ORIGINAL PAPER

Nagoya J. Med. Sci. **84**. 60–68, 2022
doi:10.18999/nagjms.84.1.60

DNA methylation is associated with muscle loss in community-dwelling older men -the Yakumo study- : a preliminary experimental study

Daisaku Kato[1], Yasuhiko Takegami[1], Taisuke Seki[1], Hiroaki Nakashima[1], Yusuke Osawa[1], Koji Suzuki[2], Hiroya Yamada[3], Yukiharu Hasegawa[4] and Shiro Imagama[1]

[1]*Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Preventive Medical Sciences, Fujita Health University School of Medicine, Toyoake, Japan*
[3]*Department of Hygiene, Fujita Health University School of Medicine, Toyoake, Japan*
[4]*Department of Rehabilitation, Kansai University of Welfare Science, Kashiwabara, Japan*

ABSTRACT

Frailty is a state of reduced muscle strength and activity in older people. DNA methylation is associated with osteoporosis and muscle loss in murine and other animal studies, but there are no epidemiological studies in humans. This study aimed to assess the association of osteoporosis and muscle loss with DNA methylation in community-dwelling older people. This cross-sectional study was performed in a rural part of Japan. We analyzed 204 subjects (98 men and 106 women). In univariate analysis, the two groups were compared according to the presence or absence of osteoporosis and of muscle loss. Logistic regression analysis was performed to determine predictors of frailty in the muscle loss group. We used age, sex, body mass index, smoking history, drinking history, serum albumin and C-reactive protein levels, diabetes, hypertension, hyperlipidemia, heart disease history, and LINE-1 DNA methylation as the factors. Probability values < 0.05 were considered to be statistically significant. The levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in men over the age of 60. LINE-1 DNA methylation levels were not associated with bone mineral density in either the men or women over the age of 60. LINE-1 DNA methylation levels in leukocytes correlated significantly with the risk of frailty in men over the age of 60. Promoting an understanding of DNA methylation may lead to a better understanding of the pathophysiology of muscle loss.

Keywords: cross-sectional study, frailty, LINE-1 DNA methylation, muscle loss, osteoporosis

Abbreviations:
BMI: body mass index
CI: confidence interval
CRP: C-reactive protein
OR: odds ratio
SMI: skeletal muscle mass index

Received: February 10, 2021; accepted: May 17, 2021

Corresponding Author: Daisaku Kato, MD

Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Tel: +81-52-741-2111, Fax: +81-52-744-2260, E-mail: dai0409kato@med.nagoya-u.ac.jp

INTRODUCTION

Frailty is a state described by the Japanese Society of Geriatrics in which "elderly people have reduced muscle strength and activity (weakness)".[1-3] Frail older people are more likely to have health problems such as dysfunction in daily living[4,5] and are more likely to be admitted to a facility,[6] at risk of falling,[7] and hospitalized.[8] In addition, their mortality rate is high.[9] Therefore, it is important to evaluate their vulnerability and apply appropriate interventions. Frailty is strongly associated with physical factors, of which muscle loss and osteoporosis are considered the significant causes.[10]

DNA methylation is a chemical reaction in which a methyl group is added to a DNA base at a cytosine base CpG island of a dinucleotide sequence CpG in DNA.[11] Methylation of CpG islands within a cluster of CpG sites in the promoter region of a gene represses that gene.[12] Methylation has the effect of directly interfering with the binding of transcription factors to the promoter region.[13] Its action is reversible and is thought to affect important biological functions such as aging and cell development.[14]

LINE-1 makes up about 17% of the human genome, is ubiquitous throughout the genome, and belongs to the family of non-long terminal repeat retrotransposons.[15] Therefore, LINE-1 methylation levels are considered to be surrogate markers for global DNA methylation.[16] It is highly methylated in normal tissue.[17] Several epidemiological studies have shown an association between global leukocyte DNA methylation levels and diseases such as cardiovascular disease and diabetes.[18,19]

DNA methylation is associated muscle loss in animal studies. DNA methylation has been shown to be essential for muscle differentiation and regeneration, and muscle regeneration was inhibited in DNA methylase knockout mice.[20] There are few epidemiological studies in humans. Gale et al showed in a cross-sectional study that there are no widespread differences in methylation patterns between physically frail and non-frail people, suggesting that blood DNA methylation is not a good biomarker of physical frailty.[21] The association between blood DNA methylation and physical frailty is still controversial. In this study, we focused on sarcopenia and osteoporosis as physical findings. This study aimed to assess the association of osteoporosis and muscle loss with DNA methylation in community-dwelling older people.

