**Perspectives to modify and counter aging**

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**Abstract**

The interpretation of aging as an adaptive and programmed phenomenon implies the existence of specific aging-causing mechanisms, genetically determined and regulated, while the opposite thesis, i.e., aging as the gradual accumulation of the effects of harmful factors only partially countered by natural selection, would be falsified by the possible existence of these mechanisms.

The subtelomere-telomere theory of aging offers what is required by the interpretation of aging as a programmed phenomenon. In fact, it is possible to expound the experimentally documented mechanisms that are part of the subtelomere-telomere theory: repression of subtelomeric sequences (TERRA sequences) consequent to the sliding of a telomeric hood over subtelomere in proportion to telomere shortening, epigenetic modifications caused by the repression of the subtelomeric sequences, gradual cell senescence, cell senescence, progressive decline of stem cells, effects of these phenomena over the whole organism.

Evidence against the interpretation of cell senescence and telomerase restrictions as defense mechanisms against cancer is reported. Consequently, the fears that telomerase activation or the elimination of senescent cells are potentially oncogenic factors should be eliminated as preconceived ideas, or limited on the basis of any available evidence in this regard.

With the rationale of the mechanisms expounded regarding the subtelomere-telomere theory, there is the logical deduction of three types of strategies that could be used to modify and counteract the mechanisms of aging: telomerase activation, elimination of senescent cells, restoration of stem cells to the number existing in young individuals.

The limits and the potential effectiveness of these methods, already the subject of active research, are briefly discussed.

**Keywords:** Aging, subtelomere, TERRA sequences, cell senescence, gradual cell senescence, stem cells

**1. Introduction**

Aging, precisely defined as:

- “a general title for the group of effects that, in various phyla, lead to a decreasing expectation of life with increasing age” [1], p. 7;

- “increasing mortality with increasing chronological age in populations in the wild” [2];

- “increasing mortality with age ... actuarial senescence”, in populations studied in the wild [3],

is the subject of two thoroughly opposite general explanations.

According to the first thesis, aging is caused by the gradual accumulation of the effects of one or more harmful factors that natural selection is only partially able to counter. As examples of the harmful factors that would cause aging, there are oxidative effects of free radicals on the whole body [4] / on mitochondria [5] / on DNA [6]. However, within this thesis, aging is a harmful phenomenon and cannot be adaptive or programmed, that is, it cannot be caused by specific mechanisms determined and regulated by genes, and natural selection always tries, with limited effectiveness, to counteract its causal factors and to procrastinate aging.

On the contrary, according to the second thesis, aging is an adaptive phenomenon, favored by selective mechanisms at supra-individual level, and which falls into the general category of phenomena defined as phenoptosis (programmed death of an organism) [7, 8] or also as programmed organismal death (POD) [9]. In nature, there are countless examples of species in which the cycle of life is determined by phenoptotic phenomena (for example, animals that reproduce or plants that bloom and die soon after [10]), which imply the existence of specific mechanisms determining them.

This work does not want to expose or discuss arguments and evidence in support or against the two theses, a topic that can be deepened in other works (e.g., see [11] and chapter 4 in [12]), while the subject proposed is the discussion about the strategies through which it might be possible to oppose aging. However, in order to counteract a phenomenon, it is essential to know the mechanisms underlying the phenomenon as a preliminary matter.

For this purpose, it should be underlined that the interpretation of aging as a programmed phenomenon, as proposed by the second thesis in utter contrast with the other thesis, implies the necessity of the existence of specific aging-causing mechanisms, genetically determined and regulated, and consequently such hypothetical mechanisms should be described and supported by scientific valid evidence.

So, it is necessary to proceed first of all to expound the experimentally documented mechanisms that appear to determine aging, implicitly proving the thesis of programmed aging and disavowing the opposite thesis. Then, on the basis of these mechanisms, it will be possible a logical deduction of the types of strategies that could be used to modify and counter the mechanisms of aging.

**2. The subtelomere-telomere theory of aging**

The general mechanism that will be expounded here as the cause of aging has been defined as the subtelomere-telomere theory of aging [13, 14]. It was proposed as a necessary transformation of the previous telomere theory of aging for which a brief mention is required that sets out both its main features and its inconsistencies with the evidence.

**2.1 – The telomere theory of aging and its limits**

In 1961, it was shown that cells had a previously negated limit in the number of duplications that could be performed [15]. In 1971, Olovnikov observed that DNA replicating enzyme allowed a partial duplication as a small terminal part was not duplicated. This resulted in a progressive shortening of the terminal part of the DNA molecule (defined as telomere), which could be the cause of the limits in cellular duplication [16]. Two years later, to explain the unlimited or numerous duplications of germ line cells and stem cells, respectively, Olovnikov observed that the existence of an enzyme with the ability to restore the non-duplicated part of the DNA was necessary [17]. This enzyme, called telomerase, was isolated in 1985 [18].

Other works confirmed the relationship between telomere length, telomerase activity and cell duplication capacity. In particular, they showed that:

- in human fibroblasts, telomeres shortened in relation to age and number of cell duplications [19];

- in *Tetrahymena* mutant strains with inactive telomerase, cell cultures had a reduced duplication capacity [20];

- in transformed human cell lines, that is, with unlimited capacity for duplications, the activity of telomerase was not restricted [21];

- the activation of telomerase allowed the cells an unlimited capacity for duplication [22].

Furthermore, it was observed that cells could pass from the condition in which they were able to duplicate to a state in which the duplication capacity was blocked and cellular functions were stereotypically altered. This radical change in their functions, to a condition defined as “cell senescence”, was related to telomere shortening and contributed to the aging of tissues and organs [19, 22], and was later described as a “fundamental cellular program” [23].

These empirical data led to the hypothesis that the limits imposed by the gradual shortening of telomeres, i.e.: (i) restrictions in cell duplication capacities and so in cell turnover; and (ii) alterations caused by the increasing number of cells in the condition of cell senescence, were the cause of a progressive decay of body’s functions, that is, the primary origin of all the alterations of aging, a concept that may be well defined as “telomere theory of aging”.

However, if this theory were true, it seemed logical to predict, in the comparison among species, a direct relationship between life span and: (i) initial telomere length (i.e., telomere mean length in germ line cells); and (ii) telomerase activity. This was contradicted by various facts:

- hamsters and mice, in comparison with our species, have much longer telomeres and shorter life spans [24];

- mice, which have a limited life span, for most somatic cells have a baseline activity of telomerase [25].

- two *Mus* strains having different telomere lengths (10 kb and 20 kb) show equal life span and aging rhythms [26], p. 60;

- among the rodents, life span shows no relationship with telomere length [27] and with telomerase activity [27, 28], while telomerase activity and body mass appear related [27];

- cloned animals obtained from somatic cells show the same timing patterns of aging, despite the fact that the original cell of the cloned animal has shorter telomeres than the germ cell of the donor animal (see [29, 30] and [26], p. 60);

- in *Mus* strains with inactivated telomerase, only when telomeres are significantly shortened (i.e., after four [31] to six [32] generations), in protected laboratory conditions, viability and fertility are compromised, while for the previous generations, despite the great difference in telomere lengths, there are no obvious differences.

Furthermore, the theory did not explain how tissues and organs in which there was a prevalence of perennial cells, i.e., cells without turnover and which therefore did not duplicate (e.g., the cerebral cortex and other parts of the central nervous system), aged like other tissues without perennial cells.

The telomere theory of aging, with these clear contradictions between its predictions and evidence, and with the aging of tissues composed mainly of perennial cells not explained, was discredited and became unsustainable. The shortening of telomeres, improperly described even in very recent works as telomere attrition, seemed to be another type of damage that accumulated over time in accordance with the thesis of aging as an inevitable and non-programmed phenomenon.

**2.2 - Preliminary considerations for the subtelomere-telomere theory of aging**

The above-said facts indicated that the telomere theory of aging either had to be radically modified or, as the only likely alternative, telomere shortening could be another of the harmful factors that explained aging as a non-adaptive phenomenon.

