Life Sciences 224 (2019) 241–248

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

DNA methylation and hydroxymethylation are associated with the degree of coronary atherosclerosis in elderly patients with coronary heart disease

Dan Jiang[a,b], Min Sun[a], Linna You[a], Kai Lu[a], Lei Gao[a,b], Chunxiao Hu[a,b], Shiyong Wu[a,b], Guanglei Chang[a], Hongmei Tao[a], Dongying Zhang[a,*]

[a] Department of Cardiology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China
[b] Laboratory Research Center, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

ARTICLE INFO

Keywords:
Methylation
Hydroxymethylation
Coronary heart disease
Elderly

ABSTRACT

Aims: DNA methylation and hydroxymethylation are significantly related to the occurrence and development of coronary heart disease (CHD) and atherosclerosis (AS). 5-Methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) are used to assess DNA methylation and hydroxymethylation levels, respectively. However, 5-mC and 5-hmC levels associated with CHD remain controversial. In the present study, we aimed to investigate the association of the peripheral blood levels of 5-mC and 5-hmC and the degree of coronary atherosclerosis in elderly CHD patients.

Main methods: 5-mC and 5-hmC levels in peripheral blood mononuclear cells (PBMCs) were measured in 44 CHD patients and 42 matched control subjects by ELISA and dot blot analysis. Immunohistochemical staining was used to observe 5-mC, 5-hmC and TET2 expression in human aortic tissue. Gensini score was used to evaluate the degree of coronary atherosclerosis.

Key findings: 5-mC and 5-hmC levels in PBMCs from CHD patients and in human aortic atherosclerosis plaque were both higher than those in control subjects and in tissue samples. TET2 expression was significantly up-regulated in CHD patients compared with control subjects, while only an increasing trend in the expression of DNMT1, DNMT3A and all the other TET genes were found. Spearman correlation analysis demonstrated that 5-mC and 5-hmC levels were positively correlated with Gensini score. 5-mC and 5-hmC were considered as the risk factors for CHD after adjustment.

Significance: DNA methylation and hydroxymethylation levels in PBMCs from elderly CHD patients were significantly increased, showing a positive correlation with the degree of coronary atherosclerosis.

1. Introduction

Recent studies have reported that age-related clonal hematopoiesis is associated with an increased risk of cardiovascular disease. The majority of the variants occurred in three genes related to DNA methylation and hydroxymethylation: DNMT3A, TET2 and ASXL1 [1,2]. DNA methylation is the process of converting cytosine (C) to 5-methylcytosine (5-mC) that is catalysed by a member of DNA methyltransferase (DNMT) family comprising DNMT1, DNMT3A and DNMT3B; DNA hydroxymethylation is the oxidation process of 5-mC to 5-hydroxymethylcytosine (5-hmC) in the presence of ten-eleven translocation (TET) enzymes, including TET1, TET2 and TET3 [3,4]. Somatic mutations in DNMT and TET genes can modify DNA methylation and hydroxymethylation levels.

Accumulating evidence suggests an important role for DNA methylation and hydroxymethylation in the development of coronary heart disease (CHD) and atherosclerosis (AS). DNA methylation and hydroxymethylation modifications can alter the genes expression without changing their sequences. Exploring 5-mC and 5-hmC signatures linked to CHD and AS could contribute to better understanding of their mechanisms and to the definition of new diagnostic markers [5]. Previous studies have focused on DNA methylation, but the results were inconsistent, with hypomethylation [6–11] and hypermethylation [12–15] associated with CHD and AS. Few studies have investigated the correlation of DNA hydroxymethylation with CHD and AS [16]. Heart tissue is the best model used to study epigenetic changes in CHD. Since the difficultly access to heart tissue, peripheral blood is much more easily accessible. Somatic mutations increased with age and the

* Corresponding author at: Department of Cardiology, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Rd, Yuzhong, Chongqing 400016, China.
E-mail address: zdy202284@126.com (D. Zhang).

https://doi.org/10.1016/j.lfs.2019.03.021
Received 21 December 2018; Received in revised form 8 March 2019; Accepted 9 March 2019
Available online 10 March 2019
0024-3205/ © 2019 Elsevier Inc. All rights reserved.

incidence was > 10% among persons over 70 years old. No report of 5-mC and 5-hmC levels in elderly CHD patients was published.

Therefore, the aims of the present study were 1) to determine the peripheral blood levels of 5-mC and 5-hmC in elderly CHD patients, and 2) to investigate the relationship between DNA methylation and hydroxymethylation and the degree of coronary atherosclerosis.

2. Patients and methods

2.1. Study population

This study was approved by the Human Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (No. 2016-39) and registered on Clinical Trials (No. NCT03462277). Additionally, written informed consent was obtained from all subjects. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. A total of 86 subjects were enrolled in the present study, including 44 CHD patients (23 acute myocardial infarction patients and 21 angina pectoris patients) and 42 control subjects matched by age and gender. Blood samples were collected from all participants. The CHD group was defined as severe stenosis of at least one lesion in a coronary artery or branches. The control group was defined as people with negative findings in coronary angiograms.

2.2. Gensini score

Gensini score is most widely used to evaluate the degree of coronary atherosclerosis by coronary angiography; it is a scoring system that evaluates the degree of luminal narrowing and the geographic importance of its location [17]. The score of a lesion equals the severity coefficient of the lesion segment multiplies by the score of stenosis degree as described previously [17–19]. The final Gensini score of a patient equals the sum of the scores of all the lesions.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood

Blood samples were obtained from all patients before coronary angiography. PBMCs were obtained by centrifugation of whole blood (~5 ml) in a mononuclear cell separation solution (Tian Jin Hao Yang Biological Manufacture Co., Ltd., Tianjin, China) at 2050 rpm for 25 min at room temperature (RT). Then, the mononuclear cell fractions were removed, washed twice with PBS, washed once with Erythrocyte lysate (Beijing Solarbio Technology Co., Ltd., Beijing, China) and centrifuged at 3000 rpm for 10 min at RT. Separated mononuclear cell samples were stored at −80 °C and were not thawed until use.

2.4. Quantification of DNA methylation and hydroxymethylation

Cytosine is converted to 5-mC in a reaction catalysed by DNMT genes, while 5-mC is converted into 5-hmC under the catalysis of TET genes. Genomic DNA was extracted from PBMCs using a Blood Genomic DNA Purification Kit (GMbiolab Co., Ltd., Taiwan, China) and quantified by a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The genomic levels of 5-mC and 5-hmC in CHD patients and control subjects were determined by a colorimetric enzyme-linked immunosorbent assay (ELISA) using a 5-mC DNA ELISA Kit and a Quest 5-hmC™ DNA ELISA Kit (Zymo Research, Irvine, CA, USA), respectively [20,21]. The kits are the most cited ELISA-based global 5-mC and 5-hmC quantification kits in the literature and provide scientists with a quick, cost-effective and reliable alternative to chromatographic methods (e.g., LC-MS/MS, HPLC) [22,23]. The assays were performed according to the manufacturer's instructions, loading 100 ng of DNA per well. The absorbance at 405 nm was captured using a Multiskan Spectrum (Thermo Electron Corporation, Waltham, MA, USA).

