European Journal of Epidemiology (2019) 34:451–462
https://doi.org/10.1007/s10654-019-00493-7

MORTALITY

Oxidative stress and epigenetic mortality risk score: associations with all-cause mortality among elderly people

Xu Gao[1,2,7] · Xīn Gào[1,2,3] · Yan Zhang[1] · Bernd Holleczek[4] · Ben Schöttker[1,3] · Hermann Brenner[1,3,5,6]

Received: 19 January 2018 / Accepted: 7 February 2019 / Published online: 15 February 2019

Abstract

Oxidative stress (OS) has been found to be related to accelerated aging and many aging-related health outcomes. Recently, an epigenetic "mortality risk score" (MS) based on whole blood DNA methylation at 10 mortality-related CpG sites has been demonstrated to be associated with all-cause mortality. This study aimed to address the association between OS and MS, and to assess and compare their performance in the prediction of all-cause mortality. For 1448 participants aged 50–75 of the German ESTHER cohort study, the MS was derived from the DNA methylation profiles measured by Illumina HumanMethylation450K Beadchip and the levels of two urinary OS markers, 8-isoprostane (8-iso) and oxidized guanine/guanosine [including 8-hydroxy-2′-deoxyguanosine (8-oxo)], were measured by ELISA kits. Associations between OS markers and the MS were evaluated by linear and ordinal logistic regression models, and their associations with all-cause mortality were examined by Cox regression models. Both OS markers were associated with the MS at baseline. The 8-iso levels and MS, but not 8-oxo levels, were associated with all-cause mortality during a median follow-up of 15.1 years. Fully-adjusted hazard ratios (95% CI) were 1.56 (1.13–2.16) for the 4th quartile of 8-iso levels compared with the 1st, 1.71 (1.27–2.29) and 2.92 (2.03–4.18) for the moderate and high MS defined by 2–5 and > 5 CpG sites with aberrant methylation compared with a MS of 0–1, respectively. After controlling for 8-iso levels, the hazard ratios of MS remained essentially unchanged while the association of 8-iso levels with mortality was attenuated. This study demonstrates that OS is highly associated with the epigenetic MS, and the latter at the same time has a higher predictive value for all-cause mortality.

Keywords Oxidative stress · DNA methylation · Mortality risk score · Epigenetic epidemiology · Aging · All-cause mortality

Introduction

According to the free radical/oxidative stress theory of aging, the increased production of reactive oxygen species (ROS) may cause oxidative stress (OS) when it cannot be balanced by anti-oxidative capacities of tissues [1]. OS has

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10654-019-00493-7) contains supplementary material, which is available to authorized users.

✉ Hermann Brenner
h.brenner@dkfz-heidelberg.de

1 Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 581, 69120 Heidelberg, Germany

2 Medical Faculty Heidelberg, University of Heidelberg, Im Neuenheimer Feld 672, 69120 Heidelberg, Germany

3 Network Aging Research, University of Heidelberg, Bergheimer Straße 20, 69115 Heidelberg, Germany

4 Saarland Cancer Registry, Präsident Baltz Strasse 5, 66119 Saarbrücken, Germany

5 Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Im Neuenheimer Feld 460, 69120 Heidelberg, Germany

6 German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

7 Present Address: Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

been found to be an important contributor to accelerated senescence of cells and aging, and to be associated with the developments of aging-related health outcomes, including various forms of cancer, cardiovascular disease (CVD), cognitive decline and mortality [2–4]. Given the very short half-life of ROS [5], OS is commonly indirectly estimated by proxy markers, such as 8-isoprostane (8-iso) levels [6, 7] and 8-hydroxy-2′-deoxyguanosine (8-oxo) levels [8], which are best measured in urine samples.

Recently, the oxidative damage triggered by the ROS has been shown to be associated with smoking-related DNA methylation changes in whole blood samples [9]. Modification of DNA methylation, the most studied and stable form of epigenetic modification, has been identified to be associated with aging and aging-related health outcomes [10, 11], and has been recognized as a highly predictive indicator of increased risk of all-cause and disease-specific mortalities [12]. In a recent epigenome-wide association study with approximately 1900 older adults followed up for 14 years and an external validation with 1727 participants, 58 CpG sites within 19 chromosomes were identified to be associated with all-cause mortality [13]. A "mortality risk score" (MS) based on 10 out of the 58 loci was found to be a robust and informative predictor of all-cause, CVD and cancer mortality.

Considering the relationship between OS and DNA methylation changes, we hypothesized that OS might be associated with the MS. In the present study, we performed a comprehensive investigation to determine the cross-sectional association of OS defined by both urinary 8-iso and 8-oxo levels with the MS in a large population-based study of older adults in Germany, and to examine whether the MS outperformed the two OS markers in the prediction of all-cause mortality during a median follow-up of 15.1 years.

Methods

Study design and population

Study subjects were chosen from the ESTHER study, an ongoing statewide population-based cohort study conducted in Saarland, a state located in southwestern Germany. Details of the study design and previous analyses on mortality prediction by the MS within this cohort have been reported elsewhere [13, 14]. As shown in Fig. 1, 9949 older adults (aged 50–75 years) were enrolled by their general practitioners during a routine health check-up between July 2000 and December 2002, and followed up thereafter. The cross-sectional analysis of this study is based on the data and biospecimen collected at baseline, from 1448 participants who were randomly selected for the measurements of DNA methylation profiles, among participants recruited consecutively at the start of the ESTHER study between July 2000 and March 2001 [9]. The selected participants were then regularly followed up with respect to the incidence of major chronic diseases and mortality. The study was approved by the ethics committees of the University of Heidelberg and the state medical board of Saarland, Germany. Written informed consent was obtained from all participants.

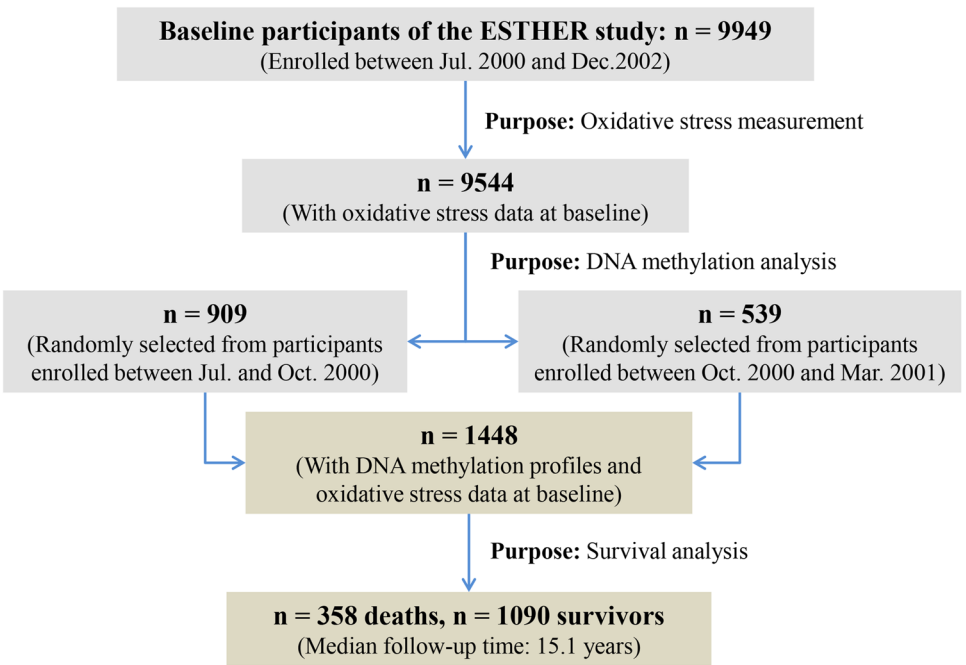

Fig. 1 Overview of the sampling procedures of participants for analysis

Data collection

Information on socio-demographic characteristics, lifestyle factors and health status at baseline was obtained by standardized self-administered questionnaires. Participants were asked about their past and present smoking behaviors and were then categorized into current, former and never smokers. Information on BMI was extracted from a standardized form filled by the general practitioners during the health check-ups. Prevalent CVD at baseline was defined by either physician-reported coronary heart disease or a self-reported history of a major cardiovascular event, such as myocardial infarction, stroke, pulmonary embolism or revascularization of coronary arteries. Prevalent diabetes was defined by physician diagnosis or the use of glucose-lowering drugs. Prevalent cancer [ICD-10 C00-C99 except non-melanoma skin cancer (C44)] was determined by self-report or record linkage with data from the Saarland Cancer Registry.

