Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : November 18, 2023
Accepted : April 11, 2024
Published online : June 30, 2024
doi:10.24659/gsr.11.2_62

Original article

Glycation and biologic clock: DNA methylation age

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Abstract

***Background and Purpose*:** Advanced glycation endproducts (AGEs) produced by glycative stress are implicated in the risk of various age-related diseases. We hypothesized that DNA methylation is involved as a mechanism linking the two. This study analyzed the relationship between DNA methylation in skin samples and physical information, especially in terms of glycation stress.

***Methods*:** Male and female Caucasian patients aged 40-75 years at Riga Stradins University were included (296 patients), consisting of two groups: 149 patients in the metabolic syndrome (MS) group and 147 in the non-MS group. Methylation age (MethylAge) was calculated by measuring hydroxymethylated DNA by LC-MS and matching with cohort data from the Reunis Institute. Glycative stress indices were measured by skin AGE fluorescence (SAF) with an AGE Reader (DiagnOptics, The Netherland). In addition, physical measurements and blood sex chemistry tests were performed.

***Results*:** Items that showed significant correlations with MethylAge were chronological age ($r = 0.594$), waist circumference ($r = 0.261$), triglyceride ($r = 0.317$), and skin aging index (SAI, $r = 0.318$, $p < 0.05$ for each). The correlation between methylation age (y) and SAF (x) was particularly high ($y = 131.9x^2 - 490.1x + 491.5$, $R^2 = 0.989$, $p < 0.001$). The items that showed significant differences in MethylAge depending on the presence or absence of disease/lesions were MS, SAI, Sebhoroic keratosis and Lentigo type of hyperpigmentation ($p < 0.05$ for each). The glycation stress index SAF showed a significant correlation with the presence or absence of MS, oxidative stress index (glutathione, superoxide dismutase), and glycolipid metabolism index (fasting plasma glucose, total cholesterol, high-density lipoprotein-cholesterol).

***Conclusion*:** Assessment of MethylAge may be an important indicator for physiological aging affected by glycative and oxidative stress. The mechanism by which these stress affects methylation age requires further investigation.

***KEY WORDS*:** DNA hydroximethylation, biological clock, advanced glycation end products (AGEs), metabolic syndrome, oxidative stress, aging, premature skin aging.

Introduction

Approximately 20-25% of the World's adult population aged 40-75 years have metabolic syndrome (MS) and they are twice as likely to die from and three times as likely to have a heart attack or a stroke in comparison to those without MS. According to the International Diabetes Federation definition[1)], a person can be characterized as having MS if he has central obesity (waist circumference for women ≥ 80 cm, man ≥ 94 cm) and additionally two of any of the following factors: raised triglyceride (TG), reduced high-density lipoprotein-cholesterol (HDL-C), raised blood pressure, raised fasting plasma glucose (FPG). The cluster of cardiovascular disease (CVD) risk factors that defy MS is now considered to be the driving force for the uprising CVD epidemic[2)]. MS maintains a chronic inflammatory reaction throughout the body due to the presence of oxidative stress. Application of early treatment strategies may result in control of symptoms and complications of MS. However, severe sequelae such as myocardial infarction, cerebral stroke and disability remain very high[3)].

MS, due to the oxidative stress, supports a chronic inflammatory reaction in the skin and in the other parts of the body[4)]. Oxidative stress is a condition of oxidant/antioxidant imbalance, in which the net amount of reactive oxygen species (ROS) exceeds the antioxidant capacity of the body. Excessive ROS can react with cellular macromolecules and cause lipid peroxidation, protein oxidation and oxidative DNA damage[5)].

The skin is a major component of the antioxidant defence system, primarily through its xenobiotic/ drug biotransformation system, ROS-scavenging system and an

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excretory system mediated via sweat and sebaceous glands. In the circumstances of chronic excessive energy uptake and inhibition of sebum secretion, excessive lipids are primarily stored as adipose tissue, whereas excessive cholesterol can accumulate in the arterial wall. Inhibition of sebum secretion increases the levels of circulating lipids and cholesterol, and subsequently the risk of dyslipidaemia and MS [6].

Nowadays we know good about ageing theories, which we define as intrinsic and extrinsic. Intrinsic ageing theories are genetically programmed events causing cellular damage that accelerates aging of the body. Extrinsic are associated less or more with environment, *i.e.*, ultraviolet (UV) damage. Ageing itself is inevitable process accompanied by progressive decline in physical functions and increased risk of disease, *i.e.*, CVD, cancer. In fact, CVD, one of the most common disease of aging, accounted for 30 % of all death worldwide per year [7].