METHODS

This study was approved by the institutional review board of our institution's Ethics Committee (NUEC; approval number, 2014-0207), and the study protocol was approved by our institutional review board. All subjects gave their written informed consent. All authors approved the publication of this study.

Subjects

This research is part of the Yakumo study we are conducting based on the health examination conducted in Yakumo-cho, Hokkaido. In 2015, a total of 525 Japanese people participated in the health examination. The study excluded those who did not participate in the musculoskeletal test, did not have enough samples to measure LINE-1 DNA methylation levels, and were under the age of 60. Persons with a history of cancer, stroke, or ischemic heart disease and who did not provide informed consent were also excluded (Table 1). Eventually, 204 eligible persons (98 men and 106 women) remained in this analysis.

Table 1 Demographic, blood test results and lifestyle habits of the participants

	>60 years	Men	Women	P value
Variable	n=204	n=98	n=106	
Age (years)	68.7±5.86	68.9±5.65	68.5±6.08	0.627[a]
SMI (kg/m^2)	6.93±0.98	7.62±0.66	6.28±0.75	<0.001[a]
Muscle loss, n	41 (20.1%)	16 (16.3%)	25 (23.6%)	0.223[b]
Back strength (kg)	75.3±29.7	96.3±25.5	55.6±17.2	<0.001[a]
BMD %YAM	77.6±14.2	82.8±15.7	72.9±10.7	<0.001[a]
Knee OA, n	72 (35.3%)	29 (29.6%)	43 (40.6%)	0.109[b]
BMI (kg/m^2)	23.4±3.13	23.9±2.44	22.8±3.59	<0.05[a]
Serum albumin (mg/dl)	4.28±0.26	4.28±0.29	4.28±0.24	0.962[a]
C-reactive protein (mg/dl)	0.12±0.33	0.15±0.42	0.09±0.20	0.162[a]
DM, n	43 (21.1%)	23 (23.5%)	20 (18.9%)	0.493[b]
Hypertension, n	80 (39.2%)	44 (44.9%)	36 (34.0%)	0.453[b]
Hyperlipidemia n	65 (31.9%)	27 (27.6%)	38 (35.8%)	0.102[b]
Heart disease, n	7 (3.4%)	6 (6.1%)	1 (0.9%)	0.0933[b]
Smoking habit				<0.05[b]
+, n	34 (16.7%)	17 (17.3%)	7 (6.6%)	
–, n	170 (83.3%)	81 (82.7%)	99 (93.7%)	
Alcohol consumption				<0.001[b]
Current, n	98 (48.0%)	72 (73.5%)	26 (24.5%)	
Former, n	7 (3.4%)	5 (5.1%)	2 (1.9%)	
Never, n	99 (48.5%)	21 (21.4%)	78 (73.6%)	

SMI: skeletal muscle mass index
BMD: bone mineral density
YAM: young adult mean
BMI: body mass index
DM: diabetes mellitus
[a]Mann-Whitney U test, [b]Fisher's exact test.

Data collection

We used a self-administered questionnaire during the health examination and obtained health information such as smoking habit (never, previously, or now), alcohol consumption (never, previously, or now), and history of significant illness (yes/no). We also obtained basic physical data such as height, weight, waist circumference, and blood pressure. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2).