2.5. Dot blot assay

Dot blot analysis was performed to detect DNA methylation and hydroxymethylation levels in PBMCs. As described previously, 100 ng of genomic DNA was denatured for 10 min at 95 °C and then neutralized for 10 min at 4 °C [24,25]. After dilution to 50 ng/μl, 2 μl of diluted genomic DNA was spotted on an N^+ membrane (Amersham Hybond). The membrane was dried at RT and incubated for 30 min at 80 °C. The membrane was then blocked with 5% milk in TBS-T for 1 h and incubated overnight with a mouse anti-5-mC monoclonal antibody (1:2000) or a rabbit anti-5-hmC polyclonal antibody (1:1000) (Zymo Research, Irvine, CA, USA) in TBS-T at 4 °C. The membrane was incubated with a secondary antibody, HRP-conjugated sheep anti-mouse IgG or sheep anti-rabbit IgG (1:5000; Abbkine, Redlands, CA, USA) in TBS-T for 1 h at RT. DNA was detected by a Bio-Rad gel imaging system (Bio-Rad, Hercules, CA, USA) using enhanced chemiluminescence (Advansta, Menlo Park, USA). The dot blot intensity was quantified by the gel imaging system.

2.6. Total RNA isolation and quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from PBMCs using the Trizol reagent (Ambion, Waltham, MA, USA). The extracted sample was reverse transcribed with a QuantiNova™ Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. qRT-PCR was performed with 2× SYBR Green PCR Master Mix (QuantiNova™ SYBR Green PCR Kit, Qiagen, Germany). The quantity of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. β-Actin was also quantified and used as a loading control. All primers (Takara Bio, Dalian, China) are listed in Supplemental Table 1.

2.7. Human aortic tissue and immunohistochemiscal staining

Human aortic atherosclerotic plaque lesions (n = 5) were obtained from autopsies from elderly people with an average age of > 70 years old. The human aortic tissues used as control subjects (n = 5) were obtained from young people who died due to accidents. All human studies strictly adhered to the principles of the Declaration of Helsinki. Their family members were provided written informed consent. As described previously [26], immunohistochemiscal (IHC) staining was performed using an IHC kit (PV-9000, Zhong Shan Golden Bridge Biotechnology Corp, Beijing, China). Sections were incubated overnight with a mouse anti-5-mC monoclonal antibody (1:250; Zymo Research, Irvine, CA, USA), a rabbit anti-5-hmC polyclonal antibody (1:250; Zymo Research, Irvine, CA, USA) or a mouse anti-TET2 monoclonal antibody (1:100, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in a moist chamber at 4 °C. Then, the sections were incubated with an HRP-labeled secondary antibody for 60 min at RT. The developed tissue sections were imaged with a light microscope attached to a microscope (Axio Imager A2, Zeiss, Germany).

2.8. Quantification of plasma interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)

Blood samples were centrifuged at 3000 rpm for 10 min immediately after collection. Separated plasma samples were stored at −80 °C until use. The plasma levels of IL-1β, IL-6 and TNF-α were measured by a human IL-1β ELISA kit, a human IL-6 ELISA kit and a human TNF-α ELISA kit (ImmunoWay Biotechnology, Plano, TX, USA), respectively. Assays were performed according to the manufacturer's instructions. The absorbance at 450 nm was determined using a Multiskan Spectrum.

Table 1
Baseline characteristics of CHD patients and control subjects undergoing coronary angiography.

Characteristics	Control (n = 42)	CHD (n = 44)	*P* value
Male/female (n)	22/20	28/16	0.290
Age (years)	70.5(67–73.5)	73.5(69–76)	0.155
Smoker (n)[a]	15(33.3%)	18(41.9%)	0.417
BMI (kg/m^2)	23.83 ± 0.54	24.88 ± 0.44	0.135
SBP (mm Hg)	134.07 ± 2.88	140.50 ± 3.89	0.154
DBP (mm Hg)	74.05 ± 1.96	76.16 ± 1.85	0.435
History of hypertension (n)[a]	23(54.3%)	31(70.5%)	0.132
History of diabetes (n)[a]	10(23.8%)	24(54.5%)	0.004
FPG (mmol/L)[b]	5.15(5.00–5.50)	5.95(5.25–7.88)	< 0.001
HbA1c (%)[b]	5.80(5.60–6.15)	6.15(5.70–7.38)	0.009
TC (mmol/L)	4.11 ± 0.13	4.23 ± 0.17	0.563
TG (mmol/L)[b]	0.94(0.75–1.18)	1.40(1.05–1.98)	< 0.001
LDL-c (mmol/L)	2.51 ± 0.12	2.80 ± 0.14	0.125
HDL-c (mmol/L)	1.39 ± 0.06	1.06 ± 0.03	< 0.001
Hs-CRP (mg/L)[b]	1.05(0.43–2.63)	2.84(0.59–11.22)	0.006
PBMCs (%)	31.49 ± 1.36	28.46 ± 1.48	0.135
Lymphocytes (%)	24.45 ± 1.34	22.15 ± 1.33	0.226
Monocytes (%)	6.70(5.55–7.93)	6.45(4.40–7.63)	0.184
Gensini score	0	63.5(40.25–92.75)	–

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HbA1c, glycosylated hemoglobin; TC: total cholesterol; TG: triglycerides; LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; Hs-CRP: high-sensitivity C-reactive protein; PBMCs: peripheral blood mononuclear cells.

[a] Data expressed as n (%).

[b] Data expressed as median (interquartile ranges).

2.9. Statistical analysis

All statistical analysis was performed with SPSS software version 22.0 (SPSS Inc., Chicago, USA). Data are presented as mean ± SEM, frequency (%) or median (interquartile ranges). Two-tailed *P* values < 0.05 were considered statistically significant. Intergroup comparisons between CHD patients and control subjects were made by the independent-samples *t*-test (normally distributed continuous variables), Mann-Whitney *U* test (nonnormally distributed continuous variables) and chi-square test (categorical variables). ANOVA and Kruskal-Wallis test were applied to compare quantitative data among patients with acute myocardial infarction (AMI), patients with angina pectoris and control subjects. Bivariate associations were described by Spearman correlation coefficients. The associations of 5-mC and 5-hmC levels with CHD prevalence were analyzed by unconditional logistic regression.

3. Results

3.1. Baseline characteristics of CHD patients and control subjects

As shown in Table 1, the gender, age, smoker, BMI and history of hypertension of CHD patients and control subjects were well matched. The average age of control subjects and CHD patients was 70 years [70.5 (67–73.5) vs. 73.5 (69–76)]. CHD patients tended to have a history of diabetes. The prevalence of diabetes in the CHD group was more than twice as high as that of the control group ($P = 0.004$). Fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c) and triglycerides (TG) were significantly higher in CHD patients than in control subjects, while high-density lipoprotein cholesterol (HDL-c) was significantly lower in the CHD group than in the control group. There was no significant difference in total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) between the control and CHD groups. The level of high-sensitivity C-reactive protein (Hs-CRP) in the CHD group was more than once higher than that of the control group. No significant difference was found between the percentages of PBMCs, lymphocytes and monocytes of the CHD and control groups. All patients underwent coronary angiography. As a consequence of different degrees of coronary atherosclerosis, CHD patients had significantly higher Gensini score than control subjects.