The process of ageing in multiple changes at both molecular and cellular level, including cellular senescence and telomere attrition, and epigenetic alteration [8]. Telomere length, which experiences progressive shortening during replication of somatic cells is remarkable characteristic of aging and linked with age-related health status [9].

Thus, various factors are intricately involved *in vivo*, and just by analysing the relationship between DNA methylation and MS, oxidative stress, glycative stress (accumulation of AGEs), and inflammation are involved. We have reported the association of AGE accumulation with skin manifestations, *i.e.*, epidermoid melanoma, seborrheic keratosis, facial cutaneous telangiectasia, as well as with increased skin aging index (SAI). We hypothesized that DNA methylation is involved as the mechanism linking the two. In this study, we analysed the association between glycative stress and biological age, indices in terms of DNA methylation.

Methods

Current prospective study was done and both gender Caucasian patients were analysed age group 30-60 years in Riga Stradins University. Altogether 296 patients were analysed. Analysed group included 2 specific categories MS and non-MS (based on IDF criterion). Waist circumference and body mass index (BMI) were conducted, as well as lifestyle factors and skin ageing signs using SAI [10].

Generally, MS (n = 149) and Non-MS (n = 147) patients were clinically and biochemically diagnosed with MS based on the IDF (2006) criteria [1]:

Essential major criteria:

- Central obesity with a waist circumference of 80 cm (women) and 94 cm (men).
- Additional 2 minor criteria out of the following:
- Raised TG > 1.7 mmol/L;
- Reduced HDL-C < 1.3 mmol/L (women) and < 1.0 mmol/L (men);
- Raised blood pressure ≥ 130 mmHg (systolic) or/and ≥ 85 mmHg (diastolic);
- Raised FPG ≥ 5.6 mmol/L;

During the study several parameters were evaluated in all patients:

Clinical examination:

- Measurement of the arterial blood pressure by Korotkov's method.
- Measurement of the waist circumference using centimeter.
- Biochemical analyses were performed in E. Gulbis laboratory; levels of total cholesterol (TC), fasting plasma glucose (FPG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and C-reactive protein (CRP).

Oxidative stress parameters (selenium, superoxide dismutase; SOD, malondialdehyde; MDA, glutathione; GPx) were determined in the RSU Biochemistry Laboratory. The principle for determination of MDA is to determine MDA alone (in hydrochloric acid) or MDA in combination with hydroxynonenal (HNE) in the presence of methanesulphonic acid. Two molecules, 4-HNE and MDA, react with N-methyl-2-phenylindole at 45°C to form a stable chromophore with an absorption peak at 586 nm [11, 12]. GPx peroxidase is detected automatically by spectrophotometry according to the manufacturer's instructions for the RX Daytona analyser (Randox laboratories, Ltd., Crumlin, UK). GPx peroxidase catalyses oxidation of GPx (GSH) in the presence of cumene hydroperoxide. Oxidised GPx (GSSG) is further converted to a reduced form, GSH, by GPx reductase (GR) and NADPH, while NADPH is oxidised to $NADP^+$. GPx peroxidase activity corresponds to a decrease in absorbance at 340 nm caused by NADP oxidation. One unit corresponds to the amount of enzyme produced by the oxidation of 1.0 µM NADPH to $NADP^+$ per minute at 340 nm at 37°C.

For glycation index, the intensity of skin fluorescence was measured by the AGE Reader™ (DiagnOptics B.V., Groningen, The Netherlands). The AGE Reader™ is a non-invasive device that measures tissue accumulation of AGEs in human skin on the forearm, and the results are expressed as values of skin autofluorescence (SAF).

The patient's clinical examination, blood pressure and waist circumference were measured. Clinical examination of the skin was performed using dermatoscopy (Dermlite DL; Derma Medical Systems, Vienna, Austria). The detected skin manifestations (seborrheic keratosis, actinic keratosis, acanthosis nigricans, and facial telangiectasia) were divided into several subtypes (mild, expressive, and extreme expressive).

Biological age was determined by measuring DNA hydroxymethylation status by LC-MS [13, 14] and correlation of the result to a reference cohort to calculate the biological age in Reunis laboratories. DNA hydroxymethylation was measured for all patients. Hydroxymethylation measurement was done using a gold standard technique (liquid chromatography + mass spectrometry), the biological age equivalent was calculated. *AGE Reader* was used to measure AGE accumulation in skin by fluorescence techniques, SAF.

Ethical Standards

This study was conducted using existing materials that were not linked to personal information. Our study was designed in conformation to the Helsinki Declaration. The study protocol was approved by the Committee of Ethics, Riga Stradins University. No conflict of interest.

