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Original article

The role of the low density lipoprotein receptor related protein 1 (LRP1) and the receptor for advanced glycation end products (RAGE) in the ageing process

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Abstract

The process of ageing is currently seen as chronic progressive, unstoppable and irreversible changing of the living being's structures and functions, which leads to the decline of its adaptive capacities and increased risk for injuries, diseases and death. The ageing process can be broken down into two closely interwoven components: biological, *i.e.* normal or physiological ageing, driven by a defined epigenetic/genetic program, and pathological or accelerated ageing, defined by the influence of primarily chronic uncontagious diseases. Presently, a number of theories are trying to answer the crucial questions, how and why this inevitable and complex biological process takes place in all living beings. On one side there are different variations of the programmed ageing theory, and on the other side there are ageing theories based on chronic damage of organism structures and functions that through time lead to irreparable damage, decline of adaptive capacities, weakening of a number of vital functions and in the end inevitable death. It is important to mention the currently actual theories: theory of reactive oxygen species effects (ROS), theory of long protein molecule cross-linking (cross linking theories), mutation theory, autoimune theory, theory of free radical effects (Harman's free radical theory of ageing), non-enzymatic glycation theory of ageing (advanced glycation end product [AGE] compounds), and the above mentioned programmed ageing theory. The most recent experimental research emphasizes epigenetics as the crucial factor in this compound, unstoppable and essentially purposeful process. The close connection between the process of ageing and Alzheimer's disease (AD), and the role of low-density lipoprotein receptor-related protein1 (LRP1) receptor, and the receptor for AGEs (RAGE) in this degenerative disease imply the question of the possible role of these two outstanding multifunctional receptors with numerous ligands in the ageing process. The LRP1 receptor is particularly sensitive to epigenetic factors. Due to abundant methylation, its gene (location 12q13.3), is suppressed with the transcription drop, accompanied by the disorder of its many functions, especially those linked to the drainage of harmful, toxic macromolecules from the brain. The continuing strong DNA methylation of the LRP1 gene promoter progressing during the life cycle, could be the crucial factor in the weakening of numerous processes, all of which have an impact in the accelerated ageing course of living beings. The exceptionally weak methylation of the RAGE promoter, also has a strong impact on the ageing acceleration. This study primarily deals with the essence of the normal biological, *i.e.* physiological ageing, and tries to avoid as much as possible the impact of the other component, pathological ageing.

KEY WORDS: biological and pathological ageing, ageing theories, Alzheimer's disease, LRP1 receptor, RAGE (Receptor for AGEs), epigenetics

Introduction

Decreased clearance from the brain of a number of potentially toxic metabolites (for example: glutamate, Amyloid β [Aβ], advanced glycation end products [AGEs]), could be a possible reason for pathophysiological events which can result in the acceleration of fundamental processes included in the ageing of the brain. The tight link between Alzheimer's disease (AD), which is marked by an accelerated rate of ageing, and decreased low-density lipoprotein receptor-related protein 1 (LRP1) receptor expression, lead to the logical conclusion that the LRP1 receptor expression decline could be the crucial factor in the brain ageing, perhaps even in the normal, physiological ageing of the whole body [1-4]. LRP1, a multifunctional endocytic and cell signalling receptor, a large endocytic receptor for more than 50 ligands, is dominantly expressed on the abluminal side of endothelial cell membranes of the blood brain barrier (BBB). It is composed of 515 kDa extracellular α-chain and 85 kDa intracellular β-chain noncovalently connected. Its crucial function in the brain is endocytosis, transcytosis and exocytosis of the mentioned toxic metabolites, among them the amyloid beta peptide (Aβ) [5]. Of all its ligands, it is necessary to mention the following: amyloid precursor protein (APP), bacterial by-products, tissue

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plasminogen activator (tPA), plasminogen activator inhibitor1 (PAI-1), apoE, α2-macroglobulin (α2M), proteases, protease inhibitor complexes, extracellular matrix proteins, growth factors, toxins, and viral proteins. LRP1 is also abundantly expressed on the membranes of many cell types, such as neurons, astrocytes, macrophages, vascular cells, cerebral glial cells, hepatocytes, fibroblasts, monocytes, adipocytes, retinal Müller glial cells, and malignant cells. It is found that LRP1 has a crucial role in many processes included in tumorigenesis and tumor progression [2, 6-13].

The *LRP1* gene promoter is enriched with CpG islands (regions where CG sequences are concentrated) and sites that induce the sensitivity of the *LRP1* gene to DNA methylation (strong epigenetic event). Related to the DNA methylation level rise, the LRP1 transcriptional activity decreases, and when CpG sites are completely methylated, its transcriptional activity completely disappears and LRP1 expression ceases (***Fig. 1***)[14]. As opposed to the *LRP1* gene, in the process of ageing and AD, there is a significant decline of DNA methylation in the promoter of the gene for the receptor for advanced glycation end products (*RAGE*). During the ageing process and in AD, around this promoter there is an intensified accumulation of AGEs, cytokines (*i.e.*, interleukin-1 [IL-1], tumor necrosis factor-α [TNF-α]) and nuclear factor-κB (NF-κB), all of which can decrease the promoter DNA methylation (blockade of the Dnmt [DNA methyltransferase] approach to the RAGE promoter), and increase *RAGE* gene expression (***Fig. 2***)[15].

RAGE, the multi-ligand and multifunctional receptor, is a 50-55 kDa glycosylated protein with three extracellular domains (res. 23-342 aa, V,C1,C2), one hydrophobic transmembrane domain (343-363 aa) and the cytoplasmic tail (364-404 aa). Residues V-C1 contain numerous positive charges of Arg and Lys residue, and they can easily bind with many RAGE ligands that have high negative regions[16]. Why is RAGE promoter protected from DNA methylation, and LRP1 promoter is not? The *RAGE* gene, by transcription, does not code the inactive precursor protein, so that the completely formed and active RAGE can promptly, even in ER (where it is translated), contact and bind with the present AGEs, and form a close protective concentration (RAGE-AGE) around the close promoter. The large Dnmt molecule can not approach the promoter. Dnmt is the largest enzyme in humans, 1,620 AA. *LRP1* gene encodes the 600 kDa inactive precursor protein.