3.2. DNA methylation and hydroxymethylation levels in PBMCs

The 5-mC percentages of the control and CHD groups were 3.05 ± 0.14% and 3.77 ± 0.12%, respectively. The result that DNA methylation level was higher in CHD patients than in control subjects is illustrated in Fig. 1A. The CHD group included patients with AMI and angina pectoris. Moreover, except for the comparison of AMI group with the angina pectoris group, the differences in the pairwise comparisons between all the other groups were statistically significant (both $P < 0.01$, Fig. 1B). The dot blot analysis that detected the 5-mC levels in PBMCs from CHD patients and control subjects further demonstrated that DNA methylation was significantly increased in the CHD group compared with the control group ($P < 0.001$, Fig. 1C and D).

DNA hydroxymethylation level in CHD patients was higher than that in control subjects (Fig. 2A). Specifically, the 5-hmC percentage of

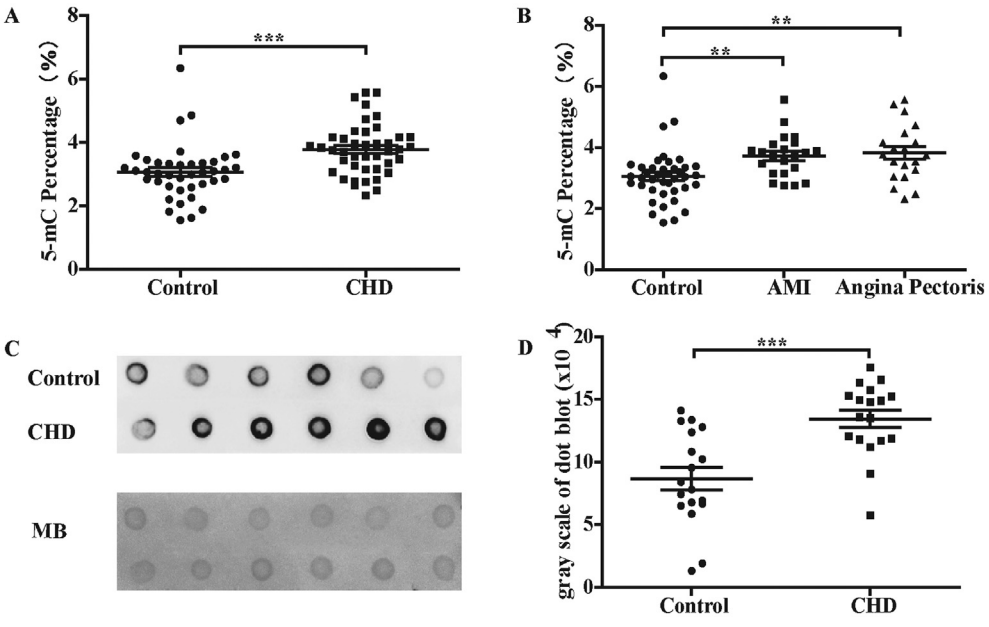


Fig. 1. DNA methylation level of PBMCs. A: The 5-mC level of control subjects and CHD patients were detected using ELISA. B: The 5-mC level among control subjects, AMI patients and angina pectoris patients were measured by ELISA. C: Dot blot analysis was performed to detect the 5-mC level in PBMCs from control subjects and CHD patients. Methylene blue (MB) staining was used as a loading control. D: The gray scales of dot blot results of CHD patients and control subjects were compared. (**, $P < 0.01$; ***, $P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

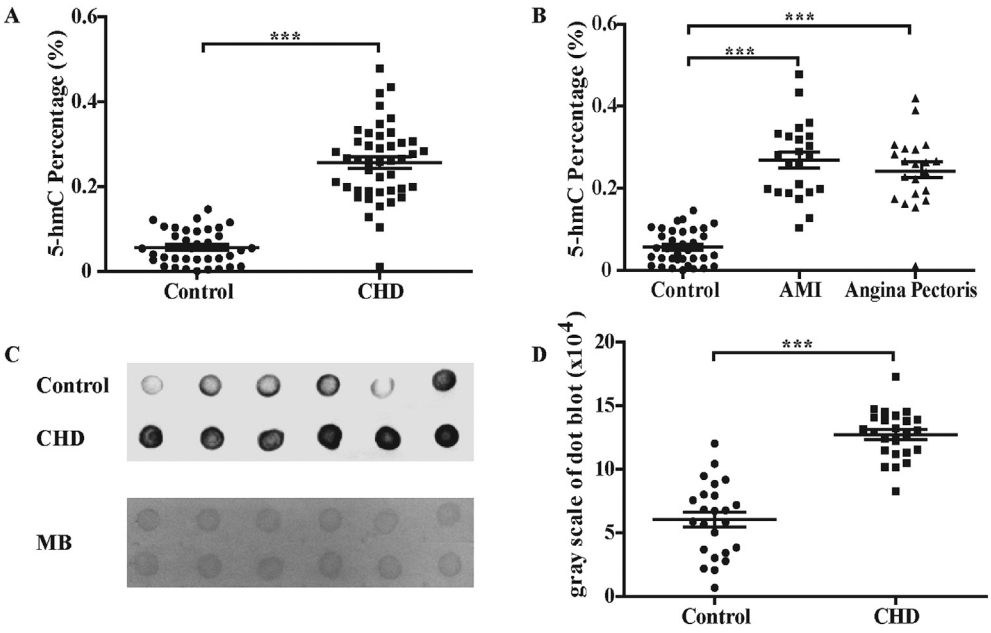


Fig. 2. DNA hydroxymethylation level of PBMCs. A: The 5-hmC level of control subjects and CHD patients were detected using ELISA. B: The 5-hmC level among control subjects, AMI patients and angina pectoris patients were measured by ELISA. C: Dot blot analysis was used to detect the 5-hmC level in PBMCs. MB staining was used as a loading control. D: The gray scales of dot blot results of CHD patients and control subjects were compared. (***, $P < 0.001$).

CHD group was more than four times that of the control group ($P < 0.001$). Moreover, the differences in the pairwise comparisons between the three groups were statistically significant (both $P < 0.001$), except for the comparison of AMI group with angina pectoris group (Fig. 2B). Furthermore, the dot blot analysis was used to detect DNA hydroxymethylation level. The result by which DNA hydroxymethylation level in PBMCs was significantly increased in CHD patients compared with control subjects was further confirmed ($P < 0.001$, Fig. 2C and D).

3.3. Relative mRNA expression of DNMT and TET genes in the PBMCs

We detected the mRNA levels of DNMT and TET genes in PBMCs from control subjects and CHD patients and found that TET2 expression was significantly upregulated in CHD patients compared with control subjects (Fig. 3). Although DNMT1, DNMT3A, TET1 and TET3 expression were increased in CHD patients, no significant difference was found between the control and CHD groups.

3.4. DNA methylation, hydroxymethylation and TET2 expression in human aortic tissue

To further investigate the expression profile of human aortic tissue, DNA methylation and hydroxymethylation levels as well as TET2 expression level were observed by IHC. As shown in Fig. 4, the expression levels of 5-mC, 5-hmC and TET2 were almost negligible in normal human aortic segments but were significantly increased in atherosclerotic plaque lesions.

