS) has been shown to identify and quantify >1,000 metabolites in large cohort studies. In addition, nontargeted metabolomics utilizes large databases and new software to annotate unidentified metabolites by their most likely chemical structures, vastly expanding the possibility to find and identify new damaged metabolites. For example, the most classic version of metabolite damage is lipid oxidation (Fig. 2), often induced by ROS that leads to hydroxylated, epoxidated or highly reactive short-chain lipids[117], including 4-hydroxynonenal (HNE)[118]. Lipid peroxidation generates electrophilic compounds, typically containing aldehydes, which form ethenoadducts with DNA, a type of DNA damage. These DNA adducts formed with lipid peroxidation products result in exocyclic additions to DNA bases[119], disrupting transcription at its own site and to adjacent pyrimidine bases. When DNA repair mechanisms fail to detect or resolve these adducts, an incorrect base may be substituted, constituting a mutagenic event. The accumulation of lipid peroxidation products can therefore reflect both a subset of damaged metabolites and a pool of adducts with the capacity to damage other macromolecules, namely DNA and proteins.

Future directions

Damage accumulates with aging at every level, from small molecules to cells. There have been many studies on specific damage types in aging, but remarkably few studies on the role of global damage in aging. This is due, in large part, to the lack of methods to accurately assess many types of damage. However, this has been changing with the development of new omics methods, increased sensitivity of existing methods and the development of computational tools for big data analyses. Many of these methods are discussed in this review and have already found applications in assessing age-related molecular damage.

It is time for systematic studies of the role of damage in human aging as well as aging of model organisms, which could proceed with omics assays and methods that are already feasible in assessing a more sizable fraction of damage (Table 1), especially in blood and biopsy tissues from existing human cohort studies. The association of candidate measurements of damage with chronological and/or biological age is a first step that is missing for almost all assays of damage included in this review. Progress can be accelerated by developing methods to measure damage burden in ways that encompass several kinds of damage, for example applying multi-omics approaches. Because cross-sectional associations with age often underestimate associations with aging, it would be ideal to describe the change in these markers of damage in serial specimens from longitudinal studies. Those measurements and types of damage that change with age would be candidates to test for associations with phenotypes of aging, such as weakness, slowness and declining cognitive performance, Frailty, as assessed by accumulated health deficits, is conceptually well aligned with premise that molecular damage accumulation drives aging[120]. In turn, this would allow the testing of hypotheses that the markers of types of damage predict the incidence of degenerative diseases, such as cardiac, vascular, mobility disability, frailty, dementia and noncancer mortality.

It may be possible to reduce the accumulation of damage with aging by slowing its generation or increasing systems of repair, clearance or replacement of damage. This approach would reduce the risk of many aging-related degenerative diseases. Reduction in the burden of damage (the deleteriome) might also mediate the benefits of treatments that reduce the risk of aging-related conditions. Testing these possibilities will require development, validation and application of an array of measurements of types of molecular damage that play an important role in aging and its consequences.

Data availability

There are no data.

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V.N.G. and S.B.K. were responsible for conception and design, drafting and substantial revisions. S.G.C., B.Z. and T.M. were responsible for drafting, creation of figures and substantial revision. A.M.C., O.F., J.P.M., M.M., R.M., E.V.S., G.T., K.W. and Y.Y. were responsible for drafting and substantial revisions. L.J.N. was responsible for drafting, creation of tables and substantial revisions. S.R.C. was responsible for conception, design, oversight, drafting and substantial revisions.

Competing interests

The authors declare no competing interests.

Additional information

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