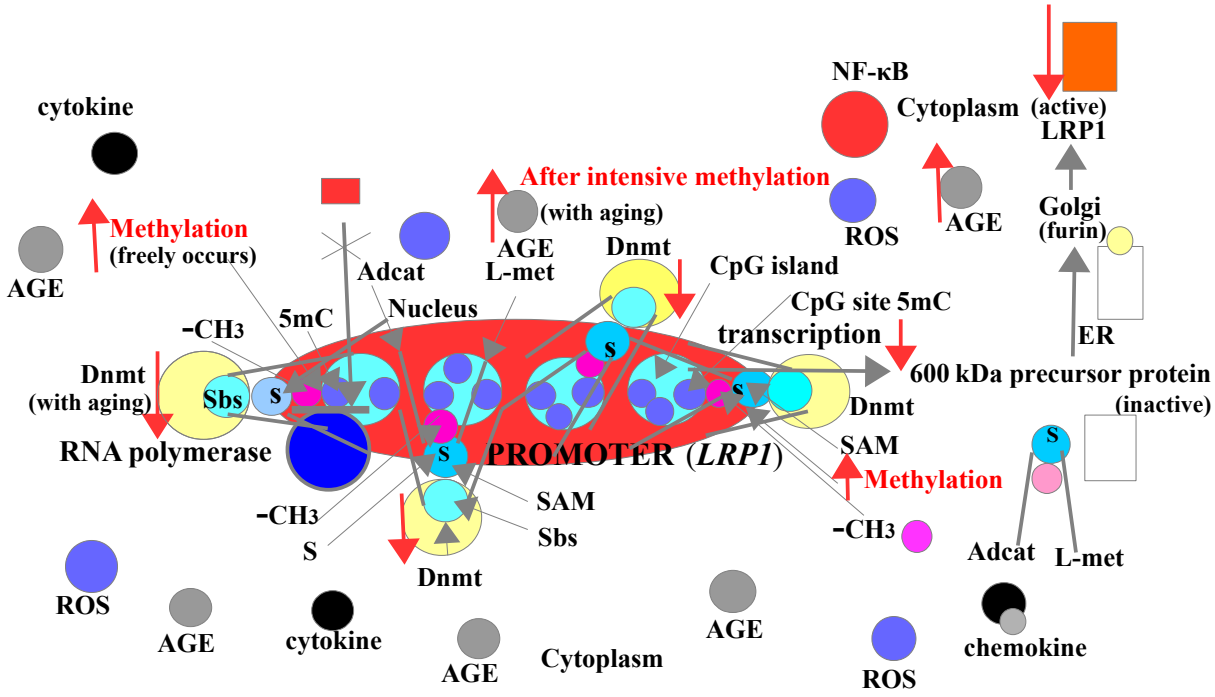

Fig. 1. **Abundant methylation of LRP1 gene promoter.**

LRP1 gene DNA methylation is abundant. RNA polymerase cannot access methylated Cm5. There exists actually a transcriptional blockade. The LRP1 gene is silent. All biochemical and physiological processes caused by these genes are weakened or prevented. Methylation adds a methyl group (-CH_3) from SAM to the substrate. Dnmt (M.Hal) catalyzes the formation of mRNA molecules. The DNA sequences that the proteins bind to, Adcat and L-met, initiate transcription of a single RNA transcript from the DNA downstream of the promoter. The LRP-1 gene encodes a 600 kDa precursor protein (inactive protein) that is processed by furin in the trans-Golgi complex after passing through ER. As a result, a 515 kDa α chain and an 85 kDa β chain are formed non-covalently (active form). This means that LRP1, which reaches the Golgi apparatus through the endoplasmic reticulum, is still inactive. No interaction with locally present AGE compounds is observed.

LRP1, low density lipoprotein receptor related protein 1; 5mC, 5-methylcytosine; CpG, regions where CG sequences are concentrated, easily methylated; SAM, S-adenosylmethionine (also known as Admet); Dnmt, DNA methyltransferase (also known as M.Hhal); Adcat, adenosyl cation; L-met, L-methionine; ER, endoplasmic reticulum; AGE, advanced glycation endproduct; ROS, reactive oxygen species ; NF-ϰB, nuclear factor-ϰB; Sbs, SAM binding site; S, sulfur.

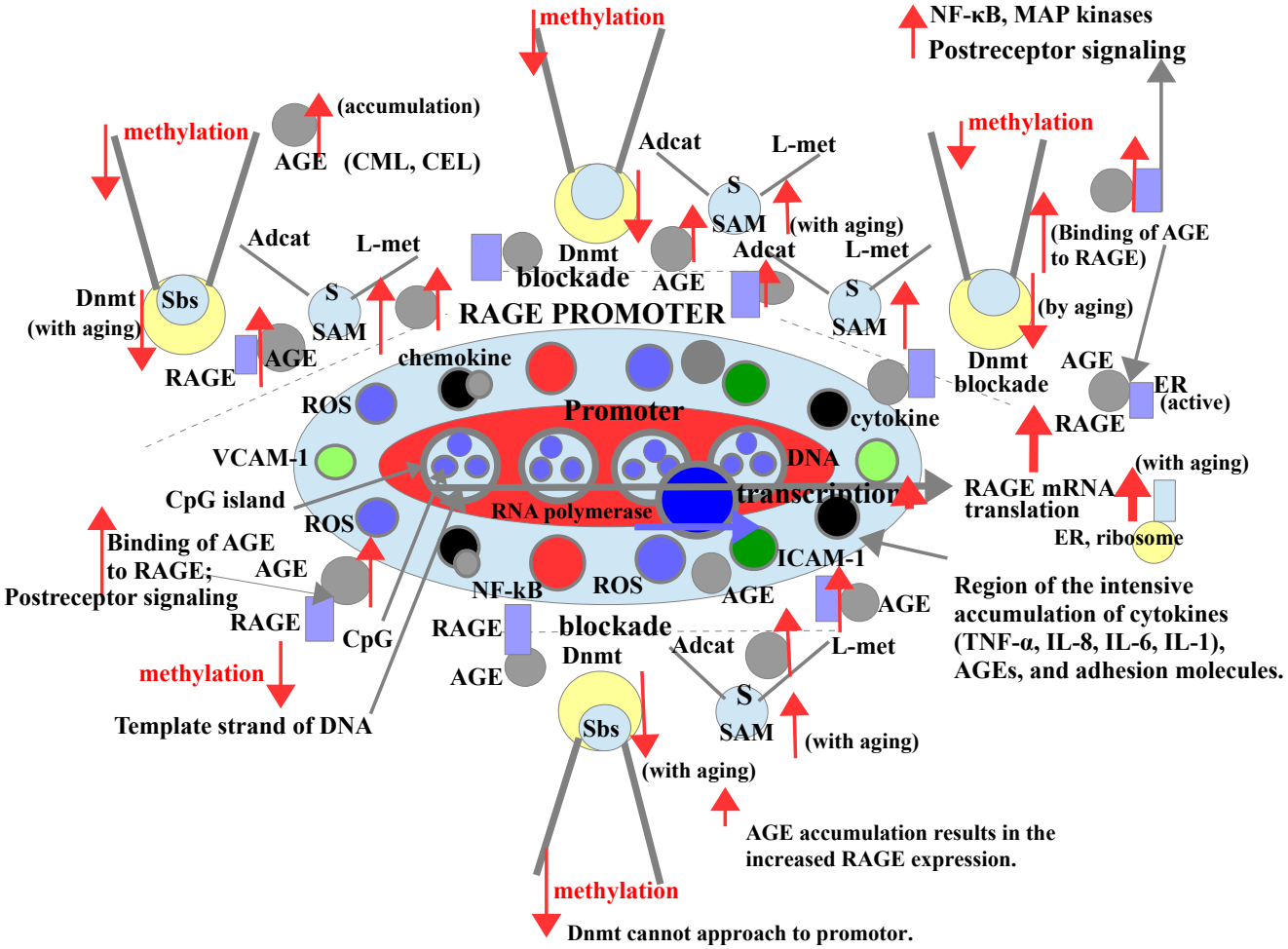

Fig. 2. **RAGE promoter-Dnmt and SAM can not approach the DNA.**

Dnmt is the largest human enzyme (1,620 amino acids). It is a key factor in carrying out methylation of DNA cytosine C5 and the consequent blockade of transcription. These events involve many biochemical compounds, which act as a barrier by staying around the promoter region.The compounds include NF-κB, AGEs, TNFα, ICAM-1, VCAM-1, cytokines, and chemokines.
Dnmt, DNA methyltransferase (also known as M.Hhal); AGE, advanced glycation end product; RAGE, receptor for AGE; CML, carboxymethyl lysine; CEL, carboxyethyl lysine; SAM, S-adenosylmethionine (also known as Admet); NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MAP, mitogen-activated protein; CpG island, regions where CG sequences are concentrated, easily methylated; LRP1, low density lipoprotein receptor related protein 1; 5mC, 5-methylcytosine; SAM, S-adenyl methionine (also known as Admet); Dnmt, DNA methyltransferase (also known as M.Hhal); Adcat, adenosyl cation; L-met, L-methionine; ER, endoplasmic reticulum; ROS, reactive oxygen species; Sbs, SAM binding site; S, sulfur.

This inactive form can not react with the locally present AGEs, and after passing across the nucleus and ER, it becomes processed by furin in the trans Golgi complex. The result is the formation of the active LRP1 form. Now, far away from the LRP1 promoter, a certain interaction of active LRP1 with AGEs is probably possible. However, around the LRP1 promoter there is no effective protective LRP1-AGE accumulation against the Dnmt approach and DNA methylation. Consequently, DNA methylation freely occurs (***Fig. 1***)[15].