Deaths occuring until the end of 2015 were retrieved by record linkage with population registries in Saarland. Study participants who could not be followed up via registries (~2%) were censored with the date of the last returned questionnaire. Information on the causes of death was obtained from death certificates provided by local public health offices and was coded with ICD-10 codes.

DNA methylation data

DNA methylation profiles were determined by the Illumina HumanMethylation450K Beadchip (Illumina, San Diego, CA, USA). As previously described [9], samples were analyzed following the manufacturer's instruction at the Genomics and Proteomics Core Facility of the German Cancer Research Center, Heidelberg, Germany. Illumina's GenomeStudio® (version 2011.1; Illumina.Inc.) was employed to extract DNA methylation signals from the scanned arrays (Module version 1.9.0; Illumina.Inc.). The methylation level of a specific CpG site was quantified as a β value ranging from 0 (no methylation) to 1 (full methylation). According to the manufacturer's protocol, no background correction was done and data were normalized to internal controls provided by the manufacturer. All controls were checked for inconsistencies in each measured plate. Probes with a detection *P* value < 0.05 were excluded. Illumina normalization and preprocessing methods implemented in Illumina's GenomeStudio® were utilized.

As described by Zhang et al. [13], 10 CpG sites (cg01612140, cg05575921, cg06126421, cg08362785, cg10321156, cg14975410, cg19572487, cg23665802, cg24704287 and cg25983901) were selected from the whole epigenome data to build the MS. Values of the 4th quartile of cg08362785 and of 1st quartiles of the other nine loci were used to define aberrant methylation for each CpG site. The ordinal MS was determined as the cumulative number of aberrantly methylated CpG sites (0–10), and the participants were further classified into three risk levels: low: MS = 0–1, moderate: MS = 2–5, and high: MS > 5 as defined by previous publications [14–16]. In addition, for dose–response relationship analyses, a continuous MS was additionally constructed as the sum of the methylation β values multiplied with the regression coefficients of each of the 10 CpG sites for all-cause mortality derived from LASSO regression [13].

Oxidative stress data

Urine samples were taken during the health check-up and temporarily stored at − 4 °C and then maintained at − 80 °C until further processing. The 8-iso levels were determined by commercial ELISA kits (8iso1) from Detroit R&D (Detroit, Michigan, USA). The 8-oxo levels were assessed by ELISA with the commercial DNA/RNA Oxidative Damage Immunoassay (EIA) kits of Cayman (Ann Arbor, Michigan, USA) by detecting all three oxidized guanine species: 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA and 8-hydroxyguanine from either DNA or RNA. To correct for differences in kidney function, both urinary OS biomarkers were standardized by urinary creatinine levels, 8-iso levels were expressed in nmol/mmol creatinine and 8-oxo levels were expressed in µg/g creatinine. A natural logarithm transformation of 8-iso and 8-oxo levels was employed for ensuring normal distribution.

Statistical analysis

First, major socio-demographic characteristics, lifestyle factors and the MS at baseline of all 1448 participants and subsets based on the quartiles of both markers of OS were summarized by descriptive statistics. Distributions of 8-iso (log) and 8-oxo (log) at baseline were assessed with respect to sex, age group (< 60, 60–70 and > 70 years) and the MS risk levels.

We then investigated the cross-sectional association of both markers of OS with the MS at baseline. Two linear regression models were used to examine the associations between the levels of each OS marker (log) with the ordinal/continuous MS, and an ordinal logistic regression model was employed to analyze the associations between the levels of each OS marker (log) and the MS (risk levels). Each analysis model increasingly controlled for potential confounding factors and treated the OS marker as the predictor and the MS as the outcome. Model 1 adjusted for age (years), sex and the leukocyte distribution estimated by the Houseman algorithm [17]. Model 2 additionally adjusted for alcohol consumption (g/day), smoking status (current/former/never smoker), BMI class [kg/m^2, underweight (< 18.5, < 1% of the study population) or normal weight (18.5 to < 25), overweight (25

to < 30), obese (≥ 30)] and physical activity [inactive (< 1 h of physical activity/week), medium or high (≥ 2 h of vigorous or ≥ 2 h of light physical activity/week), low (other)]. Model 3 additionally adjusted for the prevalence of CVD (yes/no), diabetes (yes/no) and cancer (yes/no).

Furthermore, we employed restricted cubic spline regression using the SAS macro by Desquilbet et al. [18] to evaluate the dose–response relationships of both OS markers with the continuous MS at baseline, as well as the individual associations of the OS markers and the continuous MS with all-cause mortality. All models for dose–response analyses were adjusted for all potential covariates included in Model 3. The 25th, 50th and 75th percentiles of the levels of each OS marker (log) and the continuous MS were selected as knots and their mean values were selected as the reference.

Finally, we examined the associations of both OS markers (quartile) and MS (risk levels) with all-cause mortality using three multivariate Cox regression models which increasingly adjusted for potential covariates as described in the cross-sectional analyses. In addition to models including either OS markers or MS as the predictor, we also evaluated their independent associations with all-cause mortality in models containing both predictors simultaneously. Harrell's and Uno's C statistics were employed to evaluate the predictive abilities of both indicators and their combination [19, 20], and potential overestimation by fitting to our data was corrected using bootstrap analysis with 1000 replications to quantify the degradation in model predictive accuracy. The final estimates were the averages of the results from each bootstrap sample.

Sensitivity analyses were additionally performed to explore the contribution of smoking to the predictive performance of the MS for all-cause mortality. We firstly adjusted for pack-years in the fully adjusted multivariate Cox regression model for the association between the MS and all-cause mortality and did a subgroup analysis based on smoking status (never/ever smokers). We then evaluated the performance of MS, smoking status and pack-years in the prediction of all-cause mortality. Harrell's and Uno's C statistics were also employed to evaluate the predictive abilities of the three indicators and their combination.

All aforementioned analyses were performed by SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For all statistical analyses, *P*-values less than 0.05 in two-sided tests were considered as statistically significant.

Results

Participant characteristics

Baseline characteristics of the 1448 participants and the subsets based on the quartiles of both OS markers are shown in Table 1. Overall, the average age at baseline was about 62 years. More than half of the participants were ever smokers (current or former smokers). The majority of participants were overweight or obese, consumed no or low amounts of alcohol and reported no or only low physical activity. Around 40% participants had a low MS and 45% had a moderate MS (2–5). During a median follow-up time of 15.1 years until the end of 2015, 358 participants died, of whom 129 died from CVD, 132 died from cancers and 97 died from other diseases.