Statistics

Statistical methods were used, t-test for independent samples, Spearman's correlation, Mann Whitney test. Data was documented and analysed with Microsoft Excel 2010 software.

Results

MS and MethylAge

Of a total of 296 subjects (age; 47.0 ± 11.2, BMI; 26.3 ± 4.8), 149 (50.4%) were MS (age; 51.4 ± 9.8, BMI; 29.0 ± 4.2) and 147 (49.6%) non-MS (age; 42.4 ± 10.8, BMI; 23.6 ± 3.6). The analysis was same as the previous report [15]; comparing the MS and non-MS groups, the MS group had higher BMI ($p < 0.001$), LDL-C ($p < 0.001$), TG ($p < 0.001$), MDA ($p = 0.003$) were significantly higher (***Table 1***). MethylAge was significantly higher in MS (median; 33.5) vs. non-MS (median; 61.7, $p < 0.05$, ***Fig. 1***).

MethylAge and results

Correlation analysis was performed between MethylAge and measurement results (***Fig. 2***). The items that showed significant correlation with MethylAge were actual age ($r = 0.594$, $p < 0.05$, ***Fig. 2-a***), waist circumference ($r = 0.261$, $p < 0.05$, ***Fig. 2-b***), TG ($r = 0.317$, $p < 0.05$, ***Fig. 2-c***), SAI ($r = 0.318$, $p < 0.05$, ***Fig. 2-d***).

MethylAge and skin lesions

The skin lesions were classified as seborrheic keratosis (n = 109), actinic keratosis (n = 25), acanthosis nigricans (n = 16), aging wrinkles (n = 126), gravity wrinkles (n = 34), and facial telangiectasia (n = 242).

Skin lesions that correlated with MethylAge and presence of disease were seborrheic keratosis ($r = 0.346$, $p < 0.05$) and lentigo pigmentation ($r = 0.415$, $p < 0.05$, ***Fig. 3***). There were no differences in other diseases between lesion and non-lesion areas.

SAF, oxidative stress and skin aging

For SAF, an indicator of glycative stress, it has been found to be higher in MS ($p = 0.024$); Spearman and rank correlation analysis between SAF and measures also showed that FPG ($r = 0.345$, $p = 0.036$), TC ($r = 0.328$, $p = 0.023$), HDL-C ($r = -0.399$, $p = 0.019$), and CRP ($r = 0.372$, $p = 0.028$) [15].

As for the relationship with skin lesions, SAF was significantly lower in individuals with black seborrheic keratosis ($r = -0.034$, $p = 0.588$), but there were no differences between lesion and non-lesion areas in other diseases (***Fig. 4***). There was also a significant positive correlation between AGEs and SAI, indicating that the more AGE accumulation in the skin, the worse the skin condition ($p < 0.02$).

SAF was positively correlated ($p < 0.05$) with the oxidative stress GPx (***Fig. 5-a***), SOD (***Fig. 5-b)***, and vitamin D supplementation; the regression equation for the relationship between SAF (y) and SOD (x) was $y = 0.001164x + 0.219931$ ($R2 = 0.753$, $p < 0.001$). When SOD is near its minimum value of 1,145, the predicted value of SAF is 1.6; when SOD is near its maximum value of 2,240, the predicted value of AGEs is 2.8.

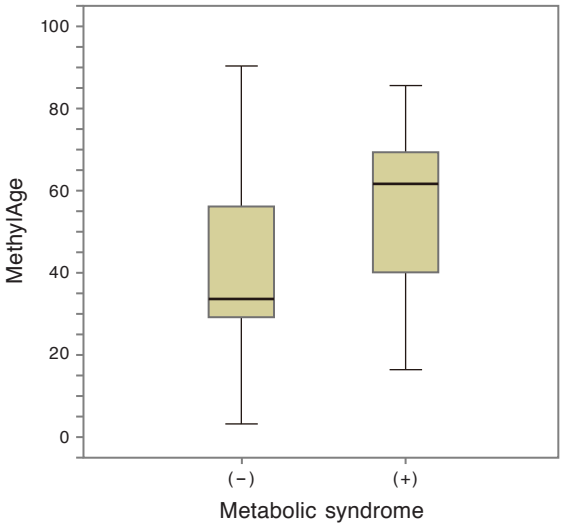
MethylAge and SAF

The correlation between age of methylation and SAF was analyzed using the Single Factor ANOVA test (trendline). A significant correlation was found between age at methylation and SAF, and the equation $y = 131.9x^2 - 490.1x + 491.5$ ($R^2 = 0.989$, $p < 0.001$), a quadratic regression equation, can be used to predict MethylAge (prognostic indicator) (***Fig. 6***). The predicted MethylAge was 36.676 years when SAF was less than 1.8 and 51.676 years when SAF was 2.2; the MethylAge increased with the SAF value, and the mean relative error of these predictions was 11%.

