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CARDIOVASCULAR DISEASE

Association of serum markers of oxidative stress with myocardial infarction and stroke: pooled results from four large European cohort studies

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Abstract

Oxidative stress contributes to endothelial dysfunction and is involved in the pathogenesis of myocardial infarction (MI) and stroke. However, associations of biomarkers of oxidative stress with MI and stroke have not yet been addressed in large cohort studies. A nested case–control design was applied in four population-based cohort studies from Germany, Czech Republic, Poland and Lithuania. Derivatives of reactive oxygen metabolites (d-ROMs) levels, as a proxy for the reactive oxygen species burden, and total thiol levels (TTL), as a proxy for the reductive capacity, were measured in baseline serum samples of 476 incident MI cases and 454 incident stroke cases as well as five controls per case individually matched by study center, age and sex. Statistical analyses were conducted with multi-variable adjusted conditional logistic regression models. d-ROMs levels were associated with both MI (odds ratio (OR), 1.21 [95% confidence interval (CI) 1.05–1.40] for 100 Carr units increase) and stroke (OR, 1.17 [95% CI 1.01–1.35] for 100 Carr units increase). TTL were only associated with stroke incidence (OR, 0.79 [95% CI 0.63-0.99] for quartiles 2–4 vs. quartile 1). The observed relationships were stronger with fatal than with non-fatal endpoints; association of TTL with fatal MI was statistically significant (OR, 0.69 [95% CI 0.51–0.93] for 100 µmol/L-increase). This pooled analysis of four large population-based cohorts suggests an important contribution of an imbalanced redox system to the etiology of mainly fatal MI and stroke events.

Keywords Oxidative stress · Myocardial infarction · Stroke · Cardiovascular disease · Cohort study

Introduction

The underlying pathology of myocardial infarction (MI) and ischemic stroke is atherosclerosis [1]. Common features of atherosclerosis include low-density lipoprotein

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(LDL) oxidation, endothelial dysfunction, and inflammation [2]. Importantly, these three features of atherosclerosis all involve reactive oxygen species (ROS) in their pathophysiology [3]. ROS can mediate oxidative modification of LDL and inflammation processes. ROS generation is increased in the endothelium of human blood vessels under condition of endothelial dysfunction [4]. Moreover, emerging data now link autophagic clearance declines to mitochondrial dysfunction and oxidative stress [5].

Nevertheless, large cohort studies linking biomarkers of oxidative stress to atherosclerotic diseases in humans are sparse because ROS are highly reactive, have a very short half-life and thus, are difficult to measure directly in serum or plasma in large population samples [6]. Recently, assays that indirectly measure ROS burden and control have been developed and operationalized for high-throughput measurement techniques: derivatives of reactive oxygen metabolites (d-ROMs) and total thiol levels (TTL). D-ROMs can be regarded as a proxy for ROS production [7] and TTL as a proxy for the redox control status of blood [8]. The d-ROMs assay detects hydroperoxide metabolites (chemical: R–O–O–H), mainly of lipids, but also of glycosides, amino acids, and proteins in the serum sample [9]. The principal carrier of lipid hydroperoxides in human plasma is HDL [10]. The oxidation of HDL causes an impairment in its antiatherogenic and anti-inflammatory capacities and is a risk factor for developing atherosclerosis. The TTL assay detects free thiol groups of the amino acids cysteine or methionine (chemical: R–S–H), which can be reversibly oxidized to disulfide bridges (chemical: R–S–S–R). The most frequent molecules in human blood with free thiol groups are glutathione, α-lipoic acid, and members of the thioredoxin protein family [9]. These molecules act as an antioxidant defense system by their ability to oppose the propagation phase of the peroxidation processes in order to maintain intracellular redox environment, which plays a key role in regulating endothelial cell function [11].

Recent applications of these assays in cohort studies showed that d-ROMs and TTL were independent predictors of cardiovascular mortality [9, 12]. The aim of this analysis in four large-scale cohort studies is a detailed analysis of the associations of d-ROMs and TTL with incident MI and stroke, including distinct analyses on fatal and non-fatal events.