The participants with the highest level of 8-iso (quartile 4) had a much larger proportion of people with a high MS compared to the remaining participants, while the distribution of the MS among the four quartiles of 8-oxo was similar (Table 1). Figure 2 further demonstrates the distributions of both OS markers according to sex, age group and the MS risk levels. Median values of both markers at baseline were higher in female than in male participants and were the highest among the oldest participants (> 70 years). Participants with the highest MS (> 5) showed higher levels of 8-iso than the remaining, but this pattern was not observed with respect to the 8-oxo levels.

Association of the mortality risk score with oxidative stress

Results of the multivariable linear and ordinal logistic regression analyses for the cross-sectional associations are shown in Table 2. Both OS markers were strongly associated with the MS after controlling for age and sex. Additional adjustment for other potential covariates attenuated the association to some extent all of which nevertheless remained statistically significant (*P*-values < 0.05). Furthermore, we explored the dose–response relationships of the levels of each OS marker with the continuous MS. As illustrated in Fig. 3, monotonic positive dose–response relationships of both OS markers with respect to the continuous MS were observed. An increase in the levels of 8-iso (log) and 8-oxo (log) by one standard deviation were roughly associated with a 0.04-unit and 0.03-unit increase in the continuous MS, respectively.

Associations of oxidative stress and the mortality risk score with all-cause mortality

Table 3 presents the associations of both OS markers and the MS with all-cause mortality of the 1448 participants. A statistically significant association with mortality was only observed for 8-iso but not for 8-oxo. The fully-adjusted hazard ratio (HR) for the top quartile of 8-iso compared with the bottom quartile was 1.56 (95% CI 1.13–2.16) and an increase by one standard deviation in the continuous 8-iso level went along with a 1.16 (95% CI 1.04–1.29) fold

Table 1 Characteristics of the ESTHER study participants at baseline with methylation risk score for total population and the subsets based on the levels of oxidative stress markers[a]

Characteristics	Total population	Subsets based on the quartiles of 8-iso (log)					Subsets based on the quartiles of 8-oxo (log)				
		Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-value	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-value
N	1448	362	362	362	362		362	362	362	362	
Age (years)	62.1 (6.51)	62.5 (6.39)	62.2 (6.38)	62.1 (6.67)	61.6 (6.58)	0.335	61.6 (6.30)	61.8 (6.65)	62.3 (6.53)	62.5 (6.54)	0.203
Sex (male)	660 (45.6%)	200 (55.3%)	178 (49.2%)	149 (41.2%)	133 (36.7%)	< 0.0001	224 (61.9%)	185 (51.1%)	145 (40.1%)	106 (29.3%)	< 0.0001
Smoking status[b]						< 0.0001					0.073
Current smoker	257 (17.8%)	35 (9.7%)	47 (13.0%)	69 (19.1%)	106 (29.3%)		149 (41.2%)	57 (15.8%)	64 (17.7%)	67 (18.5%)	
Former smoker	478 (33.0%)	145 (40.1%)	122 (33.7%)	113 (31.2%)	98 (27.1%)		143 (39.5%)	121 (33.4%)	109 (30.1%)	105 (29.0%)	
Never smoker	675 (46.6%)	171 (47.2%)	186 (51.4%)	167 (46.1%)	151 (41.7%)		69 (19.1%)	170 (47.0%)	178 (49.2%)	178 (49.2%)	
Body mass index[c]						0.221					0.067
Underweight or normal weight (< 25.0)	382 (26.4%)	93 (25.7%)	95 (26.2%)	94 (26.0%)	100 (27.6%)		91 (25.1%)	101 (27.9%)	79 (21.8%)	111 (30.7%)	
Overweight (25 to < 30)	676 (46.7%)	179 (49.5%)	179 (49.5%)	170 (47.0%)	148 (40.9%)		177 (48.9%)	167 (46.1%)	186 (51.4%)	146 (40.3%)	
Obese (≥ 30.0)	387 (26.7%)	89 (24.6%)	89 (24.3%)	97 (26.8%)	113 (31.2%)		94 (26.0%)	93 (25.7%)	97 (26.8%)	103 (28.5%)	
Alcohol consumption[d]						0.091					0.184
Abstainer	461 (31.8%)	105 (29.0%)	108 (29.8%)	112 (30.9%)	136 (37.6%)		99 (27.4%)	110 (30.4%)	130 (35.9%)	122 (33.7%)	
Low	771 (53.3%)	208 (57.5%)	201 (55.5%)	197 (54.4%)	165 (45.6%)		200 (55.3%)	196 (54.1%)	185 (51.1%)	190 (52.5%)	
Intermediate	78 (5.4%)	13 (3.6%)	20 (5.5%)	23 (6.4%)	22 (6.1%)		23 (6.4%)	22 (6.1%)	13 (3.6%)	20 (5.5%)	
High	19 (1.3%)	5 (1.4%)	5 (1.4%)	3 (0.8%)	6 (1.7%)		8 (2.2%)	4 (1.1%)	5 (1.4%)	2 (0.6%)	
Physical activity[e]						0.017					0.122
Inactive	295 (20.4%)	58 (16.0%)	83 (22.9%)	67 (18.5%)	87 (24.0%)		69 (19.1%)	67 (18.5%)	77 (21.3%)	82 (22.7%)	
Low	671 (46.3%)	172 (47.5%)	153 (42.3%)	168 (46.4%)	178 (49.2%)		163 (45.0%)	170 (47.0%)	156 (43.1%)	182 (50.3%)	
Medium or high	479 (33.1%)	131 (36.2%)	125 (34.5%)	126 (34.8%)	97 (26.8%)		130 (35.9%)	125 (34.5%)	127 (35.1%)	97 (26.8%)	
Prevalence of major diseases											
Cardiovascular disease	305 (21.1%)	84 (23.2%)	73 (20.2%)	70 (19.4%)	78 (21.6%)	0.599	78 (21.6%)	59 (16.3%)	78 (21.6%)	90 (24.9%)	0.042
Diabetes[f]	233 (16.1%)	62 (17.1%)	47 (13.0%)	52 (14.4%)	72 (19.9%)	0.055	60 (16.6%)	58 (16.0%)	47 (13.0%)	68 (18.8%)	0.217
Cancer	88 (6.1%)	24 (6.6%)	19 (5.3%)	20 (5.5%)	25 (6.9%)	0.739	21 (5.8%)	15 (4.1%)	32 (8.8%)	20 (5.5%)	0.059
Mortality risk score (category)						0.0001					0.320
0–1/Low	592 (40.9%)	157 (43.4%)	153 (42.3%)	145 (40.1%)	137 (37.9%)		148 (40.9%)	162 (44.8%)	140 (38.7%)	142 (39.2%)	
2–5/Moderate	660 (45.6%)	169 (46.7%)	173 (47.8%)	170 (47.0%)	148 (40.9%)		166 (45.9%)	157 (43.4%)	167 (46.1%)	170 (47.0%)	
> 5/High	196 (13.5%)	36 (9.9%)	36 (9.9%)	47 (13.0%)	77 (21.3%)		48 (13.3%)	43 (11.9%)	55 (15.2%)	50 (13.8%)	

[a]Mean values (SD) for continuous variables and n (%) for categorical variables; Differences among subgroups of TL quintiles were tested for statistical significance by Kruskal–Wallis test (continuous variables) and Chi square test (categorical variables)