3.5. Plasma levels and relative mRNA expression levels of IL-1β, IL-6, and TNF-α

Fig. 5 shows that the plasma levels and relative mRNA expression levels of IL-1β and IL-6 in CHD patients were higher than those in control subjects. Although the plasma level of TNF-α in CHD patients was not significantly different from control subjects, the relative mRNA expression of TNF-α was significantly increased in the CHD group compared with the control group ($P < 0.01$).

3.6. Correlation analysis of 5-mC, 5-hmC and TET genes expression with selected covariates

The Spearman correlation coefficients between selected cardiovascular risk factors, Gensini score and 5-mC, 5-hmC are shown in Table 2. The 5-mC and 5-hmC levels were both positively correlated with FPG and TG, while HDL-c was inversely correlated with 5-mC and 5-hmC. It was shown that the 5-hmC level was positively associated with HbA1c and Hs-CRP. There were significantly positive correlations between the levels of plasma IL-6, IL-1β expression, TNF-α expression and the 5-mC level. Increased 5-hmC was strongly associated with increased plasma levels of IL-1β and IL-6 and with increased relative mRNA expression levels of IL-6, TNF-α and TET2. 5-mC and 5-hmC were positively associated, but the 5-hmC level was more significantly correlated with Gensini score than the 5-mC level with significant difference (5-hmC:

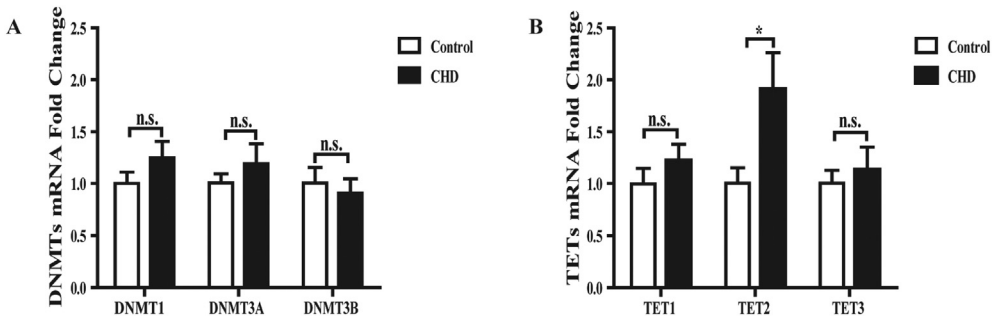


Fig. 3. Relative mRNA expression of DNMT and TET genes in PBMCs from control subjects and CHD patients measured by qRT-PCR and normalized to β-actin. (*, $P < 0.05$; n.s., not significant).

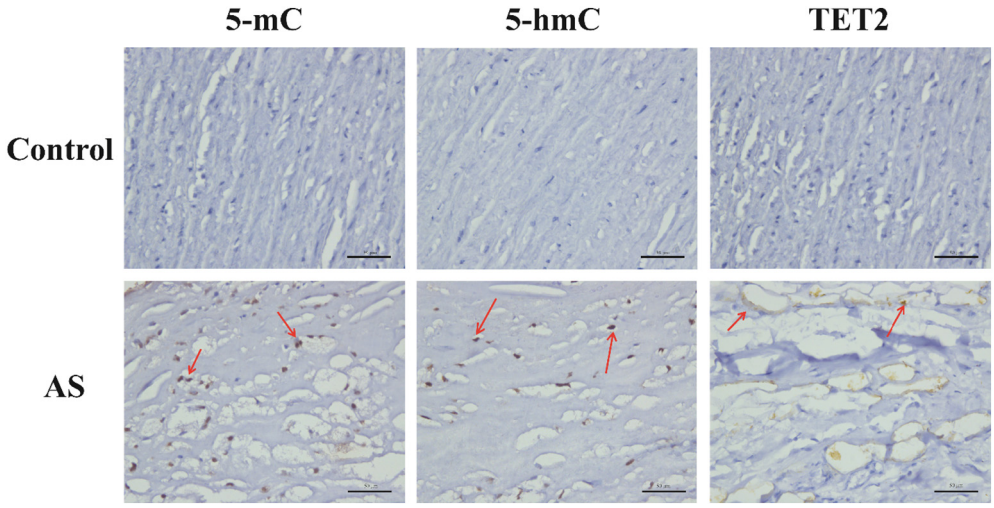


Fig. 4. Representative IHC images (red arrows indicated) of 5-mC, 5-hmC and TET2 expression in human aortic tissue (×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

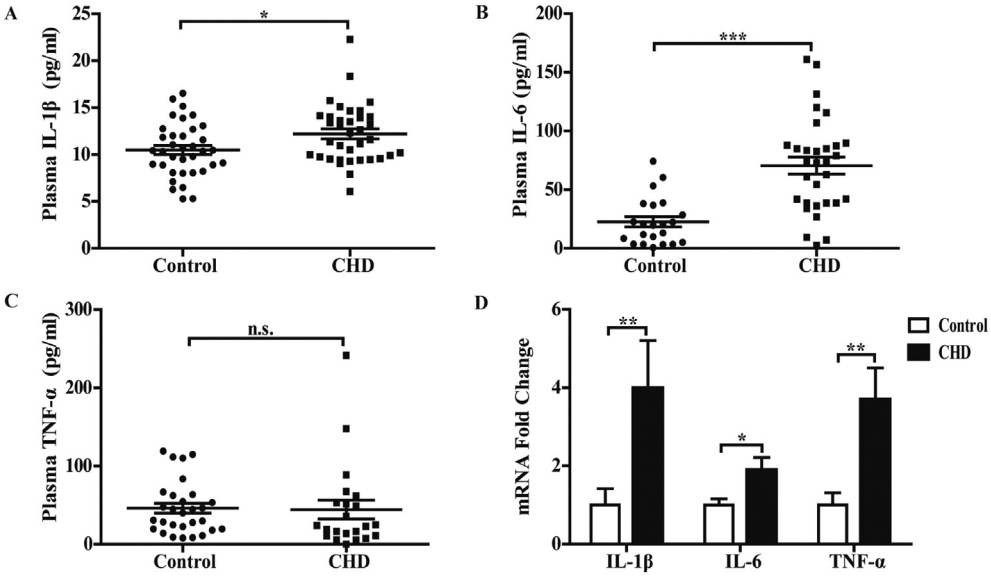


Fig. 5. Plasma levels and relative mRNA expression levels of IL-1β, IL-6 and TNF-α. A–C: The plasma levels of IL-1β, IL-6 and TNF-α in control subjects and CHD patients. D: Relative mRNA expression of IL-1β, IL-6 and TNF-α in PBMCs from control subjects and CHD patients normalized to β-actin. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant).

Table 2
Spearman correlation coefficients of 5-mC and 5-hmC with selected covariates.