Tohgi H. *et al.*[17], using the bisulfite method, the polymerase chain reaction (PCR) and the direct sequencing of PCR products have found a significant age-related decline of methylated cytosine in the RAGE promoter region during the human cortex autopsy. This reduction in the number of methylcytosines (5mC) at transcription factor binding sites increases the expression of RAGE, which may in turn play a role in the ageing of the brain.

The enhanced RAGE expression due to the significantly enhanced AGE accumulation in the course of ageing results in the protein kinase C (PKC) activation, with the activation of nicotinamide adenine dinukleotide phosphate oxidase (NADPH oxidase), and oxidative stress (accumulation of reactive oxygen species [ROS]; O_2^{*-}, H_2O_2, *OH). Oxidative stress activates the NF-κB cascade, and induces the elevation of the following: platelet-derived growth factor (PDGF),

vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule 1 (E-selectin), monocyte chemoattractant protein-1 (MCP-1), macrophage colony-stimulating factor (M-CSF), cyclooxygenase-2 (COX-2), matrix metalloproteinase (MMP), TNF-α, and interleukins (ILs) [1, 15].

Kan S. *et al.* [18], analysed the effects of 5-aza-2'-deoxycytidine (5'-aza-dC) demethylating agens on the methylation status of the RAGE gene promoter in peripheral blood mononuclear cells (PBMCs) of type-2 diabetes mellitus retinopathy. The inhibition of the RAGE promoter DNA methylation by 5-aza-dC resulted in a significant rise of IL-1β, IL-6 and TNF-α in blood samples. The explanation lies in the intensified RAGE receptor expression and its effects. Oxidative stress induces astrocyte dysfunction and the NF-κB cascade with signs of atherogenesis [1, 5, 15, 19]. It is necesary to emphasize that *LRP1* gene deletion (gene knockout) is lethal for the embryo in mice [8, 12, 13, 20].

LRP1 is also included in a number of biological processes as lipoprotein metabolism, degradation of proteases, activation of lysosomal enzymes, LRP1-mediated endocytosis, activation of cell signalling, phagocytosis of myelin debris, phagocytosis of apoptotic cells, and cellular entry of bacterial toxins and viruses. Gonias SL *et al.* [8] and Nichols CE *et al.* [11], using murine models, have investigated the effect of genetic disruption of the *LRP1* gene in smooth muscle cells on the pulmonary function in naive animals and after exposure to bacterial lipopolysacharides (LPS) or house dust mite extract. They have found an increase in tissue resistance, elastance, and tissue elastance at the baseline. The dysregulation of LRP1 in smooth muscle cells affects the baseline pulmonary function and airway responsivenes.

LRP1 expression decline is typical for ageing. Analysing the levels of *LRP1* gene mRNA in isolated cerebral microvessels, Osgood D. *et al.* [2] have found that these levels were significantly reduced in ageing. This indicates that the *LRP1* gene transcriptional activity is also reduced. Considering the crucial role of DNA methylation and demethylation in the regular embrional and later life cycle, the *LRP1* gene functions could indicate the LRP1 essential role in the complete ageing process. Why would it not be possible that the *LRP1* gene is the crucial factor in accelerated ageing?

LRP1 receptor is very sensitive to oxidative stress (oxidative attack), which is otherwise increased in the brains of the aged. The resulting LRP1 receptor oxidation results in the alteration of a number of biological processes related to this receptor [1, 7].

Actually, we do not know the crucial mechanisms involved in the biology of ageing that is composed of complex genetic and epigenetic events. A number of data indicate that one universal program determined by evolution surely exists, where epigenetic factors, interrelated with the genome, have a vital role. Therefore it is possible to implicate the LRP1 effects in this universal program, but, with the assumption that LRP1 effects are not the crucial ageing program *per se*.

LRP1 genes and their receptors are found in all living beings, including mammals, reptiles, amphibia, fish, birds, plants, fungi, mold, bacteria and viruses. All living beings are susceptible to the ageing process and death. This study deals exclusively with the ageing of mammals, and humans in particular. The close functional link of AD and accelerated ageing indicates that in the case of ageing without AD, as well as without the genetic predisposition for this disease, the LRP1 gene and its receptor can be included in the mechanism of programmed ageing and inevitable death. This is especially credible if we consider epigenetic mechanisms, particularly DNA methylation and demethylation. It has already been emphasized that gene promoter LRP1 is inclined to DNA methylation [14]. All this indicates that the permanent programmed DNA methylation/demethylation can be the component of programmed ageing and death (as emphasized before, without the presence of AD or its genetic predisposition). However, the available literature does not give direct facts about the possibility that the programmed ageing is linked to the LRP1 gene and its receptor.

DNA methylation and demethylation related to the life cycle

In the above text, it has already been stressed that epigenetics does not deal with genetic mutations, but exclusively with specific phenomena such as histone acetylation and deacetylation, histone methylation and demethylation, and especially DNA cytosine methylation and demethylation. All these epigenetic phenomena are linked to specific enzymes. The epigenetics of histones is not analysed in this study; however, epigenetic phenomena of DNA methylation and demethylation should be explained. DNA methylation is induced by DNA cytosine methyltransferase (Dnmt) enzyme, and DNA demethylation is induced by ten-eleven translocation (TET) family of dioxygenases and thymine DNA glycosylase (TDG). By the epigenetic mechanism, Dnmt enzyme transports the methyl group ($-CH_3$) from S-adenosylmethionine (SAM, also known as AdoMet, SAMe) on the C5 cytosine position with the 5-methylcytosine formation. Methylation of *LRP1* gene promoter CpG islands leads to the disturbances of transcription factors binding the accumulation of repressive methyl binding proteins and the silencing gene expression and transcription. DNA demethylation is achieved by the interplay of DNA oxidative reactions and repair mechanisms [21].

Bernstein C. *et al.* [22], in their study show epigenetic events in the early embrional phase of mice on a graph where the paternal level curve drastically falls in the fertilized female gamete (oocyte, now transformed into zygote) within the first six hours after fertilisation The paternal chromosomes within these six hours are almost 100 % actively demethylated (TET3 and repair activity; there are no TET1 and TET2 effects). Maternal DNA methylation level in the zygote (fertilised female gamete) drops more slowly due to active demethylation and achieves the exceptionally low paternal level only after 4-5 days. The initial value of maternal DNA methylation level in the moment of fertilisation was under 50 % of the value in paternal chromosomes. Only after 4-5 days, both DNA methylation values grow slowly in parallel.

Slieker R.C. *et al.* [23] give a graphic presentation of the relation between the level of the DNA methylation promoter *ELOVL2* gene and the examinees' age. The graph shows the relation of age and the total blood DNA methylation as well as the linear statistically significant positive regression line. There is a regular rise of the DNA methylation level beginning approximately at age 10 up to almost 85 years.

The DNA methylation level at the proximal age of 10 is about 40 % and at about 85 years is 75 %. The analysis of certain brain regions, excluding the minimal methylation in the region of cerebellum, practically shows the same results as the analysis of the blood.

Hernandez D.G. *et al.*[24] analysed the DNA methylation levels of certain CpG sites (10 CpG sites are shown) in the cerebellum, frontal cortex, pons, and in temporal cortex, and found a significantly marked increase of DNA methylation in relation to age in all examined regions. The research includes the age from 10 to 100 years. In fact, even the oldest groups are affected by DNA methylation.

Osgood D. *et al.*[2] point out that in the course of ageing there is a significant LRP1 reduction in expression. By measuring the mRNA of isolated cerebral microvessels during the age of 3, 6, 9, 12, 15, 20, 30 and 36 months in mice, the authors found a considerable drop of *LRP1* gene transcription. Presently, very little is known about the cause of this phenomenon. However, the obtained data indicate that the gene transcription is disturbed during the ageing process by some upstream events rather than being post-transcriptional. The graphic presentation of LRP1 mRNA expression indicates its constant growth from the 3rd to the 15th month of life, then a constant drop to the 36th month. This also relates to the receptor synthesis.