Table 1. Profiles of the subjects.

	unit	MS (n = 149)	Non-MS (n = 147)	p value
Age	year	51.4 ± 9.8	42.4 ± 10.8	<0.001
MethylAge	year	55.0 ± 19.7	40.9 ± 20.7	0.001
BMI	kg/m^2	29.0 ± 4.2	23.6 ± 3.6	<0.001
Waist circumference	cm	99.4 ± 10.6	81.8 ± 10.3	<0.001
LDL-C	mmol/L	3.6 ± 1.0	3.0 ± 0.7	<0.001
HDL-C	mmol/L	1.6 ± 0.4	1.7 ± 0.4	0.001
TG	mmol/L	1.9 ± 1.4	1.0 ± 0.5	<0.001
CRP	mg/L	2.8 ± 3.6	1.8 ± 2.7	0.059
MDA	mM	3.0 ± 0.8	2.6 ± 0.6	0.003

Results are expressed as mean ± SD. MS, metabolic syndrome; MethylAge, DNA methylation age; BMI, body mass index; LDL-C, low-density-lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride, CRP, C-reactive protein; MDA, malondialdehyde; SD, standard deviation.



Indicator; MethylAge	Non-MS (n = 149)	MS (n = 147)
Minimum	3.0	16.4
The 1st quartile	28.5	38.6
Median	33.5	61.7
The 3rd quartile	56.6	70.0
Maximum	90.4	85.6

Fig. 1. **MS and MethylAge.**

Horizontal line indicates median, Bar shows minimum and maximum, box shows from the 1st to the 3rd quartile. MS, metabolic syndrome; MethylAge, DNA methylation age.