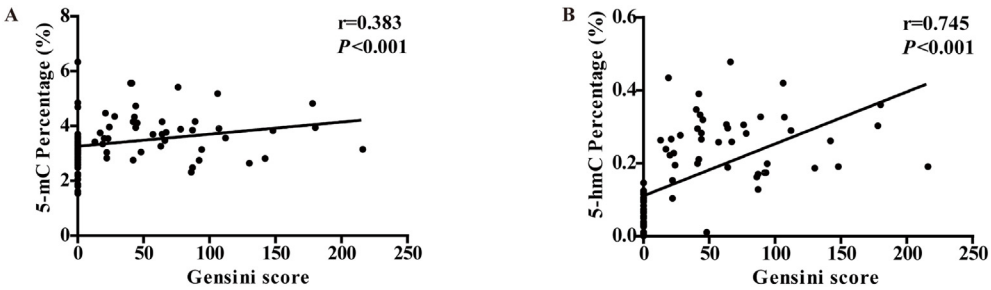
Variable	5-mC (%)		5-hmC (%)	
	r	*P*	r	*P*
FPG (mmol/l)	0.240	0.040	0.377	0.001
HbA1c (%)	0.148	0.187	0.263	0.019
TG (mmol/l)	0.233	0.038	0.266	0.018
HDL-c (mmol/l)	−0.411	< 0.001	−0.415	< 0.001
Hs-CRP (mg/l)	0.211	0.060	0.313	0.005
IL-1β (pg/ml)	0.156	0.190	0.273	0.024
IL-6 (pg/ml)	0.339	0.013	0.510	< 0.001
TNF-α (pg/ml)	−0.069	0.615	0.019	0.892
IL-1β expression	0.445	0.002	0.207	0.195
IL-6 expression	0.232	0.121	0.356	0.022
TNF-α expression	0.504	< 0.001	0.346	0.025
TET2 expression	0.239	0.105	0.386	0.012
PBMCs (%)	−0.055	0.628	−0.162	0.157
Lymphocytes (%)	−0.041	0.720	−0.153	0.181
Monocytes (%)	−0.038	0.737	−0.112	0.328
5-mC (%)	–	–	0.618	< 0.001
Gensini score	0.383	< 0.001	0.745	< 0.001

$r = 0.754$, $P < 0.001$ vs. 5-mC: $r = 0.383$, $P < 0.001$; Fig. 6A and B). Without adjustment, the odds ratios of 5-mC and 5-hmC levels for CHD were 3.064 (95% CI 1.559–6.020, $P = 0.001$) and 1.460 (95% CI 1.226–1.740, $P < 0.001$), respectively, which indicated that 5-mC and 5-hmC levels were the risk factors for CHD. After adjustment for various cardiovascular risk factors, especially history of diabetes, FPG and HbA1c, the association of 5-mC and 5-hmC levels with CHD remained significant (Fig. 6C).

The results of the correlation analyses examining relative mRNA expression of IL-1β, IL-6, TNF-α and TET genes are displayed in Table 3. It was positively associated with relative mRNA expression of IL-1β and TET1, TET2. Relative TNF-α mRNA expression was positively correlated with only TET2 mRNA expression.

3.7. Relationship of DNA methylation and hydroxymethylation with diabetes mellitus (DM)

As shown in Fig. 7A and B, the 5-mC and 5-hmC levels were significantly increased in the CHD group compared with the control group in elderly patients with and without DM. Without adjustment, the odds ratios of 5-mC and 5-hmC levels for DM were 1.357 (95% CI 0.823–2.237, $P = 0.231$) and 1.033 (95% CI 0.995–1.072, $P = 0.091$),



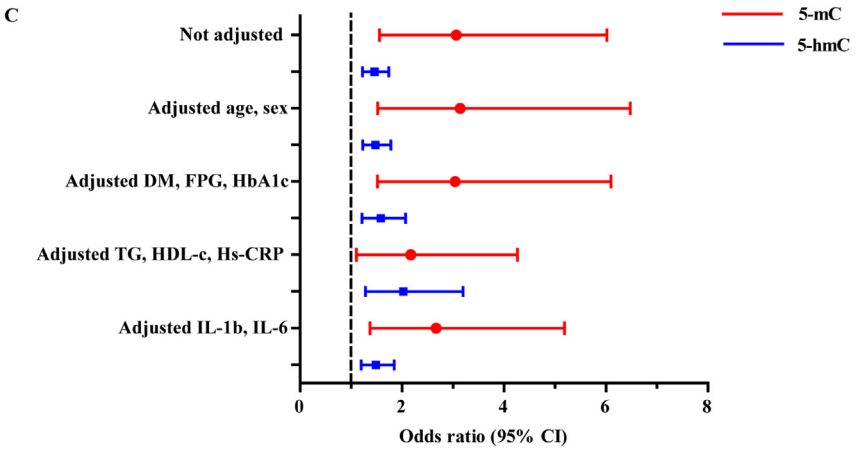


Fig. 6. Correlation analysis of DNA methylation and hydroxymethylation with coronary atherosclerosis. A–B: Correlation analyses of 5-mC (A) and 5-hmC (B) with Gensini score. C: Association of 5-mC and 5-hmC levels with CHD risk. An odds ratio > 1 indicates that 5-mC and 5-hmC levels are the risk factors. The odds ratio of 5-hmC represents an altered risk of coronary artery disease per 0.01% change in 5-hmC level.

Table 3
Spearman correlation analysis of relative mRNA expression of IL-1β, IL-6, TNF-α and TET genes.

Variable	TET1 expression		TET2 expression		TET3 expression	
	r	*P*	r	*P*	r	*P*
IL-1β expression	0.368	0.014	0.316	0.032	−0.022	0.883
IL-6 expression	−0.118	0.452	0.132	0.386	0.001	0.997
TNF-α expression	0.269	0.074	0.401	0.005	−0.048	0.746

respectively. After adjustment for various DM risk factors, the association of 5-mC and 5-hmC levels with DM was not significantly different (Fig. 7C).

4. Discussion

The role of epigenetics, especially DNA methylation and hydroxymethylation modification in CHD is a topic of intense research interest. Human tissue is the most intuitive model for studying epigenetics. Since precious human tissue, such as heart and brain, is difficult to access, the peripheral blood has become a good substitute. It has already been confirmed that the global methylation level detected in peripheral blood is consistent with the global methylation of tissue [27,28]. There are many methods to quantify DNA methylation and hydroxymethylation, such as high performance liquid chromatography-ultraviolet (HPLC-UV), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), ELISA, LINE-1 pyrosequencing and luminometric methylation assay (LUMA); however, ELISA-based methods are quick, cost-effective and reliable alternatives that have already been verified by several published studies [20–23,29].

Our data showed that the 5-mC and 5-hmC levels of PBMCs from CHD patients were higher than those from control subjects, and these results were further demonstrated by dot blot analysis. We obtained a consistent conclusion after examining human aortic tissue. Moreover, only TET2 expression was significantly upregulated in CHD patients compared with control subjects. The levels of IL-1β and IL-6 in CHD patients were higher than those in control subjects. Most importantly, 5-mC, 5-hmC and IL-1β, IL-6 levels were positively correlated with Gensini score, which indicated that DNA methylation, hydroxymethylation and cytokines were closely associated with the degree of coronary atherosclerosis.