However, other empirical data had to be considered or were emerging:

(1) Blackburn observed [33] that the transition from a cell perfectly capable of duplication to a cell in the state of cell senescence, that is, unable to duplicate and with stereotypical alterations, was not an event activated only when a critical telomere shortening was reached. On the contrary, the triggering of cell senescence showed an increasing probability in relation to telomere shortening. In fact, the decline of growth potential in cultures did not show a sudden collapse after a certain number of duplications but a progressive increasing decline of duplication capacity [34, 35]. Blackburn proposed that the telomere was covered by a cap formed by particular molecules (“sequence-specific DNA-binding proteins”) and that the complex consisting of telomere and cap (“DNA-protein complex”) oscillated between two states: (i) “capped” telomere, in which the telomere was protected and the cell was resistant to the activation of cell senescence; and (ii) “uncapped” telomere, in which the bond was at least partly loosened, the telomere was less protected and the cell was vulnerable to cell senescence triggering. This implied that even cells with telomeres not shortened or with a small shortening had some probabilities of cell senescence activation. Furthermore, as Blackburn proposed that the strength of the bond between telomere and cap was reduced in relationship with telomere shortening, this implied that in some way there was a regulation dependent on the length of the telomere.

(2) In yeast, the insertion of a gene in a position very close to the telomere determines its repression: “yeast telomeres exert a position effect on the transcription of nearby genes, an effect that is under epigenetic control” [36]. This repression, defined as “telomeric position effect” [36], was later demonstrated for our species [37] and for other mammals [38]. Furthermore, in the study of a rare human genetic disease (ring 17 syndrome) the telomere position effect has been related to telomere shortening [39].

(3) In yeast, in relation to the telomeric position effect and the study of aging, some important phenomena were observed.

Wild strains show a telomerase that is always active and so the telomeres do not shorten when there is a duplication [40, 41].

Each yeast cell divides into two slightly different cells, mother cell and daughter cell. The cells of the daughter lineage can divide an unlimited number of times [41], while cells of mother lineage can divide to a limited extent (25-35 duplications in about 3 days [42]). In relation to the number of duplications, the cells of the mother lineage show: (i) the progressive accumulation of particular molecules, called extrachromosomal ribosomal DNA circles (ERCs), on the portion of DNA immediately adjacent to the telomere (defined as subtelomere) [43]; (ii) progressive functional alterations; and (iii) an increasing probability of the block of replicative capacity soon followed by apoptosis [44-48].

Yeast *tlc1Δ* mutants have a deficient telomerase activity and, so, both mother and daughter cells show telomere shortening at each duplication. Cells of the daughter lineage, where there is no ERC accumulation as for wild strains, show, in proportion to the number of previous duplications, a reduced resistance to stress and a transcriptome like that of mother cells of the wild strain with the same number of previous duplications [45]. The metabolic alterations shown by the mother cells of wild strains and by the daughter cells of *tlc1Δ* mutant strains could be interpreted in both cases as a consequence of subtelomere repression, a form of subtelomere position effect, which in turn causes alterations in other parts of the DNA. Subtelomeric repression and its effects in the first case are clearly determined by the accumulation of ERC [43], while in the second case, where there is no ERC accumulation, it could be caused by the sliding of a telomere cap over the subtelomere in relation to the progressive shortening of the telomere [26, 49].

(4) In the comparison among species, in addition to the differences in telomere length (which is not related to life span), it is necessary to consider that: (i) there are many telomeres in a single cell (two telomeres for each DNA molecule of which there are two copies in each chromosome; multiplying the number of chromosomes by 4 we have the number of telomeres, i.e., for a species like ours which has 23 chromosomes: number of telomeres = 23 ∙ 4 = 92); (ii) the initial telomere length (i.e., that of the germ cell) of the various telomeres, even in the same DNA molecule, is not equal “... telomere lengths within the same cell are heterogeneous and certain chromosome arms typically have either short or long telomeres.” [50]; and (iii) the differences in telomere length are hereditary and there are similar lengths in monozygotic twins but not in dizygotic twins [51, 52].

The variability and poor phylogenetic stability of the telomere length in the course of evolution is in stark contrast to the extreme phylogenetic stability of the telomeric sequence. In fact, after the discovery that, in a protozoan species, each telomere was a simple sequence of nucleotides (motif) repeated many times (TTGGGG) [53], same years later, it was shown that telomeres of mammals had a motif with a little difference (TTAGGG) [54] and that this sequence was the same for trypanosomes, molds, and other non-mammal vertebrates and organisms [55]. Indeed, the Telomeric Sequence Database [56] shows that the mammalian motif is shared by all vertebrates and also by many species that are very distant phylogenetically (e.g., *Nicotiana tabacum*, common tobacco), and that another similar motif (TTAGG) is present in various phylogenetically distant species (e.g., *Apis mellifera*, honey bee, and *Giardia lamblia*, a protozoan parasite). The extraordinary conservation of the motifs, even among species with very distant common ancestors, indicated that the telomeric structure had a pivotal importance. On the contrary, whatever the role of the telomere with regard to aging, the same data did not indicate that the initial length of the telomere was important.

**2.3 - General description of subtelomere-telomere theory of aging**

To solve the incongruities of the telomere theory of aging, it appeared essential to propose a fundamental role for the subtelomere, that is to say, the portion of DNA that follows the telomere [26, 57]. This proposal was subsequently deepened and called the “subtelomere-telomere theory” of aging [13, 14, 58, 59].

The new proposal was based on the phenomena reported above in 1-4 and on others not mentioned here for the sake of brevity. Its central idea was expressed by Fossel: “... a heterochromatin ‘hood’ that covers the telomere and a variable length of the subtelomeric chromosome ... As the telomere shortens, the hood slides further down the chromosome (the heterochromatin hood remains invariant in size and simply moves with the shortening terminus) ... the result is an alteration of transcription from portions of the chromosome immediately adjacent to the telomeric complex, usually causing transcriptional silencing, although the control is doubtless more complex than merely telomere effect through propinquity ... These silenced genes may in turn modulate other, more distant genes (or set of genes). There is some direct evidence for such modulation in the subtelomere ...” [26], p. 50.

In short, the subtelomere-telomere theory of aging proposed that:

(i) each telomere is covered by a telomeric cap (or hood), which has a size defined in the first cell of the organism, not respecting a fixed size but adapting it to the length of the telomere;

(ii) the size of each telomere does not vary in any subsequent duplication even if the telomere shortens due to insufficient or zero telomerase activity;

(iii) in proportion to telomere shortening, the hood becomes longer than the telomere and the excess part inhibits a growing part of the subtelomere in which there are hypothetical regulatory sequences, which were defined as “r-sequences”. This was also described as the sliding of the hood over the subtelomere. In yeast, for the cells of mother lineage, the repression of the subtelomere was determined by the progressive accumulation of ERCs, while in the cells of the daughter line of the *tlc1Δ* mutants the repression of the subtelomere appeared to have the same mechanism before described;

(iv) the aforementioned r-sequences were also hypothesized to have direct or mediated regulatory effects on other regulatory sequences that caused a general alteration in the functions of the cell;

(v) among the progressive alterations caused by the repression of the r-sequences, there were: (i) gradual alterations of cellular function defined as “gradual cell senescence”; and (ii) reduction of the stability of the telomere-subtelomeric hood complex with consequent increasing probability of activation of the cell senescence program and so an increasing number of cells in replicative senescence.

The subtelomere-telomere theory overcame various difficulties of the telomere theory:

- Even though the telomeres were of different lengths, the cap was modeled on the initial telomere length. The subsequent repression of the subtelomeres depended on the shortening of the telomeres and not on the initial absolute lengths of the telomeres. Consequently, there was no prediction of correlation between initial (mean) telomere length and life span.

- The activation of the cell senescence program was not a function of a critical telomere shortening but depended on the progressive repression of the subtelomere which influenced the stability of the telomere-telomeric hood complex and so the probability of activation of the cell senescence program, in accordance with what envisaged by Blackburn [33].