[b]Data missing for 38 participants

[c]Data missing for 3 participants

[d]Data missing for 119 participants. Categories defined as follows: abstainer, low (women: 0 to < 20 g/d, men: 0 to < 40 g/d), intermediate (20 to < 40 g/d and 40 to < 60 g/d, respectively), high (≥ 40 g/d and ≥ 60 g/d, respectively)

[e]Data missing for 3 participants. Categories defined as follows: inactive (< 1 h of physical activity/week), medium or high (≥ 2 h of vigorous or ≥ 2 h of light physical activity/week), low (other)

[f]Data missing for 18 participants

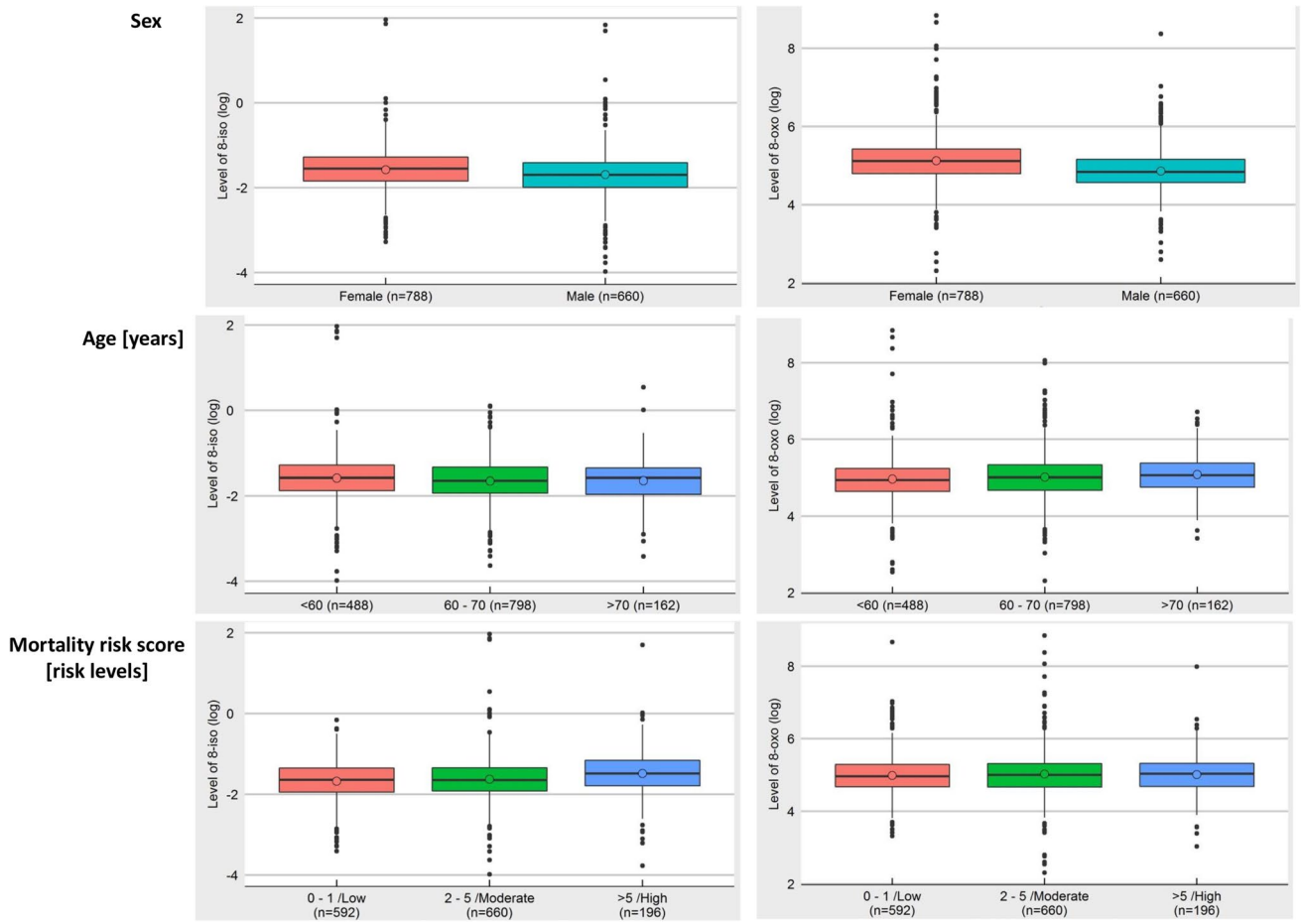

Fig. 2 Distributions of 8-iso and 8-oxo levels of the ESTHER study participants at baseline according to sex, age and mortality risk score (risk levels)

Table 2 Associations between oxidative stress markers and mortality risk score

Characteristic	Mortality risk score (ordinal)[a]		Mortality risk score (continuous)[b]		Mortality risk score (risk levels)[c]	
	Estimate (SE)	*P*-value	Estimate (SE)	*P*-value	Odds ratio (95% CI)	*P*-value
Model 1[d]						
Level of 8-iso (log, per SD)	0.315 (0.057)	< 0.0001	0.074 (0.011)	< 0.0001	1.33 (1.20–1.47)	< 0.0001
Level of 8-oxo (log, per SD)	0.155 (0.058)	0.0080	0.039 (0.012)	0.0009	1.17 (1.05–1.30)	0.004
Model 2[e]						
Level of 8-iso (log, per SD)	0.158 (0.056)	0.0045	0.044 (0.011)	< 0.0001	1.19 (1.06–1.33)	0.002
Level of 8-oxo (log, per SD)	0.131 (0.056)	0.020	0.031 (0.011)	0.0061	1.17 (1.04–1.31)	0.008
Model 3[f]						
Level of 8-iso (log, per SD)	0.146 (0.055)	0.0083	0.041 (0.011)	0.0002	1.18 (1.05–1.32)	0.004
Level of 8-oxo (log, per SD)	0.119 (0.056)	0.035	0.029 (0.011)	0.0102	1.16 (1.04–1.31)	0.010

SD standard deviation, *CI* confidence interval

[a]Ordinal mortality risk score 0–10, analyzed by linear regression

[b]Continuous mortality risk score, analyzed by linear regression

[c]Risk levels based on mortality risk score: 0–1/Low; 2–5/Moderate, > 5/High, analyzed by ordinal logistic regression

[d]Adjusted for age, sex and the leukocyte distribution (Houseman algorithm)

[e]Additionally adjusted for alcohol consumption, smoking status, BMI class and physical activity