We performed this study examining PBMCs of elderly CHD patients for the following reasons. First, PBMCs are considered to better represent the biological changes related to environmental exposures or modifications of lifestyle and behavior, which seem to be a reasonable surrogate to study the effect of age on DNA methylation profiles [30–32]. Second, somatic mutations of DNMT3A and TET2 in peripheral blood cells were reported to be related to an increased risk of CHD and were more frequent with age [1,2]. Therefore, we selected patients with an average age of > 70 years old as the research subjects.

DNA methylation is significantly associated with CHD, but DNA methylation level in CHD is controversial. Zaina et al. showed that the atherosclerotic portion of the aorta was hypermethylated across many genomic loci compared with that in the matched healthy counterpart [15]. Sharma et al. also reported that global DNA methylation was significantly higher in CAD patients than in control subjects [13], while another study suggested that the patient with vascular disease had significantly reduced genomic DNA methylation level [6]. The finding of the present study is that the DNA methylation level of PBMCs from CHD patients and human aortic atherosclerotic plaque lesions was significantly higher than those of control subjects and tissue samples, which was consistent with hypermethylation. DNMT genes are the key enzymes that determine DNA methylation level and catalyse the conversion of C to 5-mC, but only a non-significant increasing trend of DNMT1 and DNMT3A expression was found in CHD patients [3]. This discrepancy can be explained by several aspects of this study. First, our study population are elderly patients, while other studies did not have age restrictions. Sharma et al. also reported that the global DNA hypermethylation was associated with CHD, especially in aged patients

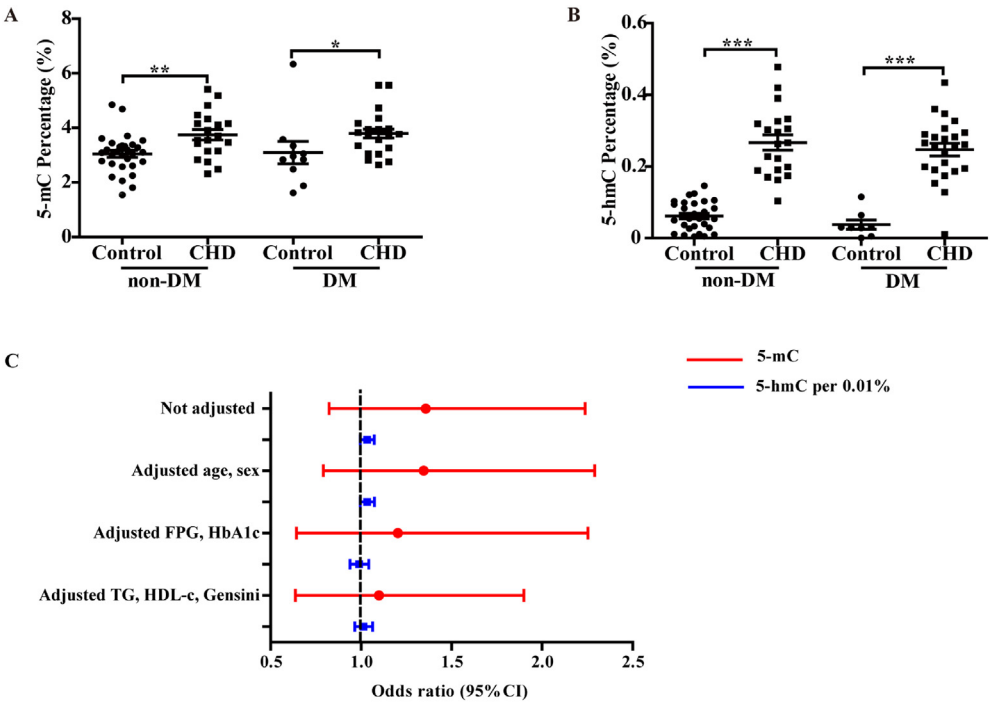


Fig. 7. Associations of DNA methylation and hydroxymethylation with DM. A–B: 5-mC (A) and 5-hmC (B) levels of control subjects and CHD patients in the non-DM and DM groups. C: Association of 5-mC and 5-hmC levels with DM risk. The odds ratios of 5-hmC represents an altered risk of diabetes per 0.01% change in the 5-hmC level. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

[13]. Second, we used different methods. Most studies analysing the association of global hypomethylation with CHD or AS measured the methylation of repetitive elements (LINE-1 or Alu) as a proxy for global methylation [5]. However, this technique assesses relatively few CpG sites [23]. We used two different methods to confirm our results.

Loss of TET2 and its product, 5-hmC, was positively correlated with the degree of injury in murine models of vascular injury and human atherosclerotic disease [33]. Fuster et al. reported that partial bone marrow reconstitution with TET2-deficient cells led to a marked increase in atherosclerotic plaque size and in IL-1β secretion [34]. In our study, we verified that the 5-hmC level was increased in PBMCs of CHD patients and human aortic atherosclerotic plaque lesions, which was accordance with the recent report by Deng [16]. TET genes are the core genes that catalyse the oxidation of 5-mC to 5-hmC. In our study, only TET2 expression was higher in CHD patients than in control subjects, but no difference in TET genes expression was found in the study by Deng [16].

DNA methylation and hydroxymethylation as well as the expression of cytokines such as IL-1β and IL-6 are involved in the formation of AS [35–37]. In murine modes, DNMT3A and TET2 somatic mutation-driven clonal hematopoiesis accelerated AS and heart failure through a mechanism involving the IL-1β and IL-6 inflammasome [34,38,39]. Our data showed that IL-1β and IL-6 levels in CHD patients, especially AMI patients were higher than those in control subjects (Supplemental Fig. 1), which was consistent with several studies reporting that increased IL-1β and IL-6 levels are associated with CHD risk [35,40]. In the Spearman correlation analysis, both 5-mC and 5-hmC were associated with increased cytokine levels or gene expression. 5-mC, 5-hmC and IL-1β, IL-6 were positively correlated with Gensini score of CHD patients (Table 2 and Supplemental Table 2), which indicated that increased DNA methylation, hydroxymethylation and cytokine levels may accelerate the development of AS.

Diabetes mellitus is closely related to CHD, which significantly promotes and aggravates the processes of CHD and AS [41]. It was confirmed that somatic mutations of DNMT3A and TET2 are associated with type 2 diabetes [1]. Our results showed that CHD patients tended to have a history of diabetes and that FPG and HbA1c levels in CHD patients were higher than in control subjects. The 5-mC and 5-hmC levels were positively correlated with FPG or HbA1c. To investigate whether diabetes was a confounding factor of the study, all patients were reclassified into non-DM and DM subgroups. The data indicated that the 5-mC and 5-hmC levels were consistently higher in CHD patients than in control subjects in patients with and without DM, which demonstrated that diabetes may be a distracting factor. We found that the association of 5-mC and 5-hmC levels with DM risk had no significant difference without or with adjustment for various DM risk factors.

We revealed that the levels of DNA methylation and hydroxymethylation in PBMCs and the levels of IL-1β and IL-6 in elderly CHD patients were significantly increased, which showed a positive correlation with the degree of coronary atherosclerosis and revealed the potential diagnostic value as the biomarkers. However, the relatively small sample is the major limitation of the present study. Future studies in larger samples are needed to validate our findings. The specific cellular components in the development of CHD or AS also need to be fully confirmed.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

We sincerely thank Tang Renkuan of the Department of Forensic Medicine, College of Basic Medicine of Chongqing Medical University and Cheng Jun of the Department of Vascular Surgery of the First Affiliated Hospital of Chongqing Medical University for their assistance with these experiments.