- In cascade, all the manifestations of aging depended on these phenomena, as will be explained in greater detail below.

However, if the life span is not related to the initial length of the telomeres but to their subsequent shortenings, it is necessary to ask how the great difference in life spans among the various species originates. The subject needs specific studies and it is likely that there are various factors that regulate the life span, including different: (i) subtelomeric structure; (ii) telomerase repression; and (iii) rate of telomere shortening (an inverse relationship between life span and rate of telomere shortening has been demonstrated [60]).

**2.4 - TERRA sequences**

However, the subtelomere-telomere theory, even if it appeared to overcome the difficulties of the telomere theory, had a big weakness. In fact, despite the plausible theoretical motivations about the existence of r-sequences having particular regulatory capabilities, as long as these sequences were only a hypothesis not confirmed by evidence, the whole construction of the theory could prove to be unfounded.

Incredibly, in the unawareness of those who proposed the subtelomere-telomere theory of aging, sequences with the precise characteristics hypothesized for the r-sequences were already known and studied by talented researchers, who in turn, not knowing the subtelomere-telomere theory, did not appear to be aware of the importance of their work in elucidating the mechanisms of aging.

In 1990, two subtelomeric sequences (TelBam3.4 and TelSau2.0) were described [61]. They show conserved regions, which are 1.6 kb and 1.3 kb long, respectively, and are described in detail in [62].

The two sequences, defined (perhaps in a partially misleading way as they presuppose a delimitation of the telomere extended to the adjacent section) as TElomeric Repeat-containing RNA, or TERRA (for brevity, “T-sequences”): (i) are subject to transcription producing RNA sequences (for brevity, “T-transcripts”); (ii) are not coding for proteins; (iii) are present in our species [63, 64], plants [65], Zebrafish and mouse [64], and yeast [66].

Moreover:

- T-sequences are “evolutionarily conserved in vertebrates.” [67], are a general characteristic in eukaryotic cells, and “are emerging as new key players in several important biological processes” [68]. This means that T-sequences have surely a pivotal function from ancestral times;

- The transcription of T-sequences, allowed by the enzyme RNA polymerase II, begins from subtelomeric promoters located on at least two-thirds of chromosome ends [62, 69, 70] and proceeds toward the repeated motifs of the telomere, including some of them in the transcription [63, 64, 71];

- “The vast majority of TERRA-binding sites were found outside of telomeres, mostly in distal intergenic and intronic regions of the genome where TERRA regulates gene expression” [68]. T-transcripts bind to many loci where there are noncoding DNA sequences that appear to have important regulatory functions for the expression of many genes [72, 73];

- “The first human subtelomeric promoters that were identified comprise CpG dinucleotide-rich DNA islands shared among multiple chromosome ends ...” [68];

- “TERRA read coverage was high within subtelomeric regions of nearly all chromosomes ... with targets being as much as tens of kilobases away from the telomeric repeat ... TERRA also bound within internal chromosomal regions and within genes, where it favored introns ... TERRA binds chromatin targets throughout the genome. ... TERRA binds both in cis at telomeres and in trans within or near genes.” [72];

- Evidence show “... significant changes in expression of TERRA targets relative to non-targets after TERRA depletion ..., indicating that TERRA target genes were more likely to be affected by TERRA depletion. ... Interestingly, subtelomeric target genes were consistently downregulated ... Internal target genes could either be up- or down-regulated ... In the mouse ES [embryonic stem] cell genome, we identified thousands of cis and trans chromatin binding sites” [72];

- “Cycling endurance exercise, which is associated with AMPK activation, increased TERRA levels in skeletal muscle biopsies obtained from 10 healthy young volunteers. The data support the idea that exercise may protect against aging.” [70];

- The blockage of T-sequences shows relationship with “defects in the capping function. With telomere-specific probes, DNA FISH analysis of metaphase spreads revealed loss of telomeric integrity after 24 hour TERRA knockdown ...” [72]. In mice, the depletion of T-transcripts in embryonic stem cells appears related to reduced telomere protection [72, 73];

- The inhibition of the transcription of T-sequences triggers the mechanisms for DNA damage response at telomeres [74]. The deletion of the 20q locus determines a collapse of T-transcripts followed by a massive DNA damage response, which appears a “demonstration in any organism of the essential role of TERRA in the maintenance of telomeres” [75];

- T-transcripts are antagonists of ATRX, which is a protein related to alpha thalassemia mental retardation X-related syndrome, and are essential for telomere protection: “TERRA and ATRX share hundreds of target genes and are functionally antagonistic at these loci. Whereas TERRA activates, ATRX represses gene expression. At telomeres, TERRA competes with telomeric DNA for ATRX binding, suppresses ATRX localization, and ensures telomeric stability.” [72].

These experimental data show that T-sequences have the features hypothesized by the subtelomere-telomere theory for r-sequences. In particular, T-sequences:

- are placed in the subtelomeres;

- are widespread even in phylogenetically distant species, appear evolutionarily conserved, and so certainly perform functions of pivotal importance;

- are repressed in proportion to telomere shortening;

- produce transcripts with regulatory functions over other regulatory sequences placed both in their immediate vicinity and in other parts, even distant ones, of the DNA molecule in which they are found, or also in other DNA molecules of the same cell;

- regulate innumerable parts of the genome, directly or indirectly, and therefore influence numberless cellular functions in various ways;

- are essential for the stability of the telomere and of the telomere-telomeric hood complex, and therefore for the probability of activation of the cell senescence program.

Consequently, in the subtelomere-telomere theory, the replacement of the hypothetical r-sequences with the T-sequences appears completely correct.

This strongly contributes to confirming the existence of a mechanism whose action is to progressively alter the cellular functions and, consequently, the overall functions of the organism, i.e., aging. This is a fundamental contribution to the thesis of aging as an adaptive and programmed phenomenon, and, at the same time, poses a serious interrogative to the opposite thesis of non-adaptive aging. In fact, wanting to defend the second thesis, how we explain the presence of T-sequences with essential importance for cellular functions in a position that is most vulnerable to the effects of telomere shortening, i.e., to what is defined as telomeric position effect?

**2.5 - Age-related epigenetic modifications and their relations with T-sequences**

Age-related epigenetic modifications (EMs) of DNA are well known:

- Age and EMs, which vary according to cell type and tissue, are strongly related [76, 77]. Cytosine-5 methylation within CpG dinucleotides (DNA methylation) is the most studied age-related EM [78, 79];

- In embryonic cells and induced pluripotent stem cells (iPSCs), EMs with DNA methylation are practically inexistent, while their frequency increases in proportion to the number of cell duplications [78, 79].

- In our species, the frequency of EMs with DNA methylation is useful in indicators aimed to assess age and the most reliable of these indicators has a correlation value with age equal to 0.96 and an error of 3.6 years [78];

- In a study about 128 mammal species (with life spans between 3.8 and 211 years and a similar range of variation for adult weight) an analogous indicator was proposed with correlation greater than 0.96 and a median relative error less than 3.5% [79];

- The reversibility of age-related EMs is shown by the transformation of adult somatic cells into iPSCs where these EMs are practically inexistent as for embryonic cells [78];

- Age-related DNA methylation in CpG sequences [80-83] is limited to particular parts of DNA molecules, defined as CpG islands (CGIs), where CpG nucleotides are 1 every 10 bp. CGIs constitute about 2% of the entire DNA [76], and often coincide with the start sites of the transcription of genes [84];

- The methylation of CGIs appears in relation with the silencing of the promoters present in them [85], while the demethylation appears to restore promoter expression [86];

- Age-related EMs with DNA methylation for CGIs in some cases appears to be hypomethylation and in others hypermethylation [82, 83, 87, 88];

- In general, CGIs appear so evolutionarily conserved to allow an index, as that before mentioned, which is reliable to assess age and is valid for mammals in general [79];

- DNA methylation is not the only known age-related EM (e.g., we have: “reduced bulk levels of the core histones, altered patterns of histone posttranslational modifications ..., replacement of canonical histones with histone variants, and altered noncoding RNA expression” [89], nucleosome remodeling, histone methylation, changes in histone marks reduction of heterochromatin [90, 91]). However, the two best indicators based on age-related EMs to assess age are the ones mentioned before regarding DNA methylation [78, 79].