[f]Additionally adjusted for the prevalence of CVD, diabetes and cancer

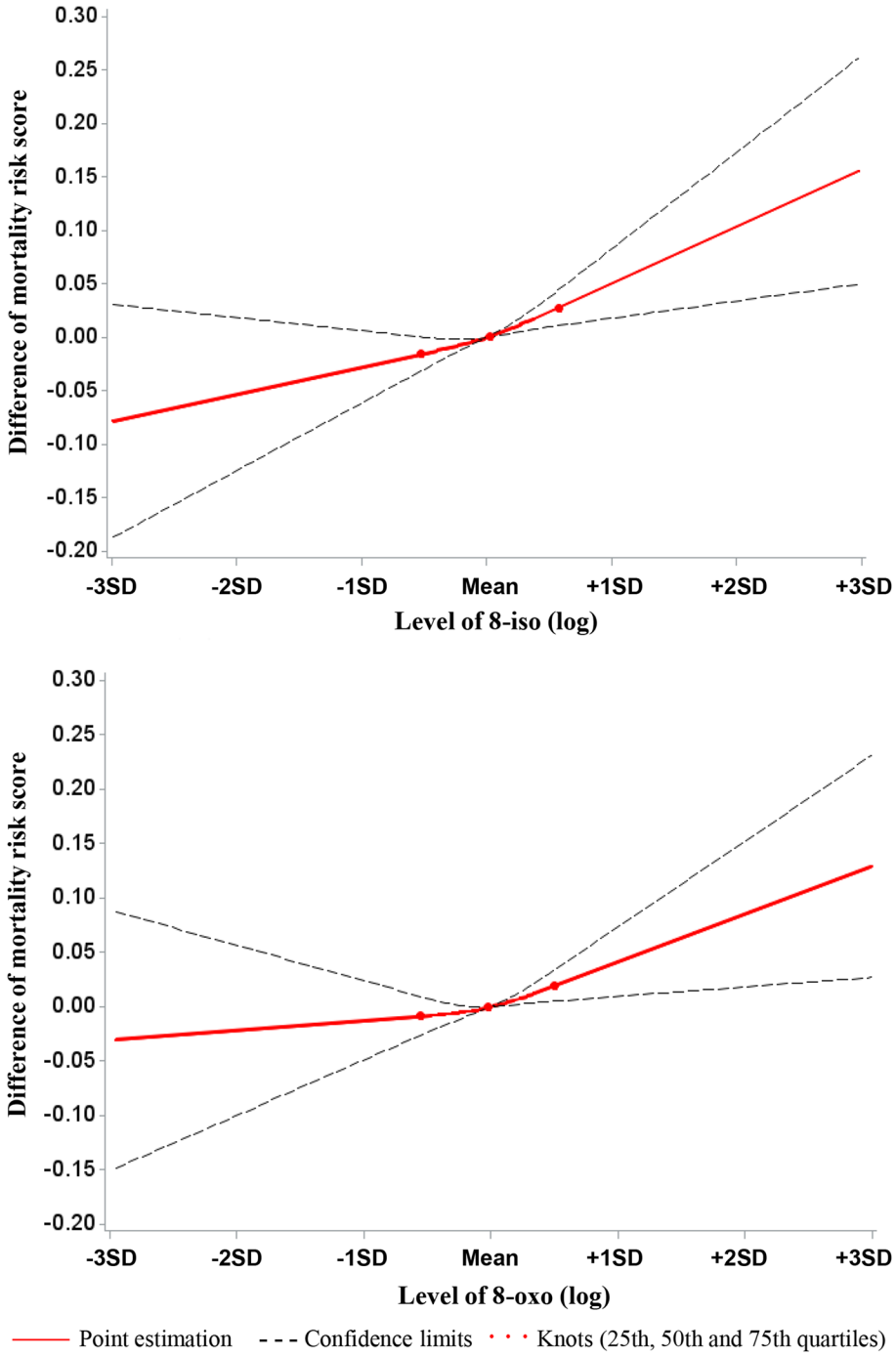

Fig. 3 Graphs of the best-fitting models for the associations of continuous mortality risk score with the levels of 8-iso (log) and 8-oxo (log). Red lines: estimation; Dashed lines: confidence limits; Red dots: knots (25th, 50th and 75th quartiles); Green lines: reference lines

increase in mortality. Figure 4 also demonstrated a clear increasing trend of mortality with increasing 8-iso levels with a similar pattern as for the MS. However, after controlling for the MS, the association between 8-iso levels (quartiles) and all-cause mortality was markedly attenuated and was no longer significant (Table 4).

By contrast, a robust association was observed between the MS and all-cause mortality, which persisted after controlling for multiple covariates (Table 3, Fig. 4). In particular, the moderate and high MS were associated with HRs of 1.71 (95% CI 1.27–2.29) and 2.92 (95% CI 2.03–4.18) compared to a MS of 0–1, respectively, and an increase by one standard deviation in the continuous MS went along with a 1.55 (95% CI 1.37–1.75) fold increase in the mortality risk after full adjustment for potential covariates (Table 3). These HRs remained essentially unchanged after additionally controlling for the levels of 8-iso or 8-oxo (Table 4).

Table 3 Associations of oxidative stress markers and mortality risk score with all-cause mortality

Characteristics	N_{total}	N_{death}	Model 1[a]		Model 2[b]		Model 3[c]	
			HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Level of 8-iso (log, quartile)								
1	362	78	Reference		Reference		Reference	
2	362	79	1.05 (0.77–1.43)	0.766	1.08 (0.77–1.51)	0.667	1.19 (0.85–1.67)	0.321
3	362	89	1.28 (0.95–1.74)	0.110	1.34 (0.97–1.87)	0.078	1.41 (1.01–1.97)	0.045
4	362	112	1.81 (1.35–2.43)	< 0.0001	1.53 (1.11–2.17)	0.009	1.56 (1.13–2.16)	0.007
Level of 8-iso (log, continuous, per SD)	1448	358	1.24 (1.13–1.37)	< 0.0001	1.18 (1.06–1.32)	0.004	1.16 (1.04–1.29)	0.006
Level of 8-oxo (log, quartile)								
1	362	90	Reference		Reference		Reference	
2	362	88	1.02 (0.76–1.37)	0.893	1.08 (0.79–1.48)	0.632	1.09 (0.80–1.51)	0.581
3	362	90	1.08 (0.80–1.45)	0.606	1.28 (0.94–1.74)	0.120	1.26 (0.93–1.73)	0.141
4	362	90	1.12 (0.83–1.51)	0.465	1.13 (0.82–1.56)	0.457	1.04 (0.75–1.43)	0.835
Level of 8-oxo (log, continuous, per SD)	1448	358	1.04 (0.93–1.16)	0.475	1.07 (0.95–1.20)	0.282	1.02 (0.91–1.15)	0.694
Mortality risk score (risk levels)								
0–1/Low	592	84	Reference		Reference		Reference	
2–5/Moderate	660	175	1.80 (1.38–2.34)	< 0.0001	1.72 (1.29–2.30)	0.0002	1.71 (1.27–2.29)	0.0004
> 5/High	196	99	3.86 (2.84–5.26)	< 0.0001	3.12 (2.18–4.47)	< 0.0001	2.92 (2.03–4.18)	< 0.0001
Mortality risk score (continuous, per SD)	1448	358	1.76 (1.58–1.95)	< 0.0001	1.60 (1.41–1.81)	< 0.0001	1.55 (1.37–1.75)	< 0.0001

SD standard deviation, *HR* hazard ratio, *CI* confidence interval

[a]Adjusted for age and sex, the leukocyte distribution (Houseman algorithm) was additionally adjusted for the models with mortality score

[b]Model 1 plus adjusted for alcohol consumption, smoking status, BMI class and physical activity

[c]Model 2 plus adjusted for the prevalence of CVD, diabetes and cancer

Summary indicators of the performance of both OS markers (quartiles) and MS (risk levels) in the prediction of all-cause mortality are presented in Table 5. The MS outperformed both OS markers in the prediction of all-cause mortality. Harrell's C statistics for the categorical MS was 0.644 (95% CI: 0.624–0.664), while the C statistics of the levels of 8-iso and 8-oxo (quartiles) were below 0.55. A very similar pattern was seen for Uno's C statistics. The combination of 8-iso levels and MS slightly increased the point estimates of both statistics, but 95% CI were overlapping.