Sources of funding

The study was supported by National Natural Science Foundation of China (No. 81570212, 31800976); Basic Research and Frontier Exploration Project of Chongqing (No. cstc2018jcyjAX0058); Science and Technology Plan Project of Chongqing Yuzhong District (No. 20140111); Chinese Medicine Science and Technology Project of Chongqing Health and Family Planning Commission (No. ZY201702073); Medical Research Project of Chongqing Health Bureau

(No. 2012-1-012); The First Affiliated Hospital of Chongqing Medical University Cultivation Fund (No. PYJJ2017-28); Cardiac Rehabilitation and Metabolic Therapy Research Foundation (No. ky-2016-12-20); Chinese Cardiovascular Association-Access found (No. 2018-CCA-AF-034, ky-2017-07-08); Medical Research Project of Chongqing Health and Family Planning Commission (No. 2015ZDXM003); Chongqing Science and Health Joint Medical Research Project (No. 2018QNXM024).

Author contributions

All authors have contributed significantly, and that all authors are in agreement with the content of the manuscript. Jiang D and Lu K: Conceptualization. Gao L, Hu C-X and Wu S-Y: Methodology. Sun M and You L: Data curation. Chang G-L and Tao H-M: Formal analysis and Validation. Jiang D and Zhang D-Y: Writing - review & editing.

Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.03.021.

References

[1] S. Jaiswal, P. Fontanillas, J. Flannick, A. Manning, P.V. Grauman, B.G. Mar, R.C. Lindsley, C.H. Mermel, N. Burtt, A. Chavez, J.M. Higgins, V. Moltchanov, F.C. Kuo, M.J. Kluk, B. Henderson, L. Kinnunen, H.A. Koistinen, C. Ladenvall, G. Getz, A. Correa, B.F. Banahan, S. Gabriel, S. Kathiresan, H.M. Stringham, M.I. McCarthy, M. Boehnke, J. Tuomilehto, C. Haiman, L. Groop, G. Atzmon, J.G. Wilson, D. Neuberg, D. Altshuler, B.L. Ebert, Age-related clonal hematopoiesis associated with adverse outcomes, N. Engl. J. Med. 371 (2014) 2488–2498.

[2] S. Jaiswal, P. Natarajan, A.J. Silver, C.J. Gibson, A.G. Bick, E. Shvartz, M. McConkey, N. Gupta, S. Gabriel, D. Ardissino, U. Baber, R. Mehran, V. Fuster, J. Danesh, P. Frossard, D. Saleheen, O. Melander, G.K. Sukhova, D. Neuberg, P. Libby, S. Kathiresan, B.L. Ebert, Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease, N. Engl. J. Med. 377 (2017) 111–121.

[3] R.M. Kohli, Y. Zhang, TET enzymes, TDG and the dynamics of DNA demethylation, Nature 502 (2013) 472–479.

[4] H. Wu, Y. Zhang, Reversing DNA methylation: mechanisms, genomics, and biological functions, Cell 156 (2014) 45–68.

[5] A. Fernandez-Sanles, S. Sayols-Baixeras, I. Subirana, I.R. Degano, R. Elosua, Association between DNA methylation and coronary heart disease or other atherosclerotic events: a systematic review, Atherosclerosis 263 (2017) 325–333.

[6] R. Castro, I. Rivera, E.A. Struys, E.E. Jansen, P. Ravasco, M.E. Camilo, H.J. Blom, C. Jakobs, I. Tavares de Almeida, Increased homocysteine and s-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease, Clin. Chem. 49 (2003) 1292–1296.

[7] S. Guarrera, G. Fiorito, N.C. Onland-Moret, A. Russo, C. Agnoli, A. Allione, C. Di Gaetano, A. Mattiello, F. Ricceri, P. Chiodini, S. Polidoro, G. Frasca, M.W.M. Verschuren, J.M.A. Boer, L. Iacoviello, Y.T. van der Schouw, R. Tumino, P. Vineis, V. Krogh, S. Panico, C. Sacerdote, G. Matullo, Gene-specific DNA methylation profiles and line-1 hypomethylation are associated with myocardial infarction risk, Clin. Epigenetics 7 (2015) 133.

[8] L. Wei, S. Liu, Z. Su, R. Cheng, X. Bai, X. Li, Line-1 hypomethylation is associated with the risk of coronary heart disease in Chinese population, Arq. Bras. Cardiol. 102 (2014) 481–488.

[9] R.T. Lin, E. Hsi, H.F. Lin, Y.C. Liao, Y.S. Wang, S.H. Juo, Line-1 methylation is associated with an increased risk of ischemic stroke in men, Curr. Neurovasc. Res. 11 (2014) 4–9.

[10] A. Baccarelli, R. Wright, V. Bollati, A. Litonjua, A. Zanobetti, L. Tarantini, D. Sparrow, P. Vokonas, J. Schwartz, Ischemic heart disease and stroke in relation to blood DNA methylation, Epidemiology 21 (2010) 819–828.

[11] R.B. Ramos, V. Fabris, S.B. Lecke, M.A. Maturana, P.M. Spritzer, Association between global leukocyte DNA methylation and cardiovascular risk in postmenopausal women, BMC Med. Genet. 17 (2016) 71.

[12] M.S. Nazarenko, A.V. Markov, I.N. Lebedev, M.B. Freidin, A.A. Sleptcov, I.A. Koroleva, A.V. Frolov, V.A. Popov, O.L. Barbarash, V.P. Puzyrev, A comparison of genome-wide DNA methylation patterns between different vascular tissues from patients with coronary heart disease, PLoS One 10 (2015) e0122601.

[13] P. Sharma, J. Kumar, G. Garg, A. Kumar, A. Patowary, G. Karthikeyan, L. Ramakrishnan, V. Brahmachari, S. Sengupta, Detection of altered global DNA methylation in coronary artery disease patients, DNA Cell Biol. 27 (2008) 357–365.

[14] M. Kim, T.I. Long, K. Arakawa, R. Wang, M.C. Yu, P.W. Laird, DNA methylation as a biomarker for cardiovascular disease risk, PLoS One 5 (2010) e9692.

[15] S. Zaina, H. Heyn, F.J. Carmona, N. Varol, S. Sayols, E. Condom, J. Ramirez-Ruz, A. Gomez, I. Goncalves, S. Moran, M. Esteller, DNA methylation map of human atherosclerosis, Circ. Cardiovasc. Genet. 7 (2014) 692–700.

[16] Q. Deng, W. Huang, C. Peng, J. Gao, Z. Li, X. Qiu, N. Yang, B. Yuan, F. Zheng, Genomic 5-mC contents in peripheral blood leukocytes were independent protective factors for coronary artery disease with a specific profile in different leukocyte subtypes, Clin. Epigenetics 10 (2018) 9.

[17] G.G. Gensini, A more meaningful scoring system for determining the severity of coronary heart disease, Am. J. Cardiol. 51 (1983) 606.