About the link between age-related EMs and the effects of T-sequences:

- The CGIs that are in subtelomere “promote transcription of TERRA molecules.” [62];

- “Subtelomeric DNA methylation is ... decreased in conjunction with telomere shortening in Terc (- / -) mice.” [92]

- In mice, there is a relation between telomere shortening and subtelomere methylation [93]. “Furthermore, the abrogation of master epigenetic regulators, such as histone methyltransferases and DNA methyltransferases, correlates with loss of telomere-length control, and telomere shortening to a critical length affects the epigenetic status of telomeres and subtelomeres.” [93];

- “Both healthy controls and sarcoidosis patients showed that long telomeres (>9.4 kb) decrease and short telomeres (<4.4 kb) increase with aging, accompanying relative increases of long telomeres with subtelomeric hypermethylation and short telomeres with subtelomeric hypomethylation. This suggested that the aging-related telomere shortening is associated with the surrounding subtelomeric hypomethylation.” [94]

- In humans, in leukocytes, “... shorter telomeres are associated with decreased methylation levels of multiple cytosine sites located within 4 Mb of telomeres ... significant enrichment of positively associated methylated CpG sites in subtelomeric loci (within 4 Mb of the telomere) (P < 0.01)” [95]. Telomere shortening is related to modifications in gene expression and an increasing risk and gravity of various age-related diseases [95].

- Cell senescence in mesenchymal stem cells (MSCs) is related to some marks of aging as DNA methylation in specific CGIs and trimethylation at particular histone targets [96];

- For the same type of cells, “expansion of MSC has a very consistent impact on DNA-methylation profiles”; “517 CpG sites were consistently differentially methylated in early versus late passages” [96];

- After various duplication, for MSCs, in some CpG sites there is hypermethylation and in others hypomethylation: “almost one third of the CpG sites reveal age-associated changes on DNA methylation, of which 60% become hypomethylated and 40% hypermethylated upon aging.” [97]

These data show clear relations between T-sequences and a series of EMs, and it is unlikely that these EMs are determined by random factors. It is therefore correct to argue that cellular aging, and the consequent general aging of the organism, is an epigenetic phenomenon caused and regulated by the repression of T-sequences. However, the definition of aging as a genetically determined and regulated epigenetic phenomenon should not be considered as a peculiarity of aging among many other functions of the organism.

In fact, the Human Genome and ENCODE Projects showed that “the protein-coding potential of the mammalian genome is extremely limited ... Although only 2% of the genome is coding, >90% is transcribed. This transcriptional activity largely produces long noncoding RNAs (lncRNA), the functions of which have remained mostly unknown.” [72] The number of proteins encoded by genes does not vary much in relation to the complexity of a species. For example, the number of protein-coding genes in our species and in a simple nematode are practically equal [98].

From this, it can be deduced that the “programs” that define any function, physiological organization, and morphological development of an organism are mostly not in protein-coding DNA but in the much longer DNA parts that regulates the whole DNA, including the protein-coding sections. This probably occurs by activating or repressive actions obtained through EMs. Therefore, it is likely that all the functions of an organism are epigenetic phenomena and aging is not an exception to this general rule.

However, this is perfectly compatible with the thesis of aging as a programmed phenomenon while it appears very difficult to explain with the opposite thesis of aging as a consequence of the accumulation of harmful random events.

**2.6 - Gradual Cell Senescence**

According to subtelomere-telomere theory, telomere shortening causes: (i) progressive subtelomeric inhibition with consequent increasing alterations of cellular functions, a phenomenon defined as “gradual cell senescence” [11]; and (ii) increasing probability of cell senescence activation, which determines both replicative senescence and pronounced alterations of cellular functionality.

In a cell culture, and also in a progressively aging tissue or organ, among the cells there will be both cases of cells in gradual cell senescence and cases of cells in the cell senescence state. However, it appears difficult to distinguish how much of the overall functional alterations of the culture is due to one or the other phenomenon. There is a tendency to confuse the cells in gradual cell senescence with those in cell senescence, as if it were the same phenomenon in different degrees: “There is substantial variability in the degree of senescence and few if any fully senescent cells, but a significant degree of altered gene expression within a percentage of partially senescent cells.” [26], p. 148.

In fact, the phenomenon of gradual cell senescence is little known or confused with cell senescence despite the existence of empirical data that clearly support the distinct existence of gradual cell senescence:

- In vitro, a culture of duplicating mesenchymal stem cells (MSCs) shows gradual changes in mRNA expression with “a consistent pattern of alterations in the global gene expression ... These changes are not restricted to later passages, but are continuously acquired with increasing passages.” [99]. Furthermore, in relationship with the number of previous duplications, MSCs show gradual changes in DNA methylation whose measurement can be used to calculate the number of duplications [100-102];

- In a study concerning the consequences of telomere shortening, the authors observed that: “Our results demonstrate that the expression of a subset of subtelomeric genes is dependent on the length of telomeres and that widespread changes in gene expression are induced by telomere shortening long before telomeres become rate-limiting for division or before short telomeres initiate DNA damage signaling. These changes include up-regulation and down-regulation of gene expression levels.” [103].

- In cultures of yeast, a unicellular organism where cell senescence causes immediate apoptosis [44, 46]: (i) cells of the mother lineage of wild strains, in which the subtelomeric repression is caused by ERC accumulation, in proportion to the number of previous duplications show increasing functional alterations and susceptibility to cell senescence [44, 46]; and (ii) cells of the daughter lineage of mutant *tlc1Δ* strains, where telomerase is inactive and telomeres shorten at each duplication, likely with progressive subtelomeric inhibition (in absence of ERC accumulation), in proportion to the number of previous duplications show functional alterations and transcriptome similar to that of cells of the mother lineage of wild strains with the same number of duplications [45].

These phenomena cannot be attributed to the casual accumulation of harmful substances or in any case to other factors that act randomly. In fact, this possibility is contradicted by various experiments that demonstrate the complete reversibility of functional alterations:

- The increasing functional alterations observed for MSCs in proportion to the number of previous duplications are canceled by the reprogramming of these cells in iPSCs [104]. These iPSCs, whatever the source of the cell and the age of the donor, showed the profile of a young cell [104].

- For induced MSCs (iMSCs), “DNA methylation, related to age, was completely erased, and iMSCs reacquired senescence-associated DNA methylation during culture in vitro.” [97]

- It is possible to get from iPSCs induced MSCs that show better cell functions and fewer epigenetic modifications [105].

As gradual cell senescence reduces the functional efficiency of the cell and therefore also of the entire organism to which the cell belongs (in the case of a multicellular organism), the phenomenon should be eliminated by natural selection at the individual level. For its existence, the action of selective mechanisms at a supra-individual level appears necessary, which is perfectly justifiable in the context of a general program aimed at the progressive reduction of survival probabilities, as proposed by the thesis of aging as an adaptive and programmed phenomenon. On the contrary, for the thesis of non-adaptive aging, the phenomenon needs an explanation.

**2.7 - Cell Senescence**

A cell in the condition of cell senescence (senescent cell) is not an old cell but a cell in which a specific cellular program [23] has been activated by various damaging factors (e.g., altered culture conditions, oxidative stress, DNA damage [106, 107]). Moreover, the program is triggered in relation to telomere shortening [108], not when a critical value is reached but with increasing probability in relation to the shortening of the telomere [33].

A senescent cell is characterized by a precise pattern of cellular alterations:

i) replicative senescence [109, 110];

ii) resistance to apoptosis [111, 112];

iii) stereotyped alterations of cellular functions [110, 112, 113] with “profound transcriptional changes” [114];

iv) stereotyped alterations of extracellular secretions, known as senescence-associated secretory phenotype (SASP) [115, 116].

The senescent cells, identified with the expression of p16Ink4a, increase in relation to age both as a fraction of the total number of cells and in their absolute number [117, 118].