Sensitivity analyses for exploring the contribution of smoking to the predictive performance of the MS for all-cause mortality were performed. As shown in Table S1, we firstly adjusted for pack-years in the fully adjusted model for the association between the MS and all-cause mortality, the estimates of MS were essentially unchanged comparing to the main results. We then did a subgroup analysis based on smoking status (never/ever smokers). Although the effect of MS was slightly attenuated in the never smokers, the MS (especially the continuous MS) was still associated with all-cause mortality in both subgroups. Finally, we evaluated the performance of MS, smoking status and pack-years in the prediction of all-cause mortality. The MS outperformed both smoking status and pack-years in the prediction of mortality (Table S2). The combination of MS and smoking status (pack-years) slightly increased the point estimates of both C-statistics, but 95% CI were overlapping.

Discussion

This study explored the associations of OS defined by two urinary biomarkers, 8-iso and 8-oxo, with an epigenetic MS in a population-based cohort study of older adults. Among 1448 participants, we found that both OS markers were significantly associated with the MS after controlling for potential confounders, and both OS markers further manifested monotonic dose–response relations with the continuous MS. Even though 8-iso was associated with all-cause mortality during a median follow-up of 15.1 years, the MS demonstrated an even stronger and robust association with mortality, which remained essentially unchanged after additionally controlling for the OS markers. This study shows the relationship between OS and the mortality-related epigenetic changes, and endorses the evidence of a potential use of the MS in the assessments of disproportional aging and mortality risks.

Previous studies have identified the association of several serum OS markers with all-cause mortality, including derivatives of reactive oxygen metabolites (d-ROM) levels,

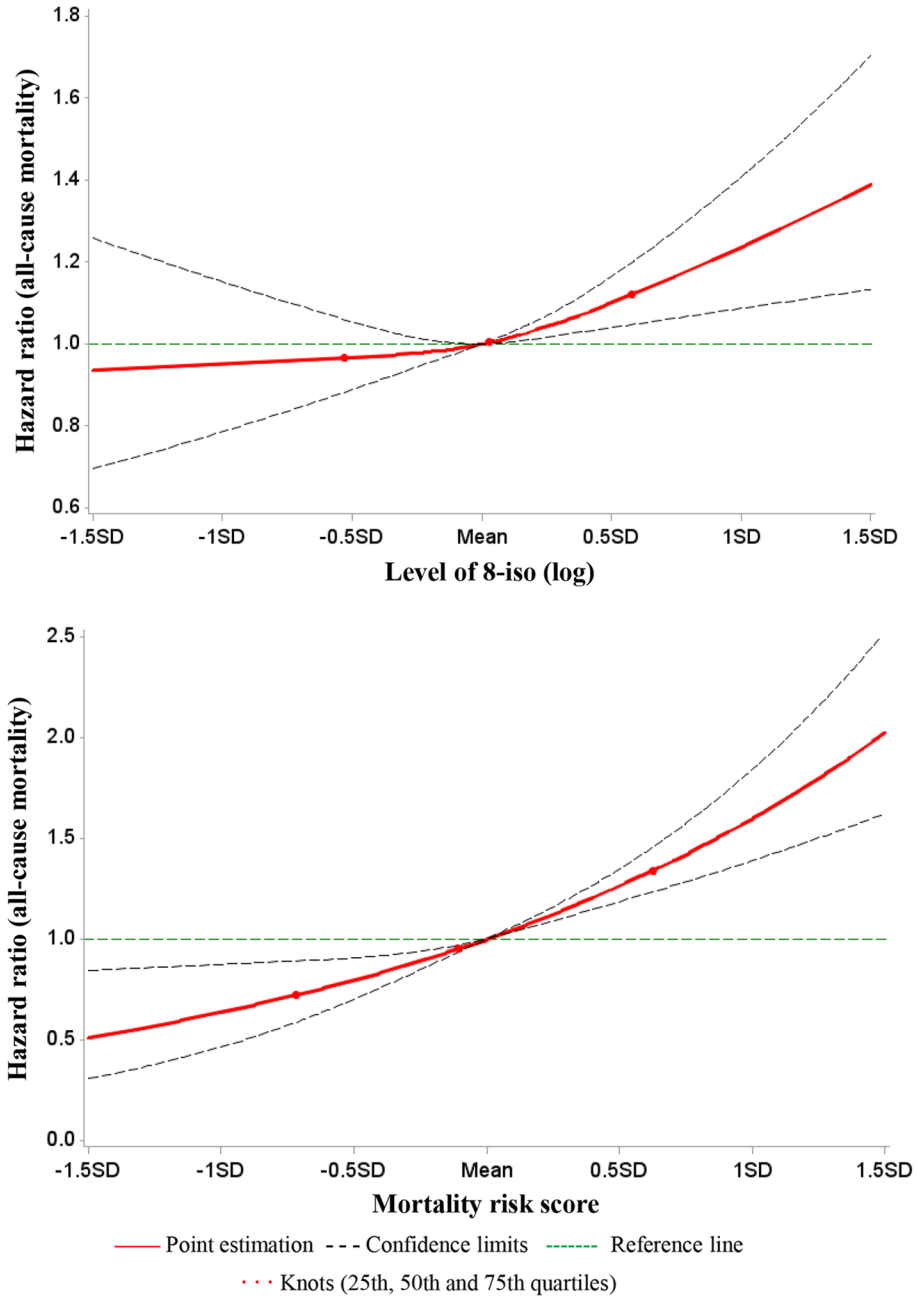

Fig. 4 Graphs of the best-fitting models for associations of the levels of 8-iso (log) and the continuous mortality risk score with all-cause mortality. Red lines: estimation; Dashed lines: confidence limits; Red dots: knots (25th, 50th and 75th quartiles); Green lines: reference lines

F2-isoprostanes and plasma malondialdehyde levels [3, 21–23]. To our best knowledge, our study is the first that identified that the level of urinary 8-iso is associated with all-cause mortality. This marker is one of the products of non-enzymatic lipid peroxidation and has been found to be particularly sensitive to smoking. In particular, positive associations with current smoking [6, 7] and also negative associations with the time after smoking cessation [9] have been demonstrated. As tobacco smoking is a well-established mortality-related lifestyle factor [24], we suggest that 8-iso levels to some extent partly reflect mechanisms linking smoking with all-cause mortality. Additionally, our sensitivity analyses with smoking status and pack-years demonstrated that our MS reflects mortality-related risks beyond those from smoking exposure.

Regarding the observational association between 8-iso levels and the mortality-related DNA methylation changes, four out of the 10 CpG sites that were used to build the MS are smoking-related loci, including cg01612140 (*6q14.1*), cg05575921 (*AHRR*), cg06126421 (*6p21.33*) and cg19572487 (*RARA*), and the last three have also been found to have the potential to reflect the smoking impact on the changes of 8-iso levels [9]. Another two CpG sites, cg08362785 (*MKL1*) and cg23665802 (*MIR19A*), have been

Table 4 Associations of oxidative stress markers (quartile) and morality risk score (risk levels) with all-cause mortality, with and without mutual adjustment for both indicators[a]