[18] K.W. Liang, W.J. Lee, W.L. Lee, C.T. Ting, W.H. Sheu, Decreased ratio of high-molecular-weight to total adiponectin is associated with angiographic coronary atherosclerosis severity but not restenosis, Clin. Chim. Acta 405 (2009) 114–118.

[19] Y. Wang, A. Zheng, Y. Yan, F. Song, Q. Kong, S. Qin, D. Zhang, Association between HMW adiponectin, HMW-total adiponectin ratio and early-onset coronary artery disease in Chinese population, Atherosclerosis 235 (2014) 392–397.

[20] R. Stoger, P.J. Scaife, F. Shephard, L. Chakrabarti, Elevated 5hmc levels characterize DNA of the cerebellum in Parkinson's disease, NPJ Parkinson's Dis. 3 (2017) 6.

[21] T. Yugendran, B.N. Harish, Global DNA methylation level among ciprofloxacin-resistant clinical isolates of Escherichia coli, J. Clin. Diagn. Res. 10 (2016) Dc27–30.

[22] A. Grelus, D.V. Nica, I. Miklos, V. Belengeanu, I. Ioiart, C. Popescu, Clinical significance of measuring global hydroxymethylation of white blood cell DNA in prostate cancer: comparison to PSA in a pilot exploratory study, Int. J. Mol. Sci. 18 (2017).

[23] S. Kurdyukov, M. Bullock, DNA methylation analysis: choosing the right method, Biology (Basel) 5 (2016).

[24] M. Zhao, J. Wang, W. Liao, D. Li, M. Li, H. Wu, Y. Zhang, M.E. Gershwin, Q. Lu, Increased 5-hydroxymethylcytosine in $CD4^+$ T cells in systemic lupus erythematosus, J. Autoimmun. 69 (2016) 64–73.

[25] Z. Jia, Y. Liang, B. Ma, X. Xu, J. Xiong, L. Duan, D. Wang, A 5-mC dot blot assay quantifying the DNA methylation level of chondrocyte dedifferentiation in vitro, J. Vis. Exp. 17 (2017) 123.

[26] S. Wu, G. Chang, L. Gao, D. Jiang, L. Wang, G. Li, X. Luo, S. Qin, X. Guo, D. Zhang, Trimetazidine protects against myocardial ischemia/reperfusion injury by inhibiting excessive autophagy, J. Mol. Med. 96 (2018) 791–806.

[27] E. Masliah, W. Dumaop, D. Galasko, P. Desplats, Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes, Epigenetics 8 (2013) 1030–1038.

[28] E. Miranda-Morales, K. Meier, A. Sandoval-Carrillo, J. Salas-Pacheco, P. Vazquez-Cardenas, O. Arias-Carrion, Implications of DNA methylation in Parkinson's disease, Front. Mol. Neurosci. 10 (2017) 225.

[29] B.J. Toghill, A. Saratzis, P.J. Freeman, N. Sylvius, M.J. Bown, Smyd2 promoter DNA methylation is associated with abdominal aortic aneurysm (AAA) and smyd2 expression in vascular smooth muscle cells, Clin. Epigenetics 10 (2018) 29.

[30] L. Delgado-Cruzata, N. Vin-Raviv, P. Tehranifar, J. Flom, D. Reynolds, K. Gonzalez, R.M. Santella, M.B. Terry, Correlations in global DNA methylation measures in peripheral blood mononuclear cells and granulocytes, Epigenetics 9 (2014) 1504–1510.

[31] S. Horvath, Y. Zhang, P. Langfelder, R.S. Kahn, M.P. Boks, K. van Eijk, L.H. van den Berg, R.A. Ophoff, Aging effects on DNA methylation modules in human brain and blood tissue, Genome Biol. 13 (2012) R97.

[32] R. Calabrese, E. Valentini, F. Ciccarone, T. Guastafierro, M.G. Bacalini, V.A. Ricigliano, M. Zampieri, V. Annibali, R. Mechelli, C. Franceschi, M. Salvetti, P. Caiafa, TET2 gene expression and 5-hydroxymethylcytosine level in multiple sclerosis peripheral blood cells, Biochim. Biophys. Acta 1842 (2014) 1130–1136.

[33] R. Liu, Y. Jin, W.H. Tang, L. Qin, X. Zhang, G. Tellides, J. Hwa, J. Yu, K.A. Martin, Ten-eleven translocation-2 (TET2) is a master regulator of smooth muscle cell plasticity, Circulation 128 (2013) 2047–2057.

[34] J.J. Fuster, S. MacLauchlan, M.A. Zuriaga, M.N. Polackal, A.C. Ostriker, R. Chakraborty, C.L. Wu, S. Sano, S. Muralidharan, C. Rius, J. Vuong, S. Jacob, V. Muralidhar, A.A. Robertson, M.A. Cooper, V. Andres, K.K. Hirschi, K.A. Martin, K. Walsh, Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice, Science 355 (2017) 842–847.

[35] D.R. Anderson, J.T. Poterucha, T.R. Mikuls, M.J. Duryee, R.P. Garvin, L.W. Klassen, S.W. Shurmur, G.M. Thiele, Il-6 and its receptors in coronary artery disease and acute myocardial infarction, Cytokine 62 (2013) 395–400.

[36] H. Kishikawa, T. Shimokama, T. Watanabe, Localization of T lymphocytes and macrophages expressing IL-1, IL-2 receptor, IL-6 and TNF in human aortic intima. Role of cell-mediated immunity in human atherogenesis, Virchows Arch. A Pathol. Anat. Histopathol. 423 (1993) 433–442.

[37] A. Steptoe, A. Wikman, G.J. Molloy, N. Messerli-Burgy, J.C. Kaski, Inflammation and symptoms of depression and anxiety in patients with acute coronary heart disease, Brain Behav. Immun. 31 (2013) 183–188.

[38] S. Sano, K. Oshima, Y. Wang, Y. Katanasaka, M. Sano, K. Walsh, Crispr-mediated gene editing to assess the roles of tet2 and dnmt3a in clonal hematopoiesis and cardiovascular disease, Circ. Res. 123 (2018) 335–341.

[39] S. Sano, K. Oshima, Y. Wang, S. MacLauchlan, Y. Katanasaka, M. Sano, M.A. Zuriaga, M. Yoshiyama, D. Goukassian, M.A. Cooper, J.J. Fuster, K. Walsh, Tet2-mediated clonal hematopoiesis accelerates heart failure through a mechanism involving the il-1beta/nlrp3 inflammasome, J. Am. Coll. Cardiol. 71 (2018) 875–886.

[40] X. Jing, S.S. Chen, W. Jing, Q. Tan, M.X. Yu, J.C. Tu, Diagnostic potential of differentially expressed homer1, il-1beta, and tnf-alpha in coronary artery disease, Int. J. Mol. Sci. 16 (2014) 535–546.

[41] G. Toperoff, D. Aran, J.D. Kark, M. Rosenberg, T. Dubnikov, B. Nissan, J. Wainstein, Y. Friedlander, E. Levy-Lahad, B. Glaser, A. Hellman, Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood, Hum. Mol. Genet. 21 (2012) 371–383.