This increase is clearly related to the extent of the manifestations of aging and age-related diseases [119, 120]. It has been shown that by selectively eliminating senescent cells there is an improvement and a reduction of these manifestations [120, 121]. Consequently, the selective elimination of senescent cells by means of appropriate drugs, defined as senolytics, today appears to be an important and realistic goal to counteract the manifestations of both aging and age-related diseases [112, 121-123].

For the subtelomere-telomere theory, and more generally for the thesis of aging as an adaptive and programmed phenomenon, senescent cells, which are clearly harmful for the individual, have an effective role in progressively reducing the efficiency of the organism and therefore the ability to survival, i.e., aging. For the opposite thesis, namely the interpretation of aging as the causal accumulation of damage resulting from multiple factors, the phenomenon of cell senescence absolutely requires a specific plausible justification.

The only explanation proposed justifies cell senescence as a general defense against the uncontrolled cell proliferation in cancer since cell senescence determines, among other things, the blockage of duplication capacity [124, 125]. Furthermore, in the awareness that cell senescence in addition to the hypothesized action against tumor proliferation certainly causes various harmful effects, this has been justified as an example of antagonistic pleiotropy, that is, a case in which positive and negative effects are clashing in natural selection [126].

There are various facts that make this justification doubtful or untenable:

- As part of the altered secretions constituting the SASP, there are “myriad factors associated with inflammation and malignancy.” [115];

- In mice, by selectively eliminating senescent cells, it was observed a delayed progression of the manifestations of induced cancer as well as minor age-related manifestations and increased life span [118];

- In humans, a relationship between cancer risk and short telomeres, which increase the probability of cell senescence, has been observed [127, 128];

- In mice, induced telomerase expression did not increase the risk of cancer risk, delayed aging manifestations and increased life span [129];

- “Senescent cells are present in premalignant lesions and sites of tissue damage and accumulate in tissues with age” [130].

- The authors of a work [131], while declare that “Cellular senescence suppresses cancer by irreversibly arresting cell proliferation.”, then observe that in cancer therapies “several chemotherapeutic drugs induce [cell] senescence”, and that “Eliminating TIS [therapy-induced senescent] cells reduced several short- and long-term effects of the drugs, including ... cancer recurrence ...”

- With a similar contradiction, the authors of another work [132], on the one hand declare that cell senescence is a “potent cancer-protective response to oncogenic events”, and then propose a model in which cell senescence is related to “an inflammatory phenotype and cancer”.

- In yeast, cell senescence determines immediate apoptosis [133]. As cancer is impossible in a monocellular specie, cell senescence cannot have any anti-cancer significance for species as yeast.

Other facts and arguments against this justification are discussed in [59, 134].

An outline of what has been expounded so far regarding the subtelomere-telomere theory of aging is illustrated in Fig. 1.

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Figure 1 – Scheme of the subtelomere-telomere theory. The T-sequences, which are progressively repressed by telomere shortening and the consequent sliding of the hood over the subtelomere, act by their transcripts (T-transcripts): (1) on near regulatory subtelomeric sequences; (2) on regulatory sequences in other parts of the chromosome; (3) on the equilibrium between capped and uncapped conditions of the telomere. The T-transcripts have analogous actions on other DNA molecules of the same cell (1', 2', and 3'). The actions 1, 1', 2, and 2’ determine modifications of the regulations of numberless genes and of other regulatory sequences and so cause progressive alterations of cellular functions (gradual cell senescence). The actions 3 and 3' reduce the vulnerability of the cell to the triggering of cell senescence program (replicative senescence + stereotypical alterations of cellular functions + resistance to apoptosis).

**2.8 - The telomeric heterochromatin hood**

The topic of the heterochromatin hood or cap that covers and protects the telomere is discussed in detail, as an important element of subtelomere-telomere theory, in [14] and to avoid repetitions only an essential point will be mentioned.

The structure of the telomeric hood can be deduced from the fact that the telomere is covered by several copies of the shelterin protein complex, which is well known both in its main components (proteins TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 [135, 136]) and in the likely arrangement of these proteins [135].

The subtelomere-telomere theory requires that: (i) the cap must have for each telomere a size determined in the first cell of the organism according to the length of the telomere, which varies greatly from telomere to telomere even in the same cell; and (ii) this size must not vary in subsequent cell duplications even when the telomere is shortened.

If the theory is true, a possible prediction is that the cellular amount of shelterin proteins should not be related to the total length of the telomeres, which vary in relation to the number of duplications, but should be constant. On the contrary, if the size of the hood changes in proportion to telomere shortening, it is expected a reduction in the amount of shelterin proteins that should be proportional to the shortening.

There is some evidence that supports the hypothesis of the invariability of the size of the telomeric hood: “We used quantitative immunoblotting to determine the abundance and stoichiometry of the shelterin proteins in the chromatin-bound protein fraction of human cells. The abundance of shelterin components was similar in primary and transformed cells and was not correlated with telomere length.” [137]

**2.9 - Cell turnover and the limits determined by aging**

Our body is largely made up of cells in continuous turnover. Cells die either from necrosis caused by accidental events or by mechanism genetically regulated of self-destruction, each of them defined as “programmed cell death” (PCD). Some cell turnovers determined by PCD are known for a long time [138] (e.g., cells of the intestinal villi detach at the top of intestinal villi and are substituted by new cells obtained from stem cells in the intestinal crypts). Apoptosis, an important type of PCD, is an ordered process of cellular self-destruction discovered quite recently, does not cause inflammation and releases cell parts that can be used by other cells [139]. Apoptosis is also shown by unicellular eukaryote species (e.g., *S. cerevisiae* [140]), is the main form of PCD that allows the turnover for many cell types in adult normal tissues and has been shown for most cell types (e.g., gliocytes [34]; hepatocytes [141]; chondrocytes [142]).

The rate of cell turnover varies greatly according to cell type [143]. Indeed, for some cell types the turnover is very slow (e.g., about 4.5 years for cardiac myocytes [144] and 10 years for bone [145]) while for other cell types it is very rapid. (e.g., 3-6 days for intestinal epithelium [145]).

A simplistic interpretation of the subtelomere-telomere theory would indicate that stem cells actively reproducing to allow cell turnover should originate cells with telomeres increasingly shortened in proportion to the speed of turnover and that an aged organism should have in most of its cells critically short telomeres. The experimental data display a more complex situation:

- A review about the reduction of telomere length over the years in humans [146] showed an annual reduction in many cell types. However, only a few cell types showed a potentially critical reduction in telomere length. For example, hepatocytes had a yearly reduction rate of 120 bp/year, passing from a telomere length of 13.7±2.5 kbp in neonates to 8.7 ± 1.4 kbp in centenarians. For most cell types the telomere length reduction rates were lower, mostly within 20-60 bp/year, while for cell types with minimal or no turnover (e.g., myocardiocytes and neurons of the cerebral cortex, respectively) there was no detectable reduction in telomere length [146]. Overall, this appeared in contrast with the homogeneity of aging in the whole organism.

- Telomere lengths are similar in different cell types of the fetus [147];

- The rates of telomere shortening were found to be similar in four types of cells and tissues (subcutaneous fat, leukocytes, skin, and skeletal muscle) despite the quite different rates of cell turnover [148];

- In hematopoietic stem cells, telomere length was shorter than that of somatic cells in tissues with low turnover [148];

For these apparently contradictory facts, a satisfactory explanation was proposed. In a first phase (expansive phase), in the growth period, the stem progenitor cells of various cell types actively proliferate, originating second level stem cells, in proportion to the subsequent necessary rates of cell turnover (for example, high rates for hematopoietic cells and minimal rate for myocardiocytes) and this corresponds to a reduction in telomere length proportional to the degree of expansion. Then, the second level stem cells originate the somatic cells with a relatively constant reduction of telomeres [148].

The explanation for the decline in cell turnover would originate not from the reduction of telomere length to critical levels but from the fact that stem cells are vulnerable to the transition to cell senescence in which replication is blocked [33]. This gradually depletes the pool of stem cells and slows down the rate of cell turnover.