Muscle mass evaluation

Skeletal muscle mass index (SMI) was measured by the bioelectrical impedance analysis method using a medical body composition meter (InBody 720, Biospace). Based on the sarcopenia diagnostic criteria of the Sarcopenia Asia Working Group, SMI <7.0 kg/m^2 for men and SMI <5.4 kg/m^2 for women were defined as muscle loss groups. The bioelectrical impedance analysis method measures the electrical resistance (impedance) of tissues by passing weak alternating current electricity through the living body and uses the fact that the electrical resistance differs

depending on muscles, bones, adipose tissue, and other tissue. A previous report described this method.[22] The subject stands on a table and holds electrodes in both hands. The measurement time is about 90 seconds, and the result can be explained to the subject immediately. This is a non-invasive and easy way to determine SMI.

Blood tests

Blood samples taken during the medical examination were mixed in tubes with ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 1500 × *g* for 10 minutes. Erythrocytes were removed from the buffy coat sample. The buffy coat was collected and treated with a lysis solution (pH 7.4) containing 150 mmol/L NH_4Cl, 14 mmol/L $NaHCO_3$, and 0.11 mmol/L EDTA-2Na in distilled water to remove erythrocytes. Extraction of DNA from peripheral leukocytes was done using a NucleoSpin® Tissue kit (Takara, Japan). The extracted DNA (500 ng, 50 ng/μL) was then converted from unmethylated cytosine to uracil. It was treated with sodium bisulfite according to the manufacturer's protocol using an EpiTect Fast DNA bisulfite kit (QIAGEN, Germany). To amplify the LINE-1 element, a polymerase chain reaction was performed using EpiTaq™ HS (for bisulfite-treated DNA) (Takara). Methylation was measured by the pyrosequencing method using the PyroMark Q24 Advanced system (QIAGEN) with sequence primers 5´-AGTTAGGTGT-GGGATATAGT-3´ and PyroMark® Q24 Advanced CpG reagent (QIAGEN). The level of LINE-1 DNA methylation was expressed as a percentage of methylated cytosine. LINE-1 DNA methylation at the three CpG sites was quantified to calculate mean LINE-1 DNA methylation levels.

Serum albumin, whole blood hemoglobin A1c, and serum lipids were measured by an automated analyzer, and serum C-reactive protein (CRP) concentrations were measured by a latex-immunoassay (LT-auto Wako CRP; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at Yakumo General Hospital.

Statistical analysis

The continuous parameters are presented as mean ± standard deviation. In the univariate analysis, the two groups were compared according to the presence or absence of osteoporosis and of muscle loss. Multivariate-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for hypermethylation were calculated by logistic regression analysis in the muscle loss groups. We used age, sex, BMI, smoking history, drinking history, serum albumin level, CRP level, diabetes, hypertension, hyperlipidemia, heart disease history, and LINE-1 DNA methylation as the factors. Probability values less than 0.05 were considered to be statistically significant. Statistical analyses were performed using EZR (Jichi Medical School, Tochigi, Japan).

RESULTS

The participants' demographic factors, blood test results, and physical performance data are listed in Table 1. BMI ($p<0.01$), back muscle strength ($p<0.001$), bone mineral density (% young adult mean) ($p<0.001$), smoking habit ($p<0.001$), and alcohol consumption ($p<0.001$) were significantly different between the men and women; however, no significant difference was noted in age or the rate of muscle loss.

The correlation between LINE-1 DNA methylation levels and the physical factors (osteoporosis and muscle loss) are shown in Table 2. In men over 60 years old, LINE-1 DNA methylation levels of the muscle loss group were lower than those in the non-muscle loss group (muscle loss group: 55.71 ± 0.77%, non-muscle loss group: 57.01 ± 2.58%, $p<0.001$) (Figure 1). In

contrast, there were no significant differences in LINE-1 DNA methylation between the muscle loss group and the non-muscle loss group in the women over 60 years (muscle loss group: 56.80 ± 2.18%, non-muscle loss group: 57.23 ± 3.10%, p=0.521). Further, the LINE-1 DNA methylation level did not differ between the osteoporosis group and the non-osteoporosis group (men over 60 years, osteoporosis group: 57.37 ± 3.33%, non-osteoporosis group: 56.81 ± 2.42%, p=0.387; women over 60 years, osteoporosis group: 57.03 ± 2.27%, non-muscle loss group: 57.19 ± 3.30%, p=0.653) (Figure 1).