Again, the actual question is if it is possible that the *LRP1* gene is the crucial component of the program that determines the process of programmed ageing. It has been emphasized that this gene is evolutionarily present in the living beings genome even from the primeval beginnings, and that it is closely linked with the evolutionary present epigenetics. The LRP1 gene is also closely linked with the accelerated ageing in AD. It has been proven that LRP1 gene deletion in the mouse embrio is incompatible with further developement and life[8,12,20].

It should be stressed that the LRP1 receptor is very sensitive to glycative and oxidative stress[1].

The biological clock, *i.e.* the biological program, determines the velocity of the DNA methylation/demethylation of particular genes, or their certain CpG sites. The regression analysis curves shown in the graphs of this study indicate that the above mentioned velocity, *i.e.* the DNA methylation level, is determined by time[22,23]. The higher velocity of DNA methylation has significantly greater regression line angles, and lower velocities have smaller ascending angles. This can be seen in the LRP1 and RAGE receptor curves that are significantly different from each other. RAGE has a much smaller ascending angle, which has also been confirmed by the analysis of its mRNA level. The graphic presentation of 10 CpG sites in the study by Hernandez DG *et al.*[24] indicates on all the curves (scatter diagrams) a significantly higher methylation level at the age of about 50-60 years, and an increased drop after that. At the age of about 100 years, there is practically no methylation. The study by Slieker RC *et al.*[23] shows similar results. The biological program that determines the mentioned velocity is most probably evolutionary determined so that the species could have optimal conditions to survive. In this respect, certain phases of human life are also determined by time. This program also determines the human maximally possible life span (about 120 years). The same applies to other living being species.

The analysis of the DNA methylation process by human DNA cytosine methyltransferase, M.Hhal, shows a remarkably ageing-related increase in SAM and SAM synthetase levels (MATs). SAM (AdoMet, S-adenosyl-methionine, methyl donor, cofactor) results from the synthesis of methionine and adenosine triphosphate (ATP) by the effect of 3 isoenzyme of MATs, SAM synthetase. During the ageing process there is an increase of SAM as well as MATs. Mammalian cells express three genes: *MAT1A* (10q22.3), *MAT2A* (2p11.2) and MAT2B (5q34). They encode a catalytic or regulatory sub-unit used in the formation of three MATs isoenzymes[24]. The above mentioned authors have found a significant rise of methyonine in aged tissues (***Fig.3***).

In the course of ageing, a weaker DNA methylation of these three gene promoters (as in the case of RAGE) should most probably be expected, which would lead to a stronger encoding of three MAT isozymes[25,26].

Xing P. *et al.*[14] emphasize that the promoter region of the *LRP1* gene is enriched with CpG islands (methylation targets) and sites that govern the sensitivity of the *LRP1* gene to DNA methylation (the importance of this promoter structure). They have found that, when CpG is completely methylated, the transcriptional activity disappears and the expression of LRP1 is silenced. CpG islands become more methylated with increasing age. LRP1 expression declines and the drainage from the brain of Aβ and other waste products drops.

Silverberg G.D. *et al.*[15], in their experiments with mice have found a decreased methylation of the *RAGE* gene promoter region during the process of ageing. This leads to a strong RAGE expression, PKC and NADPH oxidase activation, as well as a significant increase of reactive oxygen metabolites (ROS, $O_2{}^{*-}$, H_2O_2, *OH). The ROS elevation leads to oxidative stress and NF-κB cascade. The increased values of Aβ, AGEs, TNFα, and especially NF-κB prevent promoter methylation, thus intensifying *RAGE* expression and receptor transcription. Otherwise, RAGE receptor is a strong Aβ influx receptor. Oxidative stress leads to astrocyte dysfunction and the onset of atherogenesis[27].

The mentioned events related to *RAGE* receptors and RAGE genes have been presented in detail in papers by Barić N.[27,28].

The process of DNA gene promoter methylation and demethylation continually takes place during the life cycle of the living being. The curves showing the relation of gene promoter DNA methylation and the age of the living being have a continually ascending course. In humans, the scatter diagram is abundant up to 60 years, and after that it obviously thins out. The methylation of certain CpG sites occurs even after the age of 90. If the graphs of LRP1 regression curves are compared with RAGE regression curves, it is evident that the ascending LRP1 angle is markedly steep, and for RAGE the angle is has a shallow ascent. Obviously, in the LRP1 promoter, strong methylation and gene silencing occurs much erlier. This silencing continuously takes place up to an extremely old age in the living being, though gradually with less intensity. Considering the great number of LRP1 ligands and LRP1 vital functions, it is clear that parallel to the growth of its promoter DNA methylation, these functons through time