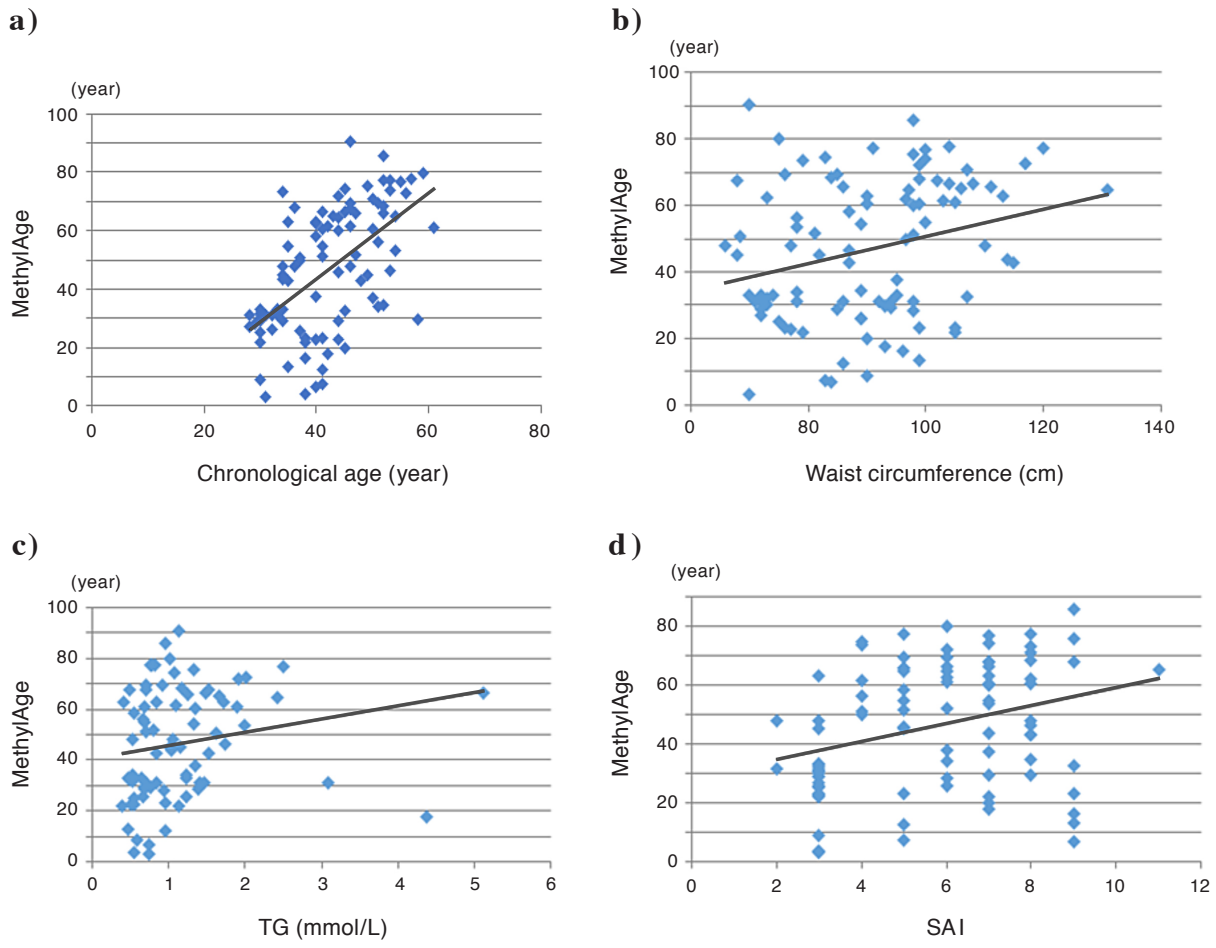


Fig. 2. **Correlation analysis with MethylAge.**

a) Chronological age, **b)** Waist circumference, **c)** TG, **d)** SAI. **a:** $r = 0.594$, $p < 0.05$, **b:** $r = 0.261$, $p < 0.05$, **c:** $r = 0.317$, $p < 0.05$, **d:** $r = 0.318$, $p < 0.05$, $n = 149$. MethylAge, DNA methylation age; TG, triglyceride; SAI, skin aging index.

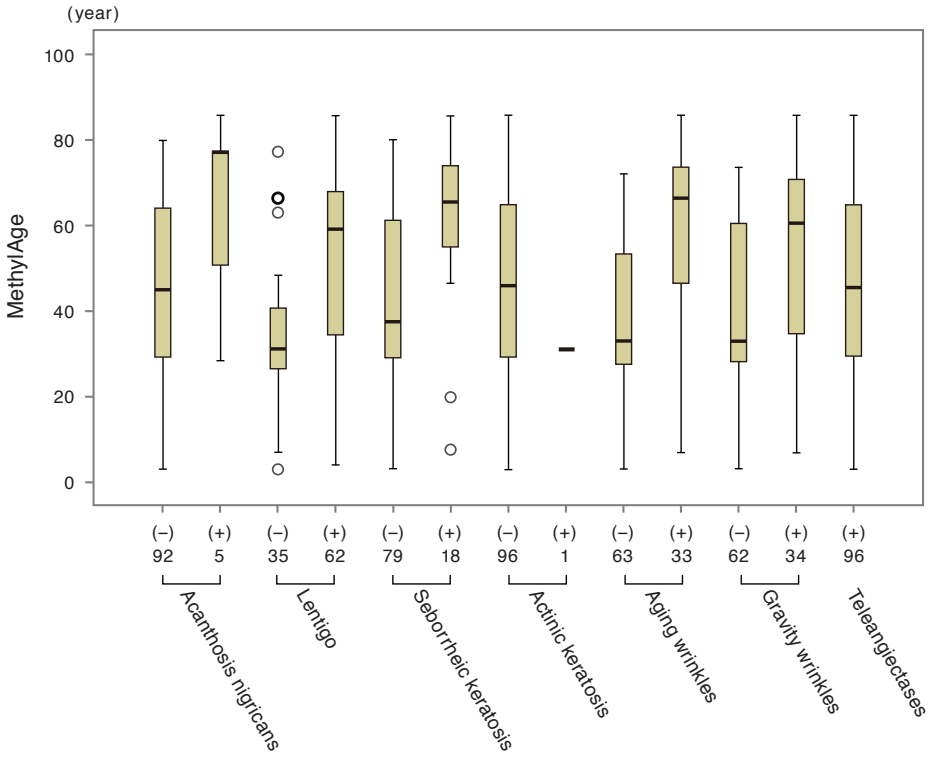


Fig.3. **MethylAge in lesion and non-lesion areas.**

Horizontal line indicates median, bar shows minimum and maximum, and box shows from the 1st to the 3rd quartile. (+) lesion aareas, (–) non-lesion areas. Digit show number of cases. All skin problems or different skin problems do not have all MethylAge data, that is why in graph we have different number of cases. MethylAge, DNA methylation age.