Regarding the aging of tissues / organs in which the main cells have no turnover, the possible explanation was proposed in two works [149, 150], where there is a detailed discussion and the necessary references.

In short, perennial cells are dependent for their viability on other cells that are subject to turnover. The decline of these satellite cells causes the served cells to decline and then die. The best documented example is that of the photoreceptors of the retina, which are a highly differentiated type of neurons. For their vitality, photoreceptors depend on the cells of the retinal pigmented epithelium, a highly differentiated type of gliocyte that is subject to turnover. The decline of these cells determines functional alterations and then death of photoreceptors, causing aging of the retina and age-related macular degeneration as the main clinical manifestation. Similarly, the decline of specialized gliocytes (microglia cells, oligodendrocytes, astrocytes), which serve neurons and their axons, determines decline and death of the neurons, resulting in the aging of nervous tissue and disorders such as Alzheimer’s disease and Parkinson’s disease as clinical manifestations.

Another perennial structure that depends on cells with turnover is the eye crystalline lens, which for its trophism depends on the cells of the lens epithelium. The decline of these cells results in cataracts. Conversely, not all neurons are perennial. For example, olfactory receptor cells, a specialized type of neurons, are subject to turnover and age similarly to other cells with turnover [149].

**2.10 - The atrophic syndrome**

The phenomena outlined above (age-related epigenetic modifications, gradual cell senescence, cell senescence, decline of cell turnover) progressively determine an “atrophic syndrome”, either directly on the cells subjected to turnover (“direct” aging) or indirectly on the cells or structures not subject to turnover but dependent on other cells subject to turnover (“indirect” aging) [12, 49, 151]. In short, the atrophic syndrome is characterized by:

- increase in the fraction of cells in gradual cell senescence, which is more or less accentuated depending on the degree of telomere shortening;

- increase in the fraction of cells in cell senescence, that is, with modified cellular functions, cell secretions altered according to a specific pattern (SASP), and replicative senescence;

- reduction of the rate of cell turnover due to the progressive decline of the stem cells;

- as a consequence of the declining cell turnover, reduction in the number of specific cells of a tissue, hypertrophy of the remaining specific cells, and replacement of specific cells with non-specific cells;

- as a consequence of the decline of specific tissue cells, the SASP of senescent cells and the altered functions of cells in gradual cell senescence, there are alterations of the intercellular fluid and of the cells that depend on the functionality of the senescent or missing cells;

- decline and alterations of perennial cells as a consequence of the decline and functional alterations of the cells on which they depend;

- as a consequence of the aforementioned progressive alterations of cells and tissues, anatomical and functional alterations of the organs and the overall functions of the organism;

- as a consequence of the reduction of telomere length, possible telomere instability and increased risk of cancer [152, 153]. It should be underlined that cancer risk and cancer are described as a possible consequence of telomere shortening and not as an evolutionary justification for telomere shortening or aging.

These alterations, exposed more fully in [12], Chapter 6 - Aging in the human species, are summarized in Figure 2.

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Figure 2 - A general scheme of the alterations produced by the atrophic syndrome in the various organs and tissues and that constitute the substrate of the progressive decline in survival capacity, i.e., aging. In the scheme, there is the distinction between “direct” and “indirect” aging (see text).

**2.11 - Distinction between aging and age-related pathologies**

It is important to emphasize and always remember the distinction between the physiological process of aging and some diseases caused by other factors (for example, smoking, excess of salts or calories or fats in the diet). As the effects and seriousness of these troubles increase over the years, they are defined as age-related diseases and often confused with the aging process. Moreover, with similar lack of distinction, the remedies adopted to combat these diseases are described improperly as anti-aging cures. However, these diseases often constitute an acceleration and aggravation of the physiological process of aging and consequently the methods that counter them should be described as cures against the pathological acceleration of aging and not as anti-aging therapies.

As example, it is useful to consider an interesting study [154], which showed: (i) endothelial cells are originated by duplication of particular cells, the endothelial progenitor cells (EPCs), from the bone marrow; (ii) the number of EPCs was negatively correlated with age and various known risk factors for cardiovascular diseases (diabetes, hypertension, smoking, hypertension, overweight and obesity); (iii) furthermore, the decline in the number of EPCs and the so-called Framingham risk score [155] had an equal predictive value for the evaluation of cardiovascular risk.

The authors suggested that: “continuous endothelial damage or dysfunction leads to an eventual depletion or exhaustion of a presumed finite supply of endothelial progenitor cell ... continuous risk-factor-induced injury may lead to eventual depletion of circulating endothelial cells” [154].

In fact, the authors showed that in physiological aging there was a gradual decline of endothelial cells with consequent possible cardiovascular diseases at older ages and that this decline together with the pathological consequences was accentuated by various risk factors and limited by some drugs. A careful review of the scientific literature showed that, in general, between the characteristic changes of aging and the decline of endothelial cells there was a parallelism: risk factors and drugs protective for endothelial cell function, as statins, angiotensin-converting-enzyme inhibitors (ACE-i) and sartans or angiotensin II receptor blockers (ARBs), were also risk factors and protective drugs, respectively, for other alterations of aging [12] (see Table 1).

On the one hand, this shows us a substantial great uniformity in the manifestations of the aging process and in the possible pharmacological remedies, but at the same time it indicates us the distinction between the physiological process of aging and its pathological acceleration caused by other factors.

Table 1 – Relations between aging dysfunctions and the harmful effect of “risk factors” or, on the contrary, the beneficial effects of “protective drugs”. This table has been inspired by Tables 7.8 and 8.2 in [12] appropriately modified. The references on which the table is based (about 400) are reported in the same work and are omitted here for the sake of brevity.

|  |  |  |
| --- | --- | --- |
|  | Effect on the risk by: | Protectiveeffect by: |
| Dysfunctionsin the elderly | Age | Hypertension | Diabetes | Smoking | Obesity / dyslipidemia | Moderate alcohol use | Alcohol abuse | Statins | ACE-i /ARBs |
| Endothelial dysfunction | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| Alopecia | **+** | **+** | **+** | **+** | **+** | **+?** | **+** | **.** | **.** |
| Atrophy of oral mucosa and salivary glands | **+** | **+?** | **+** | **+** | **.** | **.** | **+** | **.** | **.** |
| Atrophy of other sensory neuronal cells with turnover | **+** | **.** | **+** | **+** | **.** | **.** | **+** | **.** | **.** |
| Cardiac insufficiency and related diseases | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| Diabetes and impairment of glucose tolerance | **+** | **+** |  | **+** | **+** | **-** | **+** | **-/** | **+** |
| Emphysema and related diseases | **+** | **+** | **+** | **+** | **-** | **-** | **+** | **+** | **+** |
| Hepatic atrophy and related diseases | **+** | **.** | **+** | **+** | **+** | **.** | **+** | **-/** | **+** |
| Intestinal and gastric atrophy | **+** | **.** | **.** | **+** | **.** | **.** | **.** | **.** | **.** |
| Muscle atrophy | **+** | **.** | **+** | **+** | **+** | **.** | **+** | **-** | **+** |
| Olfactory dysfunction | **+** | **+** | **+** | **+** | **+** | **.** | **+** | **+** | **.** |
| Osteoporosis | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| Renal insufficiency | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| Skin atrophy | **+** | **.** | **+** | **.** | **.** | **.** | **.** | **+** | **+** |
| Testicular atrophy | **+** | **.** | **+** | **+** | **+** | **/** | **+** | **.** | **/** |
| Age-related macular degeneration\* | **+** | **+** | **+** | **+** | **+** | **-/** | **+** | **?** | **-?** |
| Alzheimer's disease\* | **+** | **+** | **+** | **+** | **+** | **-** | **+** | + | + |
| Cataract\* | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+?** | **+** |
| Hearing impairment\* | **+** | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| Parkinson's disease\* | **+** | **+/** | **+** | **-** | **+** | **-** | **+** | **+** | **+** |

Notes:

+ = increased risk or protective effect;

- = decreased risk or protective effect;

/ = unaltered risk or protective effect;

? = doubtful results;

. = no specific studyfound;

\* = cases of “indirect” aging.