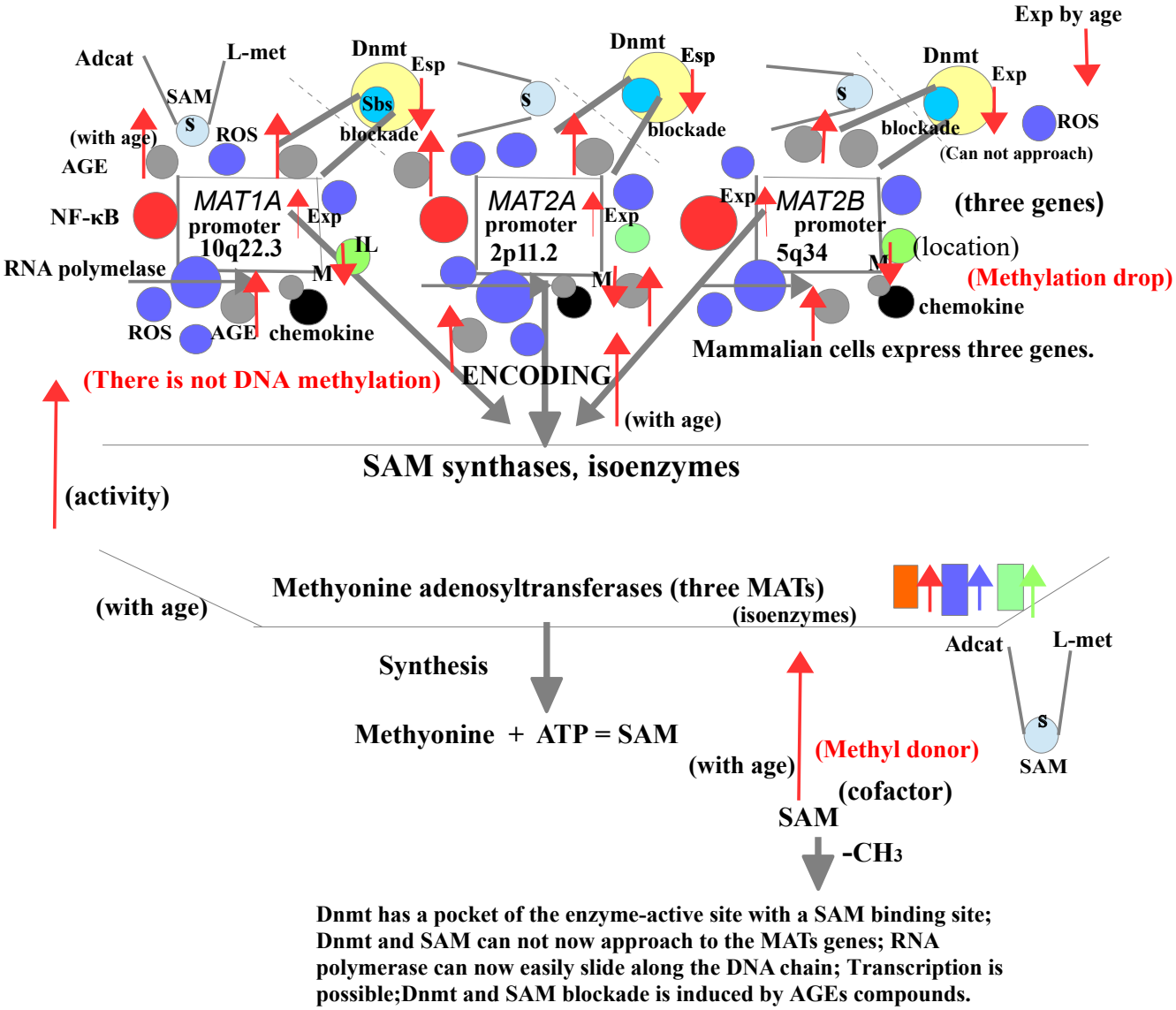

Fig. 3. **Schematic presentation of S-adenosyl-methionine (SAM) synthesis.**

SAM is synthesized intracellularly by MATs catalyzed by methionine, an essential amino acid, and ATP as substrates. As a methyl group donor, it is an essential methionine derivative for the catalytic reaction of methyltransferases. With ageing, SAM concentrations increase in the cerebrum, hippocampus, and other parts of the body (Hayashi Y et al. 25)). SAM is a coenzyme, and its presence is essential for the activity of the enzyme Dnmt. It is composed of an adenosyl cation bonded to the sulfur (S) of methionine, which plays an important role as a sulfonium functional group. MAT1A, MAT2A, and MAT2B are three genes. The MAT1A gene is expressed only in the adult liver. MAT2A and MAT2B are expressed in fetal liver and adult non-liver tissue. Three MATs isozymes, Sams, isoenzymes AdoMet synthetases, and Methyonine adenosyltransferases; SAM, S-adenosylmethionine (also known as Admet); MAT, methyonine adenosyltransferase; ATP, adenosine triphosohate; Dnmt, DNA methyltransferase (also known as M.Hhal); Adcat, adenosyl cation; L-met, L-methionine; ROS, reactive oxygen species; AGE, advanced glycation end product; IL, interleukin; exp, expression; Sbs, SAM binding site; S, sulfur.

are increasingly weakened and they are slowly exstinquished. The weakening and slow exstinguishing of these functions is actually equivalent to the process of ageing. The RAGE gene has significantly slow methylation, and its receptor, in relation to the LRP1 receptor, has a notably reversed function. For example, as to Aβ peptides, LRP1 is a significant efflux receptor, and RAGE is an influx receptor. The reduced methylation of the RAGE gene promoter actually supports its strong activity. The consequent oxidative stress, NF-κB cascade and the astrocyte dysfunction contribute to the developement of atherogenesis, and due to the positive biofeedback mechanism with Aβ and TNF-α, for the further strengthing of RAGE expression [1, 23, 24, 27, 29].

Yang J. *et al.* [30] point out that the main characteristic of DNA methylation is the transfer of the methyl group (-CH_3) from the methyl donor, SAM, onto the active site of the DNA cytosine methyltransferase enzyme, and C5 everted cytosine from one chain of the corresponding DNA molecule. It is evident that all phases of the human life cycle take place under the intensive control of the genetic program determined by evolution. However, besides this program, a parallel DNA methylation and demethylation program also takes place. These two programs mutually interweave and define the velocity and course of the ageing process. It seems that of all the genes, LRP1 and RAGE have a crucial role in this process. When the optimal period for fertilization ends, both of these genes increasingly change their expression, thus changing a whole array of biological functions that actually depend on these two genes. DNA methylation is permanently in progress, but most probably, due to the lack of DNA demethylation, it gradually suppresses biological functions linked to the *LRP1* gene over time, and encourages the negative, mostly atherogenic phenomena linked to the *RAGE* gene (***Fig. 4***).

Three methionine adenosyltransferase genes, *MAT1A* (10q22.3), *MAT2A* (2p11.2) and *MAT2B* (5q34) encode a catalytic or regulatory sub-unit used in the formation of three MATs isozymes. It has been found that the level of these three isozymes (Sams, isoenzymes AdoMet synthetases, and Methyonine adenosyltransferases; three MATs) is elevated in old age. They catalyze the interaction between methionine and ATP with the formation of SAM. SAM transmits its methyl group (-CH_3) to the Dnmt enzyme and to C5 of the DNA cytosine everted in the Dnmt enzyme pocket. SAM level is also elevated in old age. What are the reasons for these two mentioned elevations (***Fig. 3***) [29] ?

As there is no other explanation, it can be justly assumed that, in the case of DNA methylation of three MATs gene promoters, there is a similar situation as in the RAGE genes. Due to the great accummulation around the promoters, AGEs, ROS, TNF-α, cytokines, interleukines, NF-κB, PDGF, VCAM-1, and ICAM-1, the access of DNMT and SAM to the promoter is blocked as well as to CpG islands and sites. This protective ring blocks the methylation of CpG islands and sites on the promoter. However, the continually present RNA polymerase can become active and provoke the MATs isoenzymes coding. It should be emphasized that in the course of ageing there is a drop of the DNMT expression, and that the RNA polymerase is permanently attached to a sliding clamp that prevents the polymerase from falling off the DNA (***Fig. 3***) [29].

Cui D. *et al.* [31] have found that the expression levels of DNMTs decrease with age. Comparing the levels of DNMT enzymes: DNMT1 (gene location 19p13.2), DNMT3A (gene location 2p23.[3] and DNMT3B (gene location 20q11.21), in both human and mouse frontal cortex and hippocampal tissues and in young and old examinees, they have found that the levels of DNMTs are evidently decreased with age. Cui D. *et al.* [31] emphasize that the DNA methylation is an essential dynamic biochemical process during the mammalian life cycle. They also accentuate that DNMTs are abundant in the embryonic stage and significantly decrease after the terminal differentiation stage. These three enzymes have a vital role in mediating the DNA methylation process. The methyl group (-CH_3) is transfered by the DNA methylation from the methyl donors (SAM) onto other genomic DNA sequences (***Fig. 4***).

Tsang S.Y. *et al.* [32], analysing variations of the global DNA methylation levels in the ageing process, have found an increasing trend up to the age of about 55-61 years, followed by the decreasing trend up to 75 years. In this way they have obtained exact proof that the global hypomethylation in old age is a strong risk marker for a whole spectrum of age-related disorders such as cancer, cardiovascular and neurodegenerative disorders, and type 2 diabetes. The frequent AD onset at the age of about 65 years and more (LOAD) is in concordance with the steep decline of the global DNA methylation between 55 and 70 years.

Earlier in the study it has been stressed that there is a great difference between the global DNA methylation and DNA methylation connected to gene promoters and CpG islands and sites. While global DNA methylation declines with age, the latter methylation increases with age. Both methylation types are in progress during the whole life cycle of the living being. Global DNA methylation refers to the average methylation status that occurs across the whole genome. Gene-specific DNA methylation refers to the analysis of the methylation status of specific genes. This methylation increases with age resulting in the decrease of the methylated genes expression, and the decrease of a number of biological functions related to these genes [33].

It is obvious that the *DNMTs* genes are exposed to a type of age-related hypermethylation in promoter regions of specific genes, with a consequent decrease of correspondent mRNA levels and decrease of DNMT encoding (as in LRP1 genes) (***Fig. 4***) [33, 34].

Based on *in vitro* models and studies carried out in tissues and blood DNA samples, Bellizzi D. *et al.* [35] demonstrated a decrease in global cytosine methylation during ageing. On the other hand, additional studies have been reported with an age-related hypermethylation in promoter regions of specific genes, with a consequent decrease of correspondent mRNA levels. In humans, this hypermethylation primarily has been observed for genes involved in cell cycle signalling, tumor-cell invasion, DNA repair, apoptosis, metabolism and cell signalling.