Table 2 Correlation between LINE-1 DNA methylation levels and physical factors of osteoporosis and muscle loss

	r	P value
Men >60 years (n=98)		
Osteoporosis (BMD: YAM <70%)	0.0136	0.897
Muscle loss (SMI <7.0 kg/m^2)	0.0321	0.756
Women >60 years (n=104)		
Osteoporosis (BMD: YAM <70%)	0.0195	0.845
Muscle loss (SMI <5.4 kg/m^2)	–0.00573	0.954

BMD: bone mineral density
YAM: young adult mean
SMI: skeletal muscle mass index
Pearson's correlation coefficient.

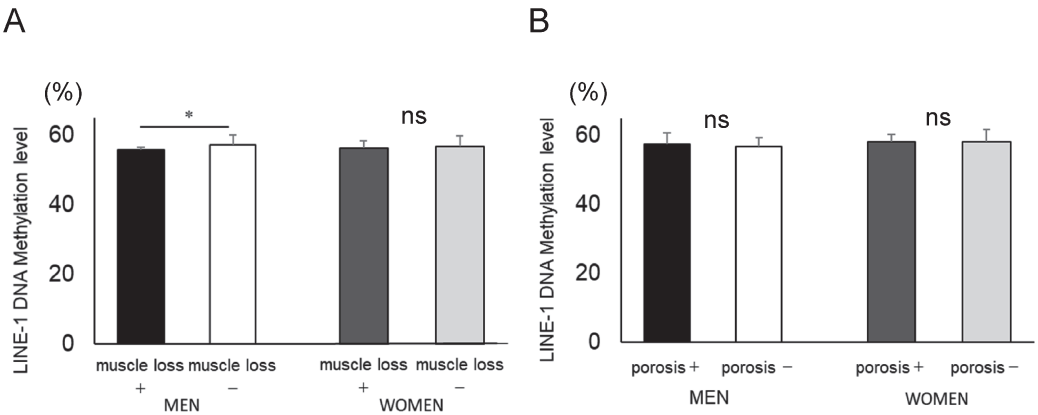

Fig. 1 Differences in LINE-1 DNA methylation levels in leukocytes according to the physical factors of osteoporosis and muscle loss

Fig. 1A: In men over 60 years old, LINE-1 DNA methylation levels of the muscle loss group were lower than those in the non-muscle loss group. In contrast, there were no significant differences in LINE-1 DNA methylation between the muscle loss group and the non-muscle loss group in women over 60 years old.

Fig. 1B: The LINE-1 DNA methylation levels did not differ between the osteoporosis group and the non-osteoporosis group for either sex. The statistical differences shown on each graph were analyzed by Student t-test.

*p<0.001. porosis osteoporosis.

Table 3 shows multivariate adjusted ORs and 95% CIs for LINE-1 DNA methylation according to muscle loss. The men over 60 years with muscle loss had significantly lower ORs for hypermethylation than those with non-muscle loss (OR, 0.49; 95% CI, 0.27–0.86, p=0.012). In this logistic regression analysis, age, sex, smoking history, drinking history, serum albumin level, CRP level, diabetes, hypertension, hyperlipidemia, and heart disease history were not significantly correlated with muscle loss, whereas BMI was correlated (OR, 0.50; 95% CI, 0.33–0.75, p<0.001).

Table 3 Multivariate adjusted ORs and 95% CIs for LINE-1 DNA methylation according to muscle loss

	Muscle loss in men >60 years old (n=98)	
	SMI ≥7.0 kg/m^2	SMI <7.0 kg/m^2
LINE-1 DNA methylation Levels (%)	57.01±2.58	55.71±0.77
n (%)	82 (83.7)	16 (16.3)
OR (95% CI)	1.00	0.49 (0.27–0.86) P=0.012

OR: odds ratio
CI confidence interval
SMI: skeletal muscle mass index

The ROC curve of the predicted value of LINE-1DNA methylation level for the presence or absence of the risk of frailty in men over 60 years of age showed an area under the curve of 0.685 (Figure 2).