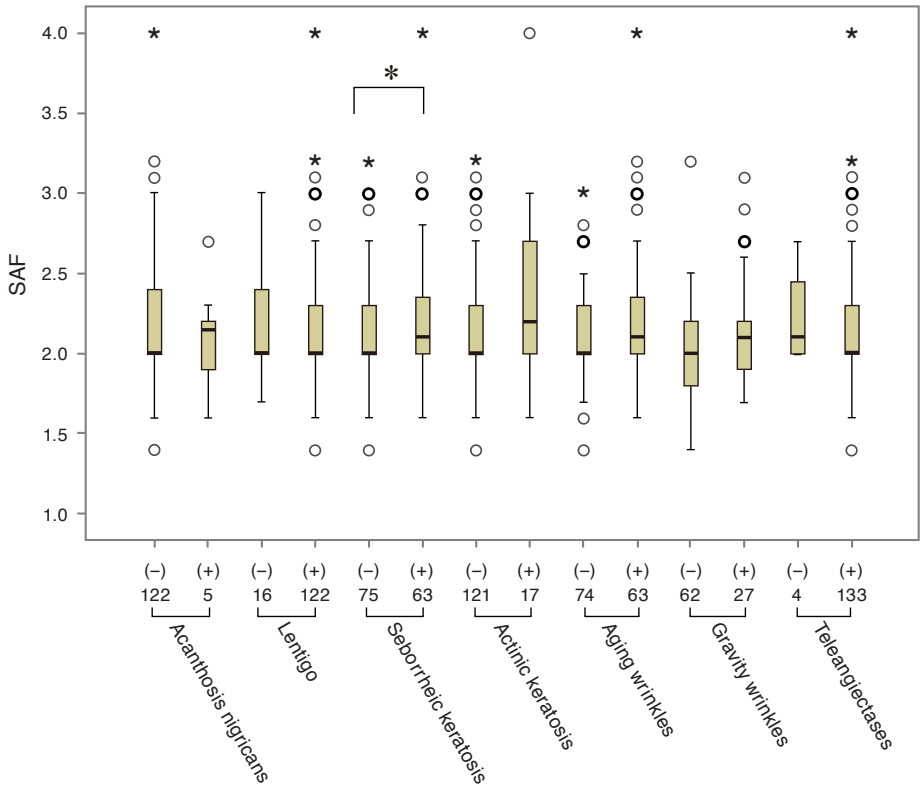


Fig.4. **SAF in lesion and non-lesion areas.**

Horizontal line indicates median, Bar shows minimum and maximum, box shows from the 1st to the 3rd quartile. Digit show number of cases. * $p < 0.05$ by t test. SAF, skin auto fluorescence.

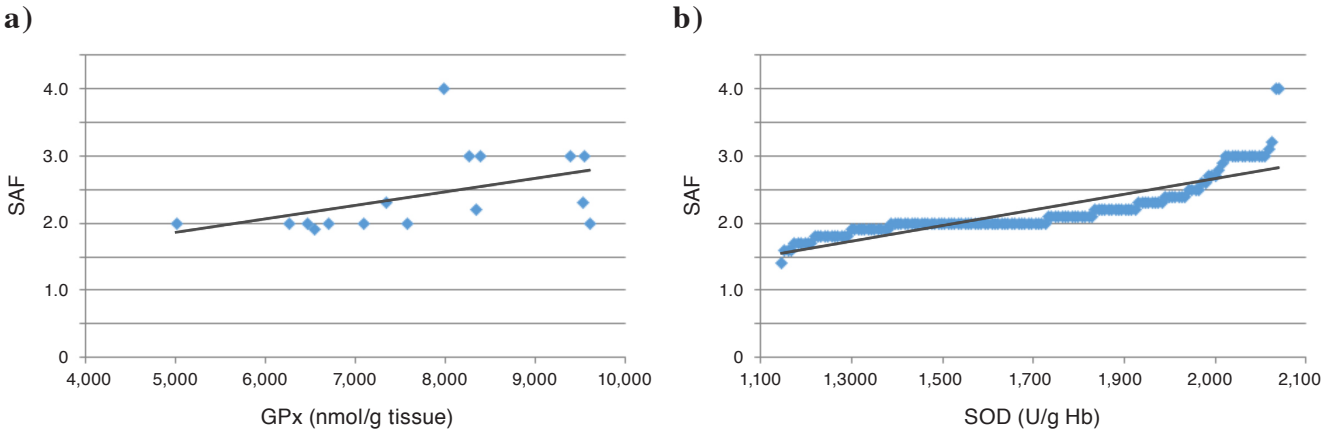


Fig. 5. **SAF and oxidative stress index: GPx, SOD.**

a) GPx, **b)** SOD. Regression line **a:** n = 16, **b:** y = 0.001164x + 0.219931, R^2 = 0.753, p < 0.001, n = 16. SAF, skin auto fluorescence; GPx, gulutathion; SOD, superoxide dysmutase.