Lin L. *et al.* [36] point out that RAGE is a pattern recognition receptor (PRR) that interacts with diverse endogenous ligands. This receptor is encoded by the *RAGE* gene. Ligation of RAGE receptors triggers a series of cellular

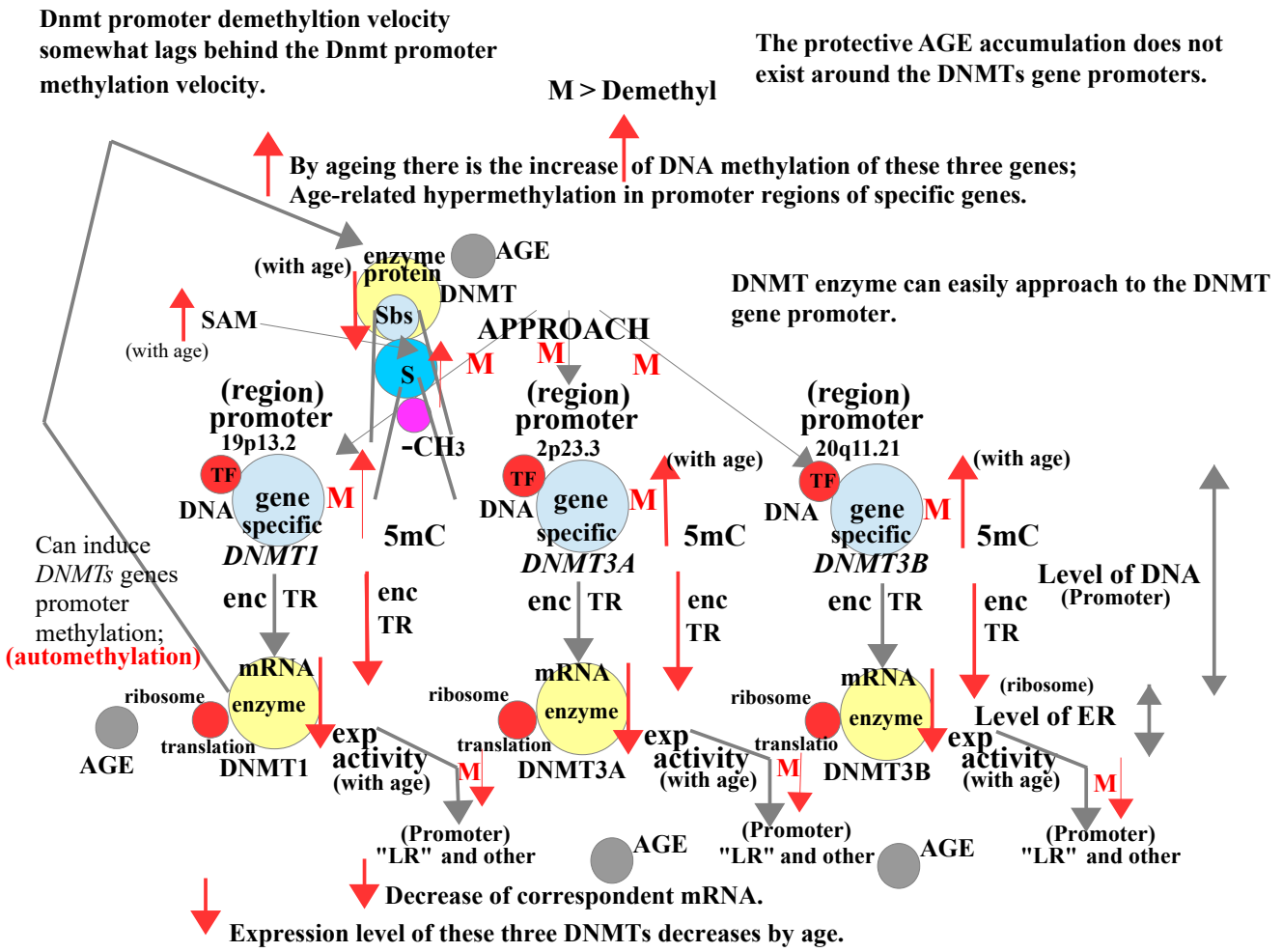

Fig. 4. **DNMT gene global cytosine methylation during ageing decreases and DNMT gene age-related hypermethylation in their promoter regions increases.**

According to *in vitro* models and studies carried out in tissues and blood DNA samples (Bellizzi D et al. 35)), demonstrated a decrease in global cytosine methylation during ageing. On the other hand, additional studies reported an age-related hypermethylation in promoter regions of specific genes, with a consequent decrease of correspondent mRNA levels. In humans, this hypermethylation has been observed for genes involved in cell cycle signalling, tumor-cell invasion, DNA repair, apoptosis, metabolism and cell signalling. This situation of silencing is practically similar to the LRP1 gene down-regulation by methylation of LRP1 CpG islands. It is important to emphasize that DNMTs genes have a lot of promoters with concentrated CpG islands and sites, and also regions without promoters, but with isolated CpG sites which can be methylated.

LRP1, low density lipoprotein receptor related protein 1; 5mC, 5-methylcytosine; CpG island, regions where CG sequences are concentrated, easily methylated; SAM, S-adenosylmethionine (also known as Admet); Dnmt, DNA methyltransferase (also known as M.Hhal); AGE, advanced glycation end product: RAGE, receptor for AGE; LR, LRP1 and RAGE; TR, transcription; M, DNA methylation; exp, expression; enc, encoding; ER, endoplasmic reticulum.

signalling events including the activation of NF-κB, leading to the production of pro-inflammatory cytokines, and causing inflammation. RAGE signalling has been implicated in multiple detrimental human illnesses including diabetes, atherosclerosis, arthritis, and Alzheimer's disease. RAGE is expressed in tissues and cell types that are critical for immune control, including lung, liver, vascular endothelium, monocytes, dendritic cells, and innate neurons. The ligand activated by RAGE interacts with integrins and facilitates the recruitment of pro-inflammatory leukocytes to the sites of inflammation and worsens, the inflammation. RAGE plays an important role in innate immunity. AGEs are the major *in vivo* ligands that activate RAGE to elicit inflammation.

Isermann B. *et al.*[37], analysing the interaction between AGEs and RAGE, have found that the interaction results in an activation of NF-κB cascade increased the expression of cytokines, chemokines, and adhesion molecules, and the induction of oxidative stress (ROS: $O_2{}^{*-}$, superoxide radical; H_2O_2, hydrogen peroxide; *OH, hydroxyl radical). They emphasize that except for RAGE, AGEs interact with a number of other receptors, while RAGE interacts with a diverse group of ligands. A detailed description of the mentioned interactions is shown in the study by Barić N[27].

Semba R.D. *et al.*[38] emphasize that AGEs in the body are permanently generated through the non-enzymatic glycation process resulting from the covalent bonding of sugar molecules, such as glucose or fructose, to protein or lipid molecules. The whole process develops slowly and continually, and without the influence of enzymes. This process is accelerated in diabetes. It has been found that the bonding of AGEs with their RAGE does not lead to the AGE clearance and degradation. On the contrary, it provokes the permanent postreceptor signallization including the NF-κB and MAP kinase activation, with prolonged cellular dysfunction and localized tissue destruction. The mentioned bonding causes the enhancement of oxidative stress and inflammatory effects. AGEs form covalent cross-links with proteins. This cross-linking of long protein molecules causes the increase of tissue density in the aorta, carotides, and other major arteries. The pulse wave velocity increases as well as the systolic and pulse pressure. In all tissues and organs, the tissue density increases and elasticity decreases. Clearly, these conditions are the ageing phenotype.

Senatus L.M. *et al.*[39] emphasize that AGEs are formed throughout life via the process of non-enzymatic glycation of proteins and lipids. Humans and animals are also exposed to exogenous sources of AGEs ingested through food-derived AGEs and tobacco products. It is evident that AGE production and accumulation strictly accompany the normal ageing process. This results in the high AGE crosslinking of collagen and elastin molecules. The consequent increased stiffness of the aorta and other conduit arteries is associated with a greater risk of ageing-associated cardio- and cerebro-vascular diseases and mortality. AGE accumulation causes upregulation of inflammation and destruction of collagen and elastin. RAGE is expressed in a number of important cell types implicated in arterial ageing. AGE interaction with RAGE provides a mechanism to link AGE-RAGE to arterial ageing and its consequences, such as stroke, hypertension, atherosclerosis, myocardial infarction, and heart failure.