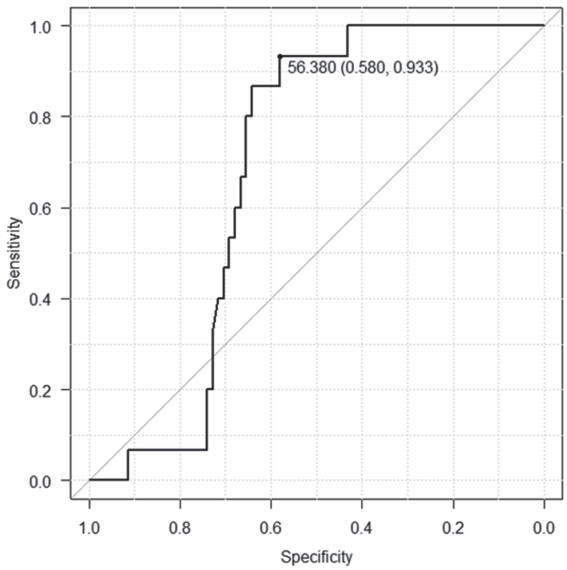

Fig. 2 The relationship between LINE-1 DNA methylation and muscle loss as shown by ROC curve
The area under the ROC curve for the predicted value of LINE-1 DNA methylation level for muscle loss in men over 60 years old was 0.683.

DISCUSSION

Few studies have investigated the relationship between the risk of frailty in the general population and LINE-1 DNA methylation in leukocytes. We showed that the levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in men over 60 years old. However, LINE-1 DNA methylation levels were not associated with bone mineral density in either men or women over the age of 60.

The levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in the men aged 60 and older. A previous study also showed that LINE-1 mRNA expression and the DNA copy count increased with age in mouse skeletal muscle.[23] Another study showed that skeletal muscle-specific Dnmt3a KO impairs skeletal muscle regeneration and causes muscle loss. At that time, the level of DNA methylation was reduced.[24]

Further, the association between muscle loss and the expression of LINE-1 methylation was present only in the men. A study reported that sex hormone-binding globulin and testosterone were associated with genome-wide DNA methylation.[25] The decrease in secreted sex hormones is significantly affected by aging, especially in men. Age-related muscle loss begins around the age of 40, and over the 35 years from 40–44 years to 75–79 years, about 11% of males and 6% of females lose limb muscle mass.[26] The higher rate of decrease in men versus women is thought to be related to age-related changes in the endocrine system, such as in the amount of testosterone secreted, which has anabolic effects.[27]

The LINE-1 DNA methylation levels were not associated with bone mineral density in either the men or women over the age of 60. The relationship between osteoporosis and LINE-1 methylation has been debated. An animal study showed the association between osteoporosis and LINE-1 methylation by DNA methylation regulation of vitamin D biosynthesis.[28] In contrast, there is a report that osteoporosis and methylation are related in Alu elements but not in LINE-1, and thus, further investigation is considered necessary.[29]

It is possible to prevent the progression of frailty if early screening can be performed. Several studies showed that LINE-1 methylation could be used as a biomarker in cardiovascular disease, dyslipidemia, and chronic liver diseases. We investigated LINE-1 DNA methylation in men over 60 years as a biomarker associated with frailty, but its AUC value was rather low at 0.683. Further consideration is thus needed regarding its usefulness as a biomarker. In recent years, other reports have shown that other epigenetic factors such as microRNA are useful as biomarkers in liver disease,[30] chronic kidney disease,[31] and osteoporosis.[32] Thus, other factors such as microRNA need to be considered for the early diagnosis of frailty.