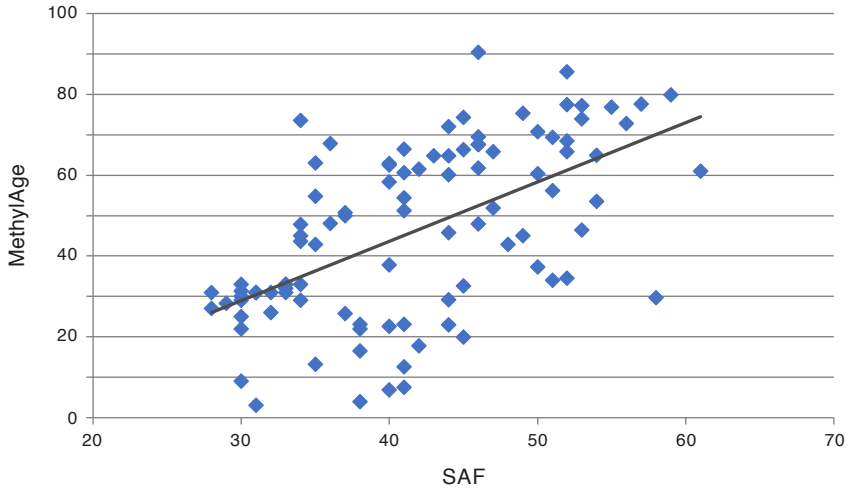


Fig. 6. **SAF and MethylAge.**

Regression line; y = 131.9x^2 – 490.1x + 491.5, R^2 = 0.989, p < 0.001, n = 150. SAF, skin auto fluorescence; MethylAge, DNA methylation age.

Discussion

In this study we measured the MethylAge and analyzed its relationship to physical information and differences by skin lesion. The items that showed significant correlation with MethylAge were chronological age, waist circumference, TG, SAI, and SAF. Skin lesions included seborrheic keratosis, actinic keratosis, epidermoid melanoma, aging wrinkles, gravity wrinkles, and facial telangiectasia, of which seborrheic keratosis and lentigo pigmentosa were the items that showed differences in methylation age between lesion and non-lesion areas.

DNA hydroxymethylation, which is the criterion for MethylAge, has been elucidated to occur as an epigenomic change and can be physiological or non-physiological. The process of non-physiological changes involves oxidation and carbonylation reactions at the molecular level, which are involved in lesion formation and aging of MethylAge. These issues are discussed below, citing the literature.

How epigenetic is linked to aging?

Epigenetics has been defined as heritable changes in gene function that take place without a change in the DNA sequence. The study of epigenetics and its involvement in metabolic diseases is still a young research field, but it is now attracting a lot of attention and growing at a fast pace. Methodological improvements, with crucial progress each year, have contributed to the current interest and advancements in the field. The epigenome includes DNA methylation, histone modifications, and non-coding RNAs, which can regulate cell differentiation, cell-specific gene expression, parental imprinting, X chromosome inactivation, as well as genomic stability and structure. DNA methylation takes place on a cytosine, mainly in CG context or the so-called CpG sites, and to a less extent in non-CG context [16].

The degree of active or inactive states of our genes is dynamic and can be impacted by environment; UV exposure, radiation, chemicals, medications, and DNA methylation.

Age dependent changes in DNA methylation include global hypomethylation Region specific hypermethylation [17]. This finding has impelled researchers to develop age predictors based on the correlation between methylation changes and chronological age.

DNA methylation, epigenetic modification, refers to the transfer of methyl (CH_3) group from S-adenosyl methionine (SAM) to the fifth position of cytosine nucleotides, forming 5-methylcytosine (5mC). Important that abnormal methylation plays an important role in the pathogenesis of autoimmune disease, metabolic syndromes, and neurological disorders [18].

Factors impacting epigenetic age we can divide into extrinsic and intrinsic. Insulin and its resistance, CRP, BMI and waist-to-hip ratio, systolic blood pressure all these factors mat impact epigenetic age.

Important factor for accelerated ageing is obesity. Telomere length is inversely correlated with lifespan, and telomere dysfunction accelerates the aging process. Obesity is associated with chronic latent inflammation and oxidative stress, that may accelerate shortening of telomeres. Human studies indicate that telomere shortening is directly correlated to adiposity, and telomere length is inversely associated with BMI [19].