**3. Possible strategies to control aging**

The knowledge of the mechanisms from which the progressive age-related alterations of the organism originate, allows the formulation of three main rational strategies to counteract aging.

**3.1 - Telomerase activation**

The use of methods aimed to telomerase activation must preliminarily overcome the obstacles generated by the idea that telomerase is potentially oncogenic and therefore its activation is an oncogenic risk to be avoided [125, 126, 156]. This idea is contradicted by various facts:

- Old individuals of animal species, as rainbow trout and lobster, with no age-related decline of survival capacity in the wild (“animals with negligible senescence” [10]), show the same telomerase activity of young individuals [157, 158], and, as they have a constant mortality at any age, cannot have an age-related increasing mortality due to cancer;

- Gradual cell senescence is determined by subtelomere inhibition consequent to telomere shortening when telomerase is inactive or partially active (see the section before dedicated to gradual cell senescence) and has no likely activity against cancer onset;

- An increasing number of cells in gradual cell senescence or in cell senescence determines a progressive weakening in the efficiency of immune system efficiency [26], which increases vulnerability to cancer and cancer incidence [159];

- Shortened telomeres causes telomere dysfunction and an increasing probability of cancer onset [152, 153, 160];

- Telomerase activation is a common feature in cancer and is clearly a cancer aggravating phenomenon, but is subsequent to cancer onset and must not be considered a cause of it [26];

- “... short telomeres can actually enhance early steps in tumor formation ... telomerase inhibition could be mutagenic in tumor cells, a lesson that should be held firmly in mind if antitelomerase treatment were being considered as a chemopreventive strategy or were to be used chronically.” [161].

Furthermore, despite the feared oncogenic risks caused by telomerase, as early as 1998 some in vitro experiments demonstrated the positive effects of telomerase activation:

- “... two telomerase-negative normal human cell types, retinal pigment epithelial cells and foreskin fibroblasts, were transfected with vectors encoding the human telomerase catalytic subunit. In contrast to telomerase-negative control clones, which exhibited telomere shortening and senescence, telomerase-expressing clones had elongated telomeres, divided vigorously, and showed reduced straining for beta-galactosidase, a biomarker for senescence. Notably, the telomerase-expressing clones have a normal karyotype and have already exceeded their normal life-span by at least 20 doublings, thus establishing a causal relationship between telomere shortening and in vitro cellular senescence.” [22]

- “... reactivation of telomerase in normal human cells leads to restoration of the length of telomeric DNA and to a highly significant increase in cellular life span. These data provide strong evidence consistent with the telomere hypothesis and indicate that elongation of telomere length by genetic manipulation might render normal human cells virtually immortal.” [162]

Similar experiments [163, 164] confirmed these results, namely that the activation of telomerase kept cells in a “phenotypically youthful state” [22]. Subsequent experiments widened these results:

- Fibroblasts aged *in vitro* and that showed “substantial alterations in gene expression” were treated with telomerase and “assessed by incorporation into reconstituted human skin”. The reconstituted skin appeared identical to that obtained using young fibroblasts [165].

-- In aged mice with blocked telomerase, and so showing short dysfunctional telomeres and typical degenerative phenotypes, telomerase reactivation extended telomeres and eliminated “degenerative phenotypes across multiple organs including testes, spleens and intestines. Notably, somatic telomerase reactivation reversed neurodegeneration with restoration of proliferating Sox2(+) neural progenitors, Dcx(+) newborn neurons, and Olig2(+) oligodendrocyte populations. Consistent with the integral role of subventricular zone neural progenitors in generation and maintenance of olfactory bulb interneurons, this wave of telomerase-dependent neurogenesis resulted in alleviation of hyposmia and recovery of innate olfactory avoidance responses.” [166]

- In normal one- and two-year-old mice, telomerase induced by adeno-associated viruses carrying the telomerase reverse transcriptase delayed aging and increased median life span of 24 and 13%, respectively, and “... had remarkable beneficial effects on health and fitness, including insulin sensitivity, osteoporosis, neuromuscular coordination and several molecular biomarkers of aging. Importantly, telomerase-treated mice did not develop more cancer than their control littermates ...” [129].

The activation or reactivation of telomerase, or the stimulation of its degree of activity, in general can be achieved through two methods.

The first is the use of specific drugs. The astragalosides, substances originated from plants, have some activity in stimulating telomerase expression [167, 168]. However, astragalosides are quite expensive and their effectiveness appears limited [169]. Other substances capable of stimulating telomerase activity are under consideration: “This study aimed to investigate the effect of natural compounds on telomerase activity in human peripheral blood mononuclear cells (PBMCs). The tested compounds included *Centella asiatica* extract formulation (08AGTLF), Astragalus extract formulation (Nutrient 4), TA-65 (containing *Astragalus membranaceus* extract), oleanolic acid (OA), maslinic acid (MA), and 3 multi-nutrient formulas (Nutrients 1, 2 and 3) at various concentrations. The mean absorbance values of telomerase activity measured following treatment with some of the above-mentioned formulations were statistically significantly higher compared to those of the untreated cells. In particular, in order of importance with respect to telomerase activation from highest to lowest, 08AGTLF, OA, Nutrient 4, TA-65, MA, Nutrient 3 and Nutrient 2, triggered statistically significant increase in telomerase activity compared to the untreated cells.” [170]

The second type of method is the one used in two studies mentioned above [129, 166], namely the expression of telomerase induced by adeno-associated viruses carrying the telomerase reverse transcriptase (TERT).

After the mandatory and precautionary procedure required for any healthcare method, for each subject its application should be repeated over time and would have potential beneficial effects:

- restoration of full functional activity for cells in gradual cell senescence;

- reduction of the frequency of passage of stem cells to the condition of cell senescence, with a consequent reduced decline in cell turnover.

The activation of telomerase, however, would have no effect on the number of senescent cells nor could it restore the number of stem cells that are present in young individuals.

**3.2 - Elimination of senescent cells**

The main features of cell senescence have been exposed before in section 2.7. With regard to the elimination of senescent cells to oppose both aging and age-related diseases, the following should be added:

- In mice, the transplantation of senescent cells around the knee joints determined alterations resembling the osteoarthritis, an age-related pathologic condition [171];

- The elimination of senescent cells through inactivation of p16Ink4a appears to reduce and improve aging manifestations [120, 121, 172];

- In general, cell senescence is described as a phenomenon characterized by a single phase and which is irreversible once activated. However: (i) when cell senescence is triggered by certain types of stress, in a first phase the phenomenon is reversible if the stress condition is eliminated or reduced; and (ii) cell senescence is reversible by artificial manipulations in vitro, (e.g., by inactivation of both p53 and p16Ink4a) [173]. This confirms that cell senescence is a regulated process, i.e., a cellular program [23], but the manipulations performed in vitro do not appear to be easily and usefully applicable in vivo. Consequently, the easiest way to achieve useful results appears not to bring senescent cells back to their normal condition but to eliminate senescent cells using appropriate drugs (defined as senolytic drugs).

It should be considered that senescent cells have their functions strongly altered and that this should usually trigger their elimination through the mechanism of apoptosis. This does not happen because, as part of the cell senescence program, there is the up-regulation or activation of particular Senescent Cell Anti-apoptotic Pathways (SCAPs), which inhibit the activation of the apoptosis mechanism. These SCAPs comprise BCL-2 / BCL-XL, PI3K / AKT, tyrosine kinase, p53 / p21 / serpines, and other pathways, which can be mutually related and can constitute broad targets for drugs capable of blocking the aforementioned SCAPs and consequently activating the apoptosis of senescent cells [112].

Dasatinib and quercetin were the first drugs that proved highly effective in eliminating senescent cells [122]. The two drugs act by inhibiting different SCAPs (dasatinib inhibits several tyrosine kinases, while quercetin acts on PI3K and some kinases and serpines, i.e., protease inhibitors) and each of them is more effective for different cell types. Consequently, the combined use of the two drugs, defined as DQ, was proposed and tested on aged mice with positive results (improvement of daily activity, walking speed, muscle strength, and food intake) [122]. DQ also appeared to attenuate various age-associated conditions (e.g., cardiovascular dysfunction) [122].