This study has some limitations. First, because it is a cross-sectional study, an association was shown but not a causal relationship. Second, the number of participants is relatively small. Third, the study targeted rural residents, who may have different living and working environments than urban residents, and this may have influenced the determination of risk levels of frailty.

In conclusion, LINE-1 DNA methylation levels in leukocytes correlated significantly with the risk of frailty in men over 60 years of age. Promoting an understanding of DNA methylation may lead to a better understanding of the pathophysiology of muscle loss.

ACKNOWLEDGMENTS

We are grateful to the staff of the Comprehensive Health Care Program held in Yakumo, Hokkaido.

SOURCES OF FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (B), 2014-2016, No. 26293144.

CONFLICTS OF INTEREST

None

REFERENCES

1 Satake S, Senda K, Hong YJ, et al. Validity of the Kihon Checklist for assessing frailty status. *Geriatr Gerontol Int*. 2016;16(6):709–715. doi:10.1111/ggi.12543.
2 Satake S, Arai H. The revised Japanese version of the Cardiovascular Health Study criteria (revised J-CHS criteria). *Geriatr Gerontol Int*. 2020;20(10):992–993. doi:10.1111/ggi.14005.
3 Fried LP, Tangen CM, Walston J, et al. Frailty in older adults: evidence for a phenotype. *J Gerontol A Biol Sci Med Sci*. 2001;56(3):M146–M156. doi:10.1093/gerona/56.3.m146.
4 Al Snih S, Graham JE, Ray LA, Samper-Ternent R, Markides KS, Ottenbacher KJ. Frailty and incidence of activities of daily living disability among older Mexican Americans. *J Rehabil Med*. 2009;41(11):892–897. doi:10.2340/16501977-0424.
5 Pahor M, Blair SN, Espelandet M, et al. Effects of a physical activity intervention on measures of physical performance: results of the lifestyle interventions and independence for Elders Pilot (LIFE-P) study. *J Gerontol A Biol Sci Med Sci*. 2006;61(11):1157–1165. doi:10.1093/gerona/61.11.1157.
6 Cegri F, Orfila F, Abellana RM, Pastor-Valero M. The impact of frailty on admission to home care services and nursing homes: eight-year follow-up of a community-dwelling, older adult, Spanish cohort. *BMC Geriatr*. 2020;6;20(1):281. doi:10.1186/s12877-020-01683-9.
7 Nowak A, Hubbard RE. Falls and frailty: lessons from complex systems. *J R Soc Med*. 2009;102(3):98–102. doi:10.1258/jrsm.2009.080274.
8 Boyd CM, Xue QL, Simpson CF, Guralnik JM, Fried LP. Frailty, hospitalization, and progression of disability in a cohort of disabled older women. *Am J Med*. 2005;118(11):1225–1231. doi:10.1016/j.amjmed.2005.01.062.
9 Nguyen AT, Nguyen TX, Nguyen TN, et al. The impact of frailty on prolonged hospitalization and mortality in elderly inpatients in Vietnam: a comparison between the frailty phenotype and the Reported Edmonton Frail Scale. *Clin Interv Aging*. 2019;20;14:381–388. doi:10.2147/CIA.S189122.
10 Greco EA, Pietschmann P, Migliaccio S. Osteoporosis and sarcopenia increase frailty syndrome in the elderly. *Front Endocrinol (Lausanne)*. 2019;10:255. doi:10.3389/fendo.2019.00255.
11 Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003;33 Suppl:245–254. doi:10.1038/ng1089.
12 Kulis M, Esteller M. DNA methylation and cancer. *Adv Genet*. 2010;70:27–56. doi:10.1016/B978-0-12-380866-0.60002-2.
13 Bayarsaihan D. Epigenetic mechanisms in inflammation. *J Dent Res*. 2011;90(1):9–17. doi:10.1177/0022034510378683.
14 Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. *Nature*. 2007;447(7143):433–440. doi:10.1038/nature05919.
15 Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860–921. doi:10.1038/35057062.
16 Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by Methy Light. *Nucleic Acids Res*. 2005;33:6823–6836. doi:10.1093/nar/gki987.
17 Yang AS, Estécio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res*. 2004;32:e38. doi:10.1093/nar/gnh032.
18 Muka T, Nano J, Voortman T, et al. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: a systematic review. *Nutr Metab Cardiovasc Dis*. 2016;26(7):553–566. doi:10.1016/j.numecd.2016.04.002.