Kim H.J. *et al.*[16] point out that RAGE binds and mediates cellular responses to a range of damage-associated molecular pattern molecules (DAMPs), such as AGEs, High Mobility Group Box1 (HMGB1-nuclear protein that binds to DNA and acts as an architecture chromatin-binding factor), and S100/calgranulins (a group of protein sensors of intracellular calcium levels). RAGE and its ligands stimulate the activation of diverse pathways, such as p38MAPK, ERK1/2, Cd42/Rac, and JNK (c-Jun amino terminal kinase). Upregulation of RAGE expression has been reported in atherosclerosis, AD, cardiovascular diseases, and immune/inflammatory diseases. As distinguished from LRP1, RAGE knockout mice are healthy and developmentally normal. This suggests partial RAGE knockdown might be a safe therapeutic strategy. RAGE receptor elevation is recognized as the hallmark of ageing. RAGE -/- mice present a pro-longevity phenotype. Once again, the authors emphasize that ageing leads to the accumulation of AGEs in tissues and plasma.

Noroozi R. *et al.*[40] emphasize that ageing, as an irretrievable phenomenon during the whole life, is marked by a progressive decline in physiological funcionality and disease variability. Epigenetic modifications, Especially DNA methylation, correlate with ageing and age-related diseases.

The analysis of the functions of both genes (LRP1 and RAGE) and their receptors clearly shows that during the ageing process and old age their functions change through the DNA methylation process. The LRP1functions increasingly weaken (*e.g.*, Aβ drainage), and RAGE functions are intensified. RAGE promoter has free access to transcription factors, thus enabling the transcription. In LRP1 genes, most CpG sites in the promoter are methylated and transcription factors (TFs) do not have direct access to CpG sites in the promoter. It should be noted that TF regulator proteins approach the beginning part of the gene. They provoke or prevent gene transcription, enabling RNA polymerasis to recognise the gene and to bind with the promoter. It should be noted that RAGE is expressed at low levels in normal tissues. The analysis of these changes indicates that they actually correspond with the damages and changes related to ageing and old age, and that they essentially correspond to the process of ageing. LRP1, RAGE, and their receptors likely play the crucial role in the primeval programme of this inevitable and purposeful process.

It is clear that the DNA methylation/demethylation processes continuously go on, faster or more slowly throughout the life cycle of the living being. In the early embrional phase, DNA methylation drastically declines with the DNA demethylation rise. In this phase, active are Ten-eleven translocation 3 (TET3) -dependent active demethylation and DNA replication-dependent passive demethylation. The volume of DNA methylation falls to the value 43% of the earlier value. After the forming of blastocyste (fourth day after fertilisation) starts the epigenetic reprogramming, and by means of DNMTs, DNA methylation is again restored. In a very small space there are everted DNA cytosines, RNA polymerase, DNMT enzymes, ATP, methionine, SAM, and MATs isoenzymes. Following is the DNA methylation with the subsequent transcription blockade (***Fig. 5***).

Epigenetic events continuously go on, and among them, DNA methylation is especially important. It correlates with ageing and age-related diseases. Using the age-related alterations in the DNA of certain CpG sites, several

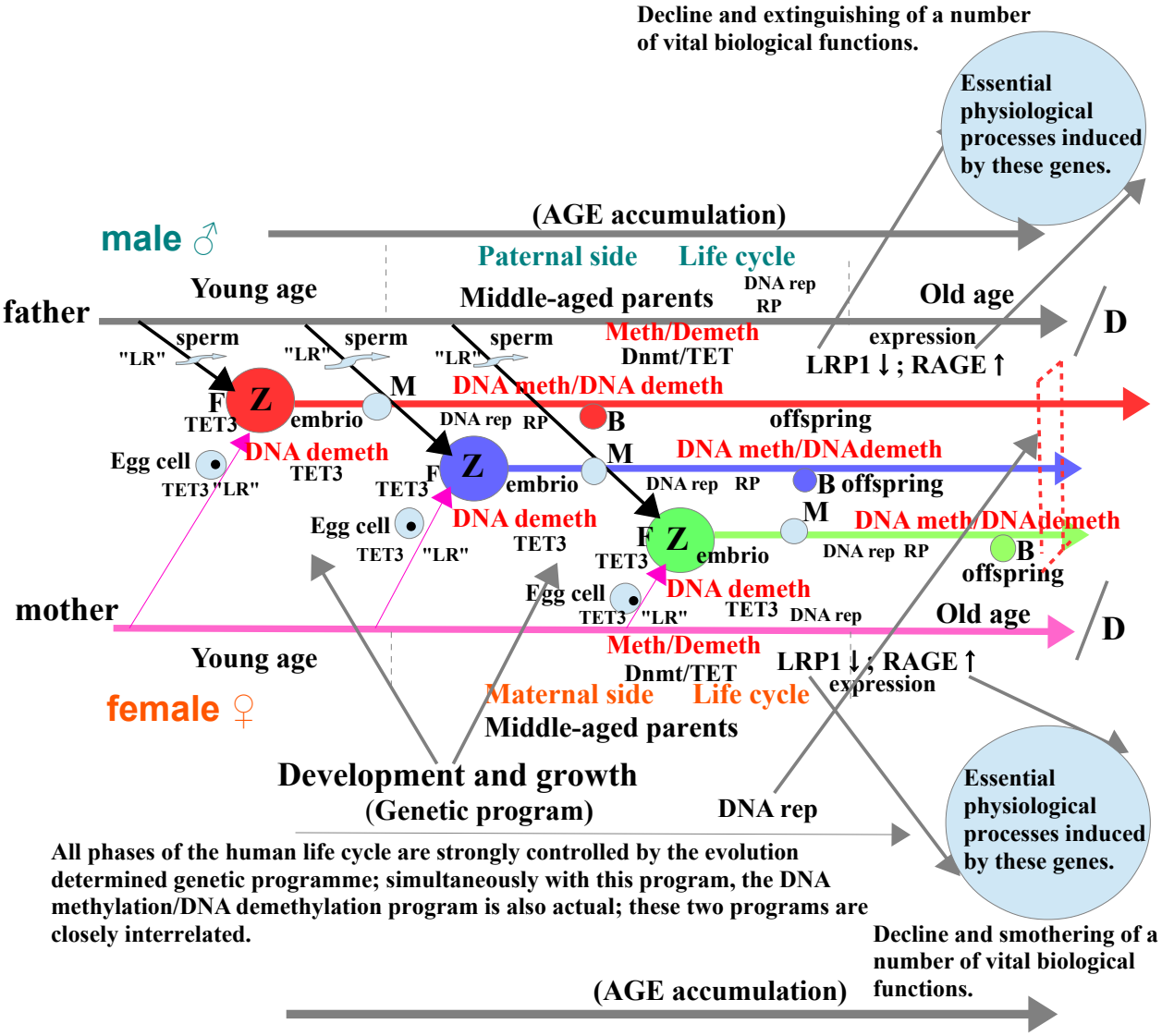

Fig. 5. **Schematic presentation of permanent DNA methylation/demethylation during the eukariotic life cycle.**

The black broad line presents the father's life cycle, and the pink broad line presents the mother's life cycle. DNA methylation and DNA demethylation are two tightly connected epigenetic events. The global DNA methylation decreases with age. Age-related hypermethylation is found in promoter regions of specific genes. The active demethylation is induced by TET family of dioxygenases. LRP1 deletion (knock-down) in the embrionic phase is incompatible with life. Events during the life cycle have an essential epigenetic biological program (biological clock).

TET3, methylcytosine dioxygenase – band 2p13.1; TET2, methylcytosine dioxygenase – band 4q24; TET1, methylcytosine dioxygenase – band 10q21.3; Thimine DNA glycosylase (TDG) – band 12q23.3; TF, transcription factor; LRP1, low density lipoprotein receptor related protein 1; AGE, advanced glycation end product; RAGE, receptor for AGE; Dnmt, DNA methyltransferase (also known as M.Hhal); TET, ten-eleven translocation; TF, transcription factor; DNA rep, DNA replication; RP, repair process; LR, LRP1 and RAGE; Meth, DNA methylation; Demeth, DNA demethylation; B, birth; F, fertilization; M, morula, 16 cells; Z, zygote; D, death.

investigations have attemped to predict chronological age[41-43].