Epigenetics and obesity

The prevalence of obesity is increasing at a rate that cannot be explained by genetic factors; rather environmental factors are the likely driver. Because of aging populations and an increasing prevalence of obesity, the number of patients with type 2 diabetes mellitus (T2DM) is increasing at alarming rates worldwide, and the current prevalence of 422 million people is expected to rise to 592 million in 2035. Of note, 5 million people die from diabetes every year, most often because of cardiovascular events [20]. Considering the rate of the obesity epidemic, it is an appealing thought that our genes are programmed to store fat or to store as much as possible of all the excess energy the body is exposed to [21]. Prospective studies comparing the DNA methylome from various tissues before and after weight loss have shown small but widespread changes across the genome and suggested that baseline DNA methylation can be used as a biomarker for the outcome, *i.e.*, the weight loss response [22]. Obesity and related phenotypes induce epigenetic dysregulation, seen as increased variability in DNA methylation. Obesity is considered a heterogeneous disease, which could influence the outcome, together with different genetic background, gender, and tissue specificity. The latter is an important limitation, as many studies are performed on blood cells, which most likely do not contribute to obesity.

DNA glycation

In recent years, the role of AGEs in promoting and exacerbating metabolic and skin abnormalities has received increasing attention. AGEs are produced when reducing sugars are non-enzymatically bound to proteins and lipids. This process is facilitating by is facilitated by the hyperglycemic and hyperlipidemic environment characteristic of many metabolic disorders, including diabetes, obesity, MS, and their complications [15]. AGEs play crucial role in the pathogenesis of CVD, kidney, Alzheimer's, neurological, T2DM and joints diseases and aging process. Depletion of cellular antioxidant GSH led to increase binding of glucose derivatives to DNA. DNA can be cross-linked with different substances, some of the nucleotide AGEs are N2-carboxymethyl oxyguanosine, 5-glycolyldeoxycytidine [23].

AGEs yield characteristics type of nucleotide adducts which are indicator of many abnormal conditions, *i.e.*, oxidative stress, arthritis. AGEs cause damage to DNA which is associated with important factors for mutagenesis, carcinogenesis and diabetes mellitus [24]. Reaction with sugars and free amino group on DNA is the first step in the evolution of these molecules through a complex series of very slow reactions in the body known as Amadori reactions, Schiff base reactions, and Maillard reactions, which may all lead to AGEs. Some AGEs are very useful compounds, but others are very reactive as they are responsible in many age-related chronic diseases what was mention above [25]. It effects the nucleic acid by modifying, mutation and breaking the strand of DNA which are the process on glycation. Glycation of DNA gives rise to characteristic nucleotide adduct, some of which have been found to increase in oxidative stress. Potent glycating agents in physiological system are a-oxoaldehydeglyoxal, methylglyoxal (MGO) and 3-deoxyglucosone (3DG) [26]. These adducts are also formed by glycation of DNA with glucose and ascorbic acid; since, glucose and other saccharide derivatives fragment to form glyoxal (GO) and MGO in the early stages of glycation reaction-the Namiki pathway of glycation [27]. Glycated DNA is a toxic compound called as glycotoxin may contribute to the toxicity of several anti-tumor agents and over expression of enzymatic anti-glycation defense with multi drug resistance in major classes of tumors [28].

AGEs, oxidative stress and carbonyl stress

AGE formation takes place under normal physiologic conditions but is accelerated in hyperglycemia. AGEs may also form from non-glucose sources including lipid and amino acid oxidation [29].

Increased level of ROS cause oxidative stress; in analogy, increased concentration of sugars (glucose, deoxyglucose, fructose, ribose and triose phosphates) and active dicarbonyl compounds (GO, MGO) can cause "carbonyl stress" resulting in the increased rate of formation of AGEs [30].

Accumulation of glycation products is associated with various diseases including, first of all, diabetes and diabetic nephropathy, microangiopathy and atherosclerosis. Regarding atherosclerosis, AGEs play a significant role in the formation and progression of atherosclerosis lesions AGE-cross link formation results in arterial stiffening with the loss of elasticity of large vessels [30]. Increased AGE accumulation in the diabetic vascular tissues has been associated with the changes in functions of endothelial cells, macrophages and smooth muscles cells [31].

Significance of the present results

In the present study, MethylAge was associated with waist circumference, hypertriglyceridemia, the presence of MS, and also with SAF, suggesting that glycative stress may play a major role. Carbohydrate-derived aldehydes, *i.e.*, GO, MGO, have been shown to be formed [32,33]. TG, once oxidized, also produces fatty acid-derived aldehydes,

i.e., MDA, HNE, acrolein. The high reactivity of these aldehydes suggests that they may induce non-physiological carbonylative modifications at the epigenomic modification sites of histone proteins or DNA. In the future, it is necessary to take into account non-physiological epigenomic changes in order to promote health and prevent pathological aging.

Conclusions

Assessment of MethylAge using skin samples may be an important indicator of the degree of physiological aging of the body undergoing epigenomic changes due to glycative stress and oxidative stress. The mechanism by which glycative stress affects MethylAge requires further investigation.

Funding

None in particular.

Conflict of interest declaration

There are no specific applicable matters.

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