Characteristics	N_{total}	N_{death}	Model with oxidative stress marker only		Model with mortality risk score only		Model with both indicators	
			HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Level of 8-iso (log, quartile)								
1	362	78	Reference				Reference	
2	362	79	1.19 (0.85–1.67)	0.321			1.22 (0.82–1.81)	0.458
3	362	89	1.41 (1.01–1.97)	0.045			1.46 (0.98–2.16)	0.110
4	362	112	1.56 (1.13–2.16)	0.007			1.31 (0.89–1.94)	0.058
Level of 8-iso (log, continuous, per SD)	1448	358	1.16 (1.04–1.29)	0.006			1.09 (0.91–1.18)	0.597
Mortality risk score (risk levels)								
0–1/Low	592	84			Reference		Reference	
2–5/Moderate	660	175			1.71 (1.27–2.29)	0.0004	1.72 (1.28–2.31)	0.0004
> 5/High	196	99			2.92 (2.03–4.18)	< 0.0001	2.88 (2.00–4.15)	< 0.0001
Mortality risk score (continuous, per SD)	1448	358			1.55 (1.37–1.75)	< 0.0001	1.60 (1.39–1.85)	< 0.0001
Level of 8-oxo (log, quartile)								
1	362	90	Reference				Reference	
2	362	88	1.09 (0.80–1.51)	0.581			1.14 (0.83–1.57)	0.428
3	362	90	1.26 (0.93–1.73)	0.141			1.20 (0.87–1.64)	0.269
4	362	90	1.04 (0.75–1.43)	0.835			0.95 (0.69–1.33)	0.780
Level of 8-oxo (log, continuous, per SD)	1448	358	1.02 (0.91–1.15)	0.694			1.04 (0.91–1.19)	0.588
Mortality risk score (risk levels)								
0–1/Low	592	84			Reference		Reference	
2–5/Moderate	660	175			1.71 (1.27–2.29)	0.0004	1.74 (1.29–2.34)	0.0003
> 5/High	196	99			2.92 (2.03–4.18)	< 0.0001	3.03 (2.10–4.38)	< 0.0001
Mortality risk score (continuous, per SD)	1448	358			1.55 (1.37–1.75)	< 0.0001	1.61 (1.40–1.86)	< 0.0001

SD standard deviation, *HR* hazard ratio, *CI* confidence interval

[a]Models are adjusted for age, sex, alcohol consumption, smoking status, BMI class, physical activity, the prevalence of CVD, diabetes and cancer. The leukocyte distribution (Houseman algorithm) was additionally adjusted for the models with mortality score; Models controlled for the categorical/continuous forms of OS markers and the MS simultaneously

Table 5 Overall Harrell's and Uno's C statistics of the oxidative stress markers (quartile) and morality risk score (risk levels) in prediction of all-cause mortality

Characteristic	Harrell's C statistics	95% CI	Uno's C statistic	95% CI
Level of 8-iso (quartiles)	0.548	0.526–0.569	0.547	0.525–0.569
Level of 8-oxo (quartiles)	0.518	0.502–0.534	0.518	0.503–0.533
Mortality risk score (3 risk levels)	0.644	0.624–0.664	0.642	0.621–0.662
Mortality risk score (3 risk levels) + Level of 8-iso (quartiles)	0.658	0.636–0.680	0.655	0.633–0.677
Mortality risk score (3 risk levels) + Level of 8-oxo (quartiles)	0.649	0.627–0.671	0.646	0.625–0.668

CI confidence interval

mapped in genomic regions that are reported to be associated with one of the most common consequence of smoking, i.e. lung cancer [25], which was also found to be related to the elevated levels of 8-iso [26]. In addition, we observed that the association of 8-iso with all-cause mortality became weaker and not statistically significant after adding the MS to the model, which suggests that the impact of OS on mortality risks may be partly mediated by the mortality-related DNA methylation changes. Further causal inference and multidisciplinary studies are required to fully understand the corresponding biological/functional connections of 8-iso with mortality-related CpG sites and aging acceleration.

As a common biomarker of DNA damage [8], 8-oxo has been found to be associated with the survival of patients

with cutaneous melanoma and non-small cell lung cancer [27, 28]. However, in this study, we did not observe an association of 8-oxo with mortality. In a previous study from our group, this marker had also not been found to be associated with smoking exposure or smoking-related DNA methylation changes [9], which are both highly related to all-cause mortality. In line with this, the levels of 8-oxo did not show any association with either cg08362785 or cg23665802, loci included in the MS that are associated with lung cancer and mortality. A possible explanation for such inconclusive findings might be inadequate statistical power due to the limited numbers of participants and deaths, which might have hindered the disclosure of pertinent associations in this study.

Regarding the mortality prediction of 8-iso levels and MS, the MS was found to be much more predictive than the 8-iso levels, and inclusion of 8-iso in a model with MS only slightly improved prediction capacity. Even though the biological connection of the MS with mortality is yet to be established in detail, this score might be useful for describing the mortality risks and for risk stratification in the general population. Another scientific question of major interest is whether the MS could reflect the accelerated biological age like another popular epigenetic-based marker for health risks, the DNA methylation age [29, 30]. Previous studies have found overlapping smoking-related CpG sites that were associated with both 8-iso levels and the DNA methylation age [9], but did not find any overlapping locus between the previously established CpG sites that are employed to determine the DNA methylation age and the mortality-related loci [13]. Given the strong association between 8-iso levels and the MS in this study, we suggest that the MS might be much more indicative for specific mortality risks contributed by OS, rather than the general "age acceleration" effect generated of OS.

One of the major strengths of this study is the availability of a broad range of covariates in addition to epigenome-wide methylation data in a population-based cohort that was comprehensively followed up for 15 years. Some limitations still have to be acknowledged in the interpretation of the study results. First, shifts of leukocyte distribution might affect the associations of DNA methylation in whole blood samples with mortality and OS [31, 32]. Hence, we adjusted for leukocyte distribution by the Houseman algorithm to restrict potential confounding from differential blood counts to the greatest possible extent [17]. Furthermore, the lack of follow-up DNA methylation profiles and OS data hinders exploration of the longitudinal association between the dynamics of OS and the MS. Future research on other proxies of OS, like d-ROM levels might enable more differentiated assessments of the relationship between OS and the mortality-related DNA methylation changes. Finally, due to the heterogeneity of populations, further studies are needed to evaluate to what extent our findings yielded from an exclusively Caucasian population (aged 50–75) can be generalized to other populations.

Conclusion

In summary, this study demonstrates that OS, defined by two urinary markers, is highly associated with the MS based on DNA methylation markers, and provides some evidence implying the possibility that the mortality-related DNA methylation changes might be triggered to some extent by oxidative damages. Although the 8-iso levels and the MS are independently associated with all-cause mortality, the MS is a much more robust indicator for predicting all-cause mortality. Further studies are warranted to elucidate the underlying pathophysiological mechanisms and potential clinical applications of the MS in routine medical care and intervention research aimed at reducing mortality.