Subsequently, DQ:

- was used for the treatment of idiopathic pulmonary fibrosis in mice. This improved pulmonary function and physical health, while lung fibrosis was not modified [174];

- has been studied in clinical trials on patients with chronic kidney disease, diabetes, idiopathic pulmonary fibrosis, and on survivors after the transplantation of hematopoietic stem cell [175].

Other natural or synthetic drugs have been proposed as senolytics [176, 177], e.g.:

- navitoclax, the BCL-XL inhibitors, ABT737, A1331852 and A1155463, which target the Bcl 2 family of anti-apoptotic factors, and fisetin, a quercetin-related flavonoid that shows less haematological toxicity than navitoclax [178];

- a senolytic compound (UBX0101) tested with positive results in transgenic mice for post-traumatic osteoarthritis [179].

- in mice with doxorubicin-induced chemotoxicity, in naturally aged mice, and in fast aging XpdTTD/TTD mice, a FOXO4 peptide that alters the interaction of FOXO4 with p53. The substance “neutralized doxorubicin-induced chemotoxicity ... restored fitness, fur density, and renal function” [180];

- the small-molecule ABT-737 and siRNAs, which act by inhibition of the anti-apoptotic proteins BCL-XL and BCL-W and have been tested in transgenic p14(ARF) mice, obtaining positive results for epidermis and lung epidermis damage [181].

- some drugs that inhibit the chaperone Heat Shock Protein 90 (HSP90) [123]. The inhibition of HSP90 appears to reduce the resistance of senescent cells to apoptosis [182], an effect tested also for cancer treatment [182].

Although several clinical trials are ongoing, to date no senolytic drugs are approved for clinical use. There is a strong need to obtain senolytic drugs that are highly selective for the elimination of senescent cells and that do not have significant side effects or contraindications. By eliminating the harmful consequences of senescent cells, senolytic drugs can reduce the manifestations of aging and also be very useful for the treatment of many age-associated diseases.

It should be remembered, however, that senolytic drugs have no action on telomere shortening nor do they solve the problem of the progressive reduction of stem cells and the consequent decline in cell turnover.

The combined use of methods to activate telomerase and drugs to eliminate senescent cells would add up the advantages of the two approaches, but would not avoid the decline in cell turnover.

**3.3 – Restoration of the number of stem cells to that of young individuals**

The decline of cell turnover is the consequence of the progressive reduction in the number of stem cells, which in turn is caused by their random passage to cell senescence state that does not allow duplication. This passage has a probability correlated with telomere shortening, and it is not zeroed even when the shortening is minimal or zero [33].

The activation of telomerase lengthens the telomeres and reduces the probability of transition to cell senescence state but does not restore the number of stem cells to that of a young individual.

Therefore, to counteract the alterations of aging, it appears essential to bring the number of stem cells back to that of young ages. As previously mentioned in section 2.5 (Age-related epigenetic modifications and their relations with T-sequences), it is possible to reprogram in vitro adult somatic cells into induced pluripotent stem cells (iPSCs) where the epigenetic modifications are practically inexistent as for embryonic cells [78] and reprogram mesenchymal stem cells (MSCs) into iPSCs [104].

As in vivo reprogramming somatic cells or MSCs into iPSCs does not appear feasible or practical, a possible procedure could include: (i) the removal of somatic cells; (ii) their reprogramming into iPSCs; and (iii) their reintroduction into the body.

Obviously, with all the experiments and precautions required for a health cure, this procedure, applied periodically, could integrate telomerase reactivation and elimination of senescent cells procedures in order to counteract aging.

**3.4 - Problems not solved by the aforementioned strategies**

There are characteristics of aging that are not resolved by the application, even if repeated, of the aforementioned three strategies:

--- The atrophic syndrome also gradually causes irreversible morphological and functional changes. For example, bones weaken to the point that in some parts they collapse and the central nervous system suffer irreversible localized injuries. The three aforementioned strategies cannot reverse these anatomical-functional alterations. However, early and repeated applications should prevent them from being reached.

--- Teeth are structures described as perennial. More precisely, in our species the teeth are renewed only once passing from deciduous dentition to permanent dentition. The limit of two dentitions is by no means mandatory for evolution. Indeed, among vertebrates there are species with many successive dentitions: for example, elephant has six dentitions [183] and alligators can substitute up to 50 times their teeth [184]. The only permanent dentition of our species may appear completely insufficient. However, in natural conditions the permanent teeth remain in good condition even in old age, while in modern conditions, despite the aid of sophisticated dental care, in the elderly the teeth are generally in poor condition. An extraordinary study of 1939 [185] shows us how in prehistoric times and in populations living in primitive conditions, caries and other affections of the teeth are practically absent while the very bad teeth conditions frequent in modern times are the consequence of diets profoundly different from that of the prehistoric era. E.g.: “... there are some excellent collections of skulls in museums in Peru, with the skulls in position where they can be readily studied for the shape of the dental arches. When we have in mind that from 25 to 75 per cent of individuals in various communities in the United States have a distinct irregularity in the development of the dental arches and facial form, the cause and significance of which constitutes one of the important problems of this study, the striking contrast found in these Peruvian skulls will be seen to constitute a challenge for our modern civilizations. In a study of 1,276 skulls of these ancient Peruvians, I did not find a single skull with significant deformity of the dental arches.” [185], p. 203; “Another important source of information regarding the Aborigines of Australia was provided by a study of the skeletal material and skulls in the museums at Sydney and Canberra, particularly the former. I do not know the number of skulls that are available there for study, but it is very large. I examined many and found them remarkably uniform in design and quality. The dental arches were splendidly formed. The teeth were in excellent condition with exceedingly little dental caries.” [185], p. 164.

One possible strategy is to adopt lifestyles that, by imitating those of primitive life in natural conditions, best preserve the teeth. To complement this strategy, the aforementioned three strategies would restore to their youthful conditions the parts of the tooth subject to turnover (dental pulp, gums, mucous membranes of the mouth).

Alternatively, or complementing this strategy, there are the solutions or palliatives offered by modern dentistry. As a distant prospect in the future, a perfect knowledge of the genetic regulation originating the morphological mechanisms of dentition will perhaps make possible, necessarily overcoming strong ethical obstacles, specific genetic modifications that will allow multiple dentitions.

--- The crystalline lens grows continuously and this among other things leads to the inability to focus on nearby objects (presbyopia) [186]. The aforementioned procedures would act on the epithelium of the lens and this would avoid the biochemical alterations of the lens body that cause cataracts [187]. However, the same procedures could not stop the growth of the lens and avoid presbyopia. In the future, it could be useful to replace the crystalline lens with an artificial lens with elastic capacity such as to allow accommodation for near vision.

**4. Conclusion**

Aging is traditionally considered a complex degenerative phenomenon that is difficult and largely useless to counter. The opposite interpretation explaining aging as a phenomenon that is physiological, adaptive and programmed, and so determined and regulated by genes, finds extraordinary support and confirmation in the mechanisms previously exposed and synthetically defined as subtelomere-telomere theory of aging.

These aging-determining mechanisms show characteristics on which actions to modify their effects are possible. These actions have been set out in their general lines and will certainly require a great many studies and experiments to become practical, reliable and safe applications.

In a very recent past, perhaps still current for many, conceiving the modification or reversal of the aging process was the object of vacuous aspirations or matter for astute charlatans. Today, more and more the aim of a modification or even a regression of aging appears as something possible on which it is useful and rational to invest time, intelligence and resources.

Furthermore, as a positive collateral action of enormous importance, the strategies indicated to combat aging should also have great efficacy for the treatment of age-related diseases that are nowadays insufficiently treated or cured with symptomatic therapies and palliatives.

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**Ethical Approval and Informed consent**

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**Consent for Publication**

Not applicable.

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