Methods

Study population and data collection

This investigation is based on the 8-year follow-up of the German ESTHER cohort (German name: "**E**pidemiologische **S**tudie zu Chancen der Verhütung, Früherkennung und optimierten **Th**erapie chronischer **Er**krankungen in der älteren Bevölkerung") and the HAPIEE cohorts (**H**ealth, **A**lcohol and **P**sychosocial factors **i**n **E**astern **E**urope) from Poland (PL), Czech Republic (CZ) and Lithuania (LT) [13, 14]. The total HAPIEE study comprises cohorts in four countries. As it was not possible to export blood samples from Russia, only three cohorts were included in the current project. An overview on the study designs of the cohorts and the baseline data collection is given in Table 1 and further details can be found elsewhere [9, 13–15]. The harmonization of variables in ESTHER and HAPIEE studies and measurement of oxidative stress markers were carried out in the framework of the Consortium on Health and Ageing: Network of Cohorts in Europe and the United States (CHANCES; www.chancesfp7.eu), which has been described elsewhere [16, 17].

Oxidative stress serum marker measurement

The assays used to measure d-ROMs levels (Diacron, Grosseto, Italy) and TTL (Rel Assay Diagnostics, Gaziantep, Turkey) were adapted to an autoanalyzer (LX20-Pro, Beckman-Coulter, Woerden, the Netherlands) at the Laboratory for Health Protection Research (Bilthoven, the Netherlands) as described previously [18]. The d-ROMs assay measures the hydroperoxide concentration in Carratelli Units (CARR U), named after the inventor of the assay, Mauro Carratelli. Each CARR U corresponds to 0.08 mg hydrogenperoxide (H_2O_2)/100 mL in the sample [19]. The TTL assay measures the concentration of free thiol groups in the sample in µmol/L. The two biomarkers have been measured from serum samples that had been stored for approx. 3–10 years in freezers with − 80 °C. TTL and d-ROMs serum levels have been shown to be stable under these conditions for at least 5 years [20]. Further information about quality controls for the measurements in the ESTHER and HAPIEE study has been described previously [9].

Outcome ascertainment

The procedures to ascertain fatal and non-fatal MI in the four cohorts are summarized in Table 1. All deaths coded with ICD-10 codes I60–I69 were considered stroke deaths and MI deaths were defined by the ICD-10 codes I21–I23. In the pooled data set, median follow-up for MI and stroke were 6.6 and 6.3 years, respectively (median follow-up ranged from 4.6 to 8.1 years in the four cohorts, see Table 1).

Table 1 Study designs of the analyzed cohorts, baseline data collection and follow-up procedures

	ESTHER 8-year follow-up	HAPIEE Poland (PL)	HAPIEE Czech Republic (CZ)	HAPIEE Lithuania (LT)
Study design aspects				
Country	Germany	Poland	Czech Republic	Lithuania
Region/town(s)	Saarland	Krakow	Havirov/Karvina, Hradec Kralove, Jihlava, Kromeriz, Liberec, and Usti nad Labem	Kaunas
Recruitment procedure	Recruitment by GPs during general health check-up	Random draw of a sample from Krakow population register, stratified by sex and 5-year age groups	Random draw of a sample from population registers of the 6 towns above, stratified by sex and 5-year age groups	Random draw of a sample from Kaunas population register, stratified by sex and 5-year age groups
Ethics approval	Medical Association of Saarland, Medical Faculty of the University of Heidelberg (Germany)	Jagiellonian University (Krakow, Poland), University College London (Great Britain)	National Institute of Public Health (Prague, Czech Republic), University College London (Great Britain)	Lithuanian University of Health Sciences (Kaunas, Lithuania), University College London (Great Britain)
Age range at baseline	56–85	45–69	45–69	45–69
Recruitment period	2008–2010	2002–2005	2002–2005	2006–2008
Sample size of total cohort	7012[a]	10,728	8857	7161
Data collection				
Socio-demographic, and lifestyle factors	Self-administered questionnaires	Self-administered questionnaires	Self-administered questionnaires	Self-administered questionnaires
History of disease data	Self-reports validated by medical records from GPs	Self-reports	Self-reports	Self-reports
Serum creatinine	Kinetic Jaffé method on a Cobas 8000 C701 (analyte from Roche)	Kinetic Jaffé method on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)	Kinetic Jaffé method on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)	Kinetic Jaffé method on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)
Serum total and HDL cholesterol	Enzymatic chromatography on a Cobas 8000 C701 (analytes from Roche)	Enzymatic chromatography on a Hitachi 917/Modular P (analytes from Roche)	Enzymatic chromatography on a Roche Cobas Mira (analytes from Roche)	Enzymatic chromatography on a Roche Cobas Mira (analytes from Roche)
Serum CRP	Immunoturbidimetry on a Cobas 8000 C701 (analyte from Roche)	Immunoturbidimetry on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)	Immunoturbidimetry on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)	Immunoturbidimetry on a LX-20 Pro, Beckman-Coulter (analyte from Beckman-Coulter)
Follow-up				
Ascertainment of deaths[b]	Local mortality registers and death certificates	Krakow city mortality register and death certificates	Country-wide mortality register and death certificates	Kaunas city mortality register and death certificates
Ascertainment of non-fatal MI and stroke events	Self-reported cases validated by medical records of GPs	Self-reported cases validated by hospital discharge reports	Self-reported cases validated by hospital discharge reports	Self-reported cases validated by hospital-based MI and stroke registers
Mortality, MI and stroke follow-up until end of	2015	2010	2011	2011
Median follow-up time (years)	5.4	7.1	8.1	4.6