Sanada F. *et al.*[43] emphasize that ageing is a complex process that results from a combination of environmental, genetic, and epigenetic factors. It is marked with a chronic low-grade inflammation in the absence of overt infection. In contrast to young individuals, aged individuals have constantly elevated levels of inflammatory cytokines, especially IL-6 and TNF-α. They have also found in visceral fat of obese individuals the accumulation of macrophages, which are a major source of low-grade persistent systemic inflammation and insulin resistance. Cigarette smoke is full of ROS with the production of IL-6, TNF-α, and IL-1β. Many tissues in the elderly are chronically inflammed, and IL-6, IL-1β, and TNF-α are known to weaken the anabolic signalling cascade, including insulin and erythropoietin signalling with sarcopenia development. Presented inflammatory cytokines are the same as cytokines connected with RAGE and its expression. No comment is necessary.

The analysis of scientific papers that comprehensively deal with the human ageing process shows remarkable concordance of detected biomarker characteristics typical of ageing and old age and the effects of LRP1 and RAGE[44-47].

Attempts to slow down the ageing process in humans

If we accept the hypothesis related to the elevated level of RAGE expression as the crucial factor in the ageing process, the question arises whether the use of RAGE blockers to slow down the course of ageing is justified. Kong Y. *et al.*[47] exclusively deal with animal models of AD therapy. They investigate therapeutic possibilities of the strong RAGE blockator, FPS-ZM1, with its toxic side effects and earlier detected biological effects. They point out the damaging effects on the human body of high RAGE expression levels. RAGE activates the NF-κB cascade with intensive secretion of proinflammatory cytokines, TNF-α, IL-6, and macrophage colony-stimulating factor 1 (CSF-1). RAGE activation leads to the production and aggregation of Aβ and the forming of neurofibrillary tangles (NFTs), as well as to the destruction of synaptic transmission and neurons. All this promotes the onset and development of AD. The above mentioned authors have found that the compound, FPS-ZM1, effectively passes across the BBB, affects the V-type region of RAGE, and after binding to the receptor, it blocks its intracranial functions. FPS-ZM1, after binding, completely restores the brain blood flow, inhibits neurotoxicity, decreases the microglial activity and the neuroinflammatory response, and improves the cognitive behavior. There are no toxic effects, even when the applied dose was 500 times higher than the therapeutic dose. The above mentioned facts are related to the experiments with mice. The FPS-ZM1 molecular formula is $C_{20}H_{22}CINO$ or N-benzyl-4-chloro-N-ciclohexylbenzamide.

Huang J. *et al.*[48] present the results of exceptional therapeutic effect of the FPS-ZM1 in the treatment of periodontal disease (gingivitis and periodontitis) in a group of patients. There were no side effects. These results give great hope for future therapeutic attempts in humans.

Deane R. *et al.*[49] synthesized the FPS-ZM1 in the year 2012. This is an extracellular polymeric substance, organic polymer, of bacterial origin that for bacteria serves as prevention from unfavourable environmental effects.

Singh H. *et al.*[50] emphasize that the accumulation of AGE compounds in the body has a significant role in the onset and course of many inflammatory health disorders, including cardiovascular diseases, diabetes mellitus, immune inflammation, cancer, and neurodegenerative disorders. The detection and binding of AGE compounds is dominantly performed by the already mentioned RAGE. The binding of AGEs, as well as other ligands, to RAGE activates multiple signalling pathways, such as STAT3 (signal transducer and activator of transcription 3), MAPK/ERK (mitogen-activated protein inase/extracellular signal-regulated kinase), and JNK, which results in the increase of transcription factors, including NF-κB. The mentioned binding is included in the pathogenesis of numerous inflammatory diseases, such as atherosclerosis, diabetes mellitus, cancer, neurodegenerative disorders, rheumatoid arthritis, and chronic renal failure. The RAGE inhibitor FPS-ZM1 has shown significant effectivenes in blocking an array of RAGE ligands, resulting in a significant drop in the oxidative stress level and in inflammatory events.

Hong Y. *et al.*[51] have found an increased level of AGEs in observed brains of AD patients. AGEs and RAGE have important roles in the pathogenesis of AD. Experimenting with rats, they have found that after intrahippocampal injection of AGEs, the intraperitoneal administration of FPS-ZM1 has significantly reduced Aβ elevated production induced by AGEs, inflammation, and oxidative stress. They conclude that FPS-ZM1 might be a novel therapeutic agent to treat AD patients. Barić N.[1], in his paper, gives a detailed review of key anti-AGE and anti-RAGE therapeutic approaches. These methods are also comprehensively presented in the study by Senatus L.M. *et al.*[39].

Kang D.E. *et al.*[52], Shibata M. *et al.*[53], and Silverberg G.D. *et al.*[15] point out that the LRP1 expression at the BBB is reduced during both normal ageing and AD. To correct the decreased LRP1 expression in the brain, Sagare A.P. *et al.*[54] suggest three sets of actions: a) lifestyle changes (*e.g.*, diet, exercise, and enriched environment), b) pharmacological agents (*e.g.*, statins and plant-based active principles), and c) gene therapy. Sagare A.P. *et al.*[54] emphasize the great importance of antioxidant nutrients (polyphenol-rich foods, vitamins A, C, and E, green tea, and extra virgin olive oil) which could protect LRP1 from oxidative damage. Physical activity can improve cerebral angiogenesis in rodents and humans. Statins are the target cells of cholesterol lowering drugs. Simvastatin and atorvastatin are especially effective in the brain. The authors stress that effective gene transfer can be achieved by viral based systems which are highly effective in mediating cell entry and transfer of genes. Adeno-associated virus (AAV) is frequently used as a vector for these therapies. The use of AAV2, which carries LRP1 DNA or its smaller fragments, leads to the restoration of LRP1 expression in the brain vascular endothelial cells in AD. The detailed explanation of the LRP1 gene deficiency gene therapy is presented in the paper by Barić N[1]. The elevated LRP1 expression in BBB is obtained by rifampicin[55]. The portal infusion of insulin leads to the evident rise of LRP1 expression on the hepatocyte plasma membrane. According to Shinohara M. *et al.*[13], the environmental enrichment improves the vascular dysfunction by increasing

angiogenesis accompanied by the upregulation of LRP1. Exercise ameliorates brain Aβ deposition and cognitive decline in APP transgenic mice with the upregulation of LRP1. Oleocanthol (a type of natural phenolic compound) from extra virgin olive oil upregulates LRP1 at the BBB and enhances Aβ clearance [1,56,57]. *Cannabis sativa* plant treatment also enhances Aβ transit at the BBB with increased LRP1 levels at the BBB [1,58]. Statin treatment can also upregulate LRP1 in the liver as well as in the brain [1,59]. Special attention in the anti-ageing treatments has recently been directed to *Ginkgo biloba*, especially to *Ginkgo biloba* leaves extract (standardized special extract, Egb761) [60-63]. Reddy V.P. *et al*. [64], in their comprehensive study, present detailed facts related to Alagebrium (ALT-711), aminoguanidine, and pyridoxamine.

Conclusion

Based on the analysis of numerous ligands linked to LRP1 receptor, RAGE, and a number of important biochemical processes that they control, these two genes, associated with DNA methylation/demethylation, could possibly be crucial factors in the program that drives the ageing process. This hypothesis probably sounds unrealistic, but many times it has happened that something so obvious has not been recognised. During the entire life cycle of eukariotes, starting from their primeval beginnings, the DNA methylation and demethylation processes, simultaneously and with a certain balance, permanently take place in their cell nuclei, thus determining the program of the biological time clock. These processes separated by fertilization into events related to parents, and events related to descendants, represent the base, primarily in parents, for the ageing process with the crucial role of LRP1 and RAGE.

Conflict of interest declaration

None.

Research grant

None in particular.

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