Acknowledgements The ESTHER study was supported by the Baden-Württemberg state Ministry of Science, Research and Arts (Stuttgart, Germany), the Federal Ministry of Education and Research (Berlin, Germany), and the Federal Ministry of Family Affairs, Senior Citizens, Women and Youth (Berlin, Germany). Measurements of the oxidative stress markers were partly funded by the German Research Foundation (DFG, Grant No.: SCHO 1545/3-1). Xu Gao and Xīn Gào are supported by the grant from the China Scholarship Council (CSC). The authors gratefully acknowledge contributions of DKFZ Genomics and Proteomics Core Facility, especially Melanie Bewerunge-Hudler and Matthias Schick, in the processing of DNA samples and performing the laboratory work, Dr. Jonathan Heiss for providing the estimation of leukocyte distribution and Ms. Chen Chen for the language assistance.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

References

1. Salmon AB, Richardson A, Perez VI. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med. 2010;48(5):642–55. https://doi.org/10.1016/j.freeradbiomed.2009.12.015.
2. Salminen A, Ojala J, Kaarniranta K, Kauppinen A. Mitochondrial dysfunction and oxidative stress activate inflammasomes: impact on the aging process and age-related diseases. Cell Mol Life Sci. 2012;69(18):2999–3013. https://doi.org/10.1007/s00018-012-0962-0.
3. Schöttker B, Saum KU, Jansen EH, Boffetta P, Trichopoulou A, Holleczek B, et al. Oxidative stress markers and all-cause mortality at older age: a population-based cohort study. J Gerontol A Biol Sci Med Sci. 2015;70(4):518–24. https://doi.org/10.1093/gerona/glu111.
4. Schöttker B, Brenner H, Jansen EH, Gardiner J, Peasey A, Kubinova R, et al. Evidence for the free radical/oxidative stress theory of ageing from the CHANCES consortium: a meta-analysis

of individual participant data. BMC Med. 2015;13(1):300. https://doi.org/10.1186/s12916-015-0537-7.
5. Stephens JW, Khanolkar MP, Bain SC. The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease. Atherosclerosis. 2009;202(2):321–9. https://doi.org/10.1016/j.atherosclerosis.2008.06.006.
6. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am J Respir Crit Care Med. 2000;162(3 Pt 1):1175–7. https://doi.org/10.1164/ajrccm.162.3.2001063.
7. Kinnula VL, Ilumets H, Myllarniemi M, Sovijarvi A, Rytila P. 8-Isoprostane as a marker of oxidative stress in nonsymptomatic cigarette smokers and COPD. Eur Respir J. 2007;29(1):51–5. https://doi.org/10.1183/09031936.00023606.
8. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2′ -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2009;27(2):120–39. https://doi.org/10.1080/10590500902885684.
9. Gao X, Gào X, Zhang Y, Breitling LP, Schöttker B, Brenner H. Associations of self-reported smoking, cotinine levels and epigenetic smoking indicators with oxidative stress among older adults: a population-based study. Eur J Epidemiol. 2017;32(5):443–56. https://doi.org/10.1007/s10654-017-0248-9.
10. Mikeska T, Craig JM. DNA methylation biomarkers: cancer and beyond. Genes. 2014;5(3):821–64. https://doi.org/10.3390/genes5030821.
11. Hedman AK, Mendelson MM, Marioni RE, Gustafsson S, Joehanes R, Irvin MR et al. Epigenetic patterns in blood associated with lipid traits predict incident coronary heart disease events and are enriched for results from genome-wide association studies. Circ Cardiovasc Genet 2017;10(1). https://doi.org/10.1161/circgenetics.116.001487.
12. Zhang Y, Schöttker B, Florath I, Stock C, Butterbach K, Holleczek B, et al. Smoking-associated DNA methylation biomarkers and their predictive value for all-cause and cardiovascular mortality. Environ Health Perspect. 2016;124(1):67–74. https://doi.org/10.1289/ehp.1409020.
13. Zhang Y, Wilson R, Heiss J, Breitling LP, Saum KU, Schottker B, et al. DNA methylation signatures in peripheral blood strongly predict all-cause mortality. Nat Commun. 2017;8:14617. https://doi.org/10.1038/ncomms14617.
14. Gao X, Zhang Y, Schöttker B, Brenner H. Vitamin D status and epigenetic-based mortality risk score: strong independent and joint prediction of all-cause mortality in a population-based cohort study. Clin Epigenet. 2018;10(1):84. https://doi.org/10.1186/s13148-018-0515-y.
15. Zhang Y, Saum KU, Schöttker B, Holleczek B, Brenner H. Methylomic survival predictors, frailty, and mortality. Aging (Albany NY) 2018. https://doi.org/10.18632/aging.101392.
16. Gao X, Zhang Y, Mons U, Brenner H. Leukocyte telomere length and epigenetic-based mortality risk score: associations with all-cause mortality among older adults. Epigenetics. 2018;13(8):846–57. https://doi.org/10.1080/15592294.2018.1514853.
17. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinform. 2012;13:86. https://doi.org/10.1186/1471-2105-13-86.
18. Desquilbet L, Mariotti F. Dose-response analyses using restricted cubic spline functions in public health research. Stat Med. 2010;29(9):1037–57. https://doi.org/10.1002/sim.3841.
19. Uno H, Cai T, Pencina MJ, D'Agostino RB, Wei LJ. On the C-statistics for evaluating overall adequacy of risk prediction procedures with censored survival data. Stat Med. 2011;30(10):1105–17. https://doi.org/10.1002/sim.4154.
20. Harrell FE Jr, Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med. 1996;15(4):361–87. https://doi.org/10.1002/(SICI)1097-0258(19960229)15:4%3c361:AID-SIM168%3e3.0.CO;2-4.
21. Schöttker B, Brenner H, Jansen EH, Gardiner J, Peasey A, Kubinova R, et al. Evidence for the free radical/oxidative stress theory of ageing from the CHANCES consortium: a meta-analysis of individual participant data. BMC Med. 2015;13(1):300. https://doi.org/10.1186/s12916-015-0537-7.
22. Kjaer LK, Cejvanovic V, Henriksen T, Petersen KM, Hansen T, Pedersen O, et al. Cardiovascular and all-cause mortality risk associated with urinary excretion of 8-oxoguo, a biomarker for rna oxidation, in patients with type 2 diabetes: a prospective cohort study. Diabetes Care. 2017;40(12):1771–8. https://doi.org/10.2337/dc17-1150.
23. Masia M, Padilla S, Fernandez M, Rodriguez C, Moreno A, Oteo JA, et al. Oxidative stress predicts all-cause mortality in HIV-infected patients. PLoS ONE. 2016;11(4):e0153456. https://doi.org/10.1371/journal.pone.0153456.
24. Centers for Disease C, Prevention. Smoking-attributable mortality, years of potential life lost, and productivity losses–United States, 2000–2004. MMWR Morb Mortal Wkly Rep 2008;57(45):1226–8.
25. Risch A, Plass C. Lung cancer epigenetics and genetics. Int J Cancer. 2008;123(1):1–7. https://doi.org/10.1002/ijc.23605.
26. Dalaveris E, Kerenidi T, Katsabeki-Katsafli A, Kiropoulos T, Tanou K, Gourgoulianis KI, et al. VEGF, TNF-alpha and 8-isoprostane levels in exhaled breath condensate and serum of patients with lung cancer. Lung Cancer. 2009;64(2):219–25. https://doi.org/10.1016/j.lungcan.2008.08.015.
27. Shen J, Deininger P, Hunt JD, Zhao H. 8-Hydroxy-2′-deoxyguanosine (8-OH-dG) as a potential survival biomarker in patients with nonsmall-cell lung cancer. Cancer. 2007;109(3):574–80. https://doi.org/10.1002/cncr.22417.
28. Murtas D, Piras F, Minerba L, Ugalde J, Floris C, Maxia C, et al. Nuclear 8-hydroxy-2′-deoxyguanosine as survival biomarker in patients with cutaneous melanoma. Oncol Rep. 2010;23(2):329–35.
29. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013;14(10):R115. https://doi.org/10.1186/gb-2013-14-10-r115.
30. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell. 2013;49(2):359–67. https://doi.org/10.1016/j.molcel.2012.10.016.
31. Schwartz J, Weiss ST. Cigarette smoking and peripheral blood leukocyte differentials. Ann Epidemiol. 1994;4(3):236–42.
32. Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, et al. Passive and active DNA methylation and the interplay with genetic variation in gene regulation. Elife. 2013;2:e00523. https://doi.org/10.7554/eLife.00523.