19 Zhong J, Agha G, Baccarelli AA. The role of DNA methylation in cardiovascular risk and disease: methodological aspects, study design, and data analysis for epidemiological studies. *Circ Res*. 2016;118(1):119–131. doi:10.1161/CIRCRESAHA.115.305206.
20 Naito M, Mori M, Inagawa M, et al. Dnmt3a regulates proliferation of muscle satellite cells via p57Kip2. *PLoS Genet*. 2016;12(7):e1006167. doi:10.1371/journal.pgen.1006167.
21 Gale CR, Marioni RE, Harris SE, et al. DNA methylation and the epigenetic clock in relation to physical frailty in older people: the Lothian Birth Cohort 1936. *Clin Epigenetics*. 2018;10:101. doi:10.1186/s13148-018-0538-4.
22 Tanaka S, Ando K, Kobayashi K, et al. Low bioelectrical impedance phase angle is a significant risk factor for frailty. *Biomed Res Int*. 2019:6283153. doi:10.1155/2019/6283153.
23 De Cecco M, Criscione SW, Peterson AL, Neretti N, Sedivy JM, Kreiling JA. Transposable elements become active and mobile in the genomes of aging mammalian somatic tissues. *Aging*. 2013;5(12):867–883. doi:10.18632/aging.100621.
24 Hatazawa Y, Ono Y, Hirose Y, et al. Reduced Dnmt3a increases Gdf5 expression with suppressed satellite cell differentiation and impaired skeletal muscle regeneration. *FASEB J*. 2018;32(3):1452–1467. doi:10.1096/fj.201700573R.
25 Arathimos R, Sharp GC, Granell R, Tilling K, Relton CL. Associations of sex hormone-binding globulin and testosterone with genome-wide DNA methylation. *BMC Genet*. 2018;19(1):113. doi:10.1186/s12863-018-0703-y.
26 Yamada M, Moriguch Y, Mitani T, Aoyama T, Arai H. Age-dependent changes in skeletal muscle mass and visceral fat area in Japanese adults from 40–79 years of age. *Geriatr Gerontol Int*. 2014;14 Suppl 1:8–14. doi:10.1111/ggi.12209.
27 Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab*. 2001;86(2):724–731. doi:10.1210/jcem.86.2.7219.
28 Liu J, Liu J, Duan S, at al. Reprogrammed epigenetic landscape-prophesied functions of bioactive polysaccharides in alleviating diseases: a pilot study of DNA methylome remodeling in astragalus polysaccharide (APS)-improved osteoporosis in a rat model. *J Agric Food Chem*. 2020;68(52):15449–15459. doi:10.1021/acs.jafc.0c06483.
29 Rerkasem K, Rattanatanyong P, Rerkasem A, et al. Higher Alu methylation levels in catch-up growth in twenty-year-old offsprings. *PLoS One*. 2015;25;10(3):e0120032. doi:10.1371/journal.pone.0120032.
30 Yamada H, Suzuki K, Ichino N, et al. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. *Clin Chim Acta*. 2013;424:99-103. doi:10.1016/j.cca.2013.05.021.
31 Fujii R, Yamada H, Munetsuna E, et al. Associations of circulating MicroRNAs (miR-17, miR-21, and miR-150) and chronic kidney disease in a Japanese population. *J Epidemiol*. 2020;5;30(4):177–182. doi:10.2188/jea.JE20180233.
32 Nakashima H, Ando K, Kobayashi K, et al. Associations of serum microRNA with bone mineral density in community-dwelling subjects: The Yakumo Study. *Biomed Res Int*. 2020;2020:5047243. doi:10.1155/2020/5047243.