CRP C-reactive protein, *ESTHER* Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung (German), *GPs* general practitioners, *HAPIEE* Health, Alcohol and Psychosocial factors in Eastern Europe; HDL, high-density cholesterol; MI, myocardial infarction

[a]The 8-year follow-up of ESTHER cohort was considered as baseline in this analysis. From initiation date to 8-year follow-up, 499 individuals deceased, 505 individuals were no longer able to participate due to poor health and 680 had declined further participation. From the remaining 8265 participants, 6061 (73%) sent back a questionnaire and 4637 (56%) donated a blood sample in the office of their GP. The GPs of the study participants also completed questionnaires about the health status of 5997 (73%) study participants. Finally, information about incident diseases from either a questionnaire filled by the study participant or his/her GP was available for a total of 7012 (85%) individuals

[b]The registries were complete for all participants that did not move outside the covered region of the registry. Loss to follow-up due to migration was 4% in the HAPIEE cohorts (combined) and 2% in the ESTHER study

Table 2 Baseline characteristics of study participants by case/control status

Baseline	Myocardial infarction		Stroke	
Characteristics	Cases (n = 476)	Controls (n = 2380)	Cases (n = 454)	Controls (n = 2270)
Age (years)	63.9 (57.8; 68.8)	64.0 (57.8; 68.7)	66.3 (61.0; 70.3)	66.2 (61.0; 70.3)
45–< 60	153 (32.1)	765 (32.1)	99 (21.8)	492 (21.7)
60–< 65	107 (22.5)	537 (22.6)	99 (21.8)	493 (21.7)
65–< 70	147 (30.9)	730 (30.7)	132 (29.1)	665 (29.3)
70–< 85	69 (14.5)	348 (14.6)	124 (27.3)	620 (27.3)
Male sex	334 (70.2)	1670 (70.2)	239 (52.6)	1195 (52.6)
Education				
Low	**108 (23.1)**	454 (19.2)	146 (32.3)	683 (30.6)
Medium	**266 (56.8)**	1225 (51.9)	220 (48.7)	1035 (46.3)
High	**94 (20.1)**	681 (28.9)	86 (19.0)	517 (23.1)
BMI (kg/m^2)	**28.4 (26.2; 31.7)**	28.0 (25.4; 30.8)	28.2 (25.6; 31.5)	28.0 (25.4; 31.0)
< 20	**6 (1.3)**	24 (1.0)	1 (0.2)	21 (0.9)
20–< 25	**73 (15.4)**	500 (21.1)	90 (20.0)	469 (20.7)
25–< 30	**218 (46.1)**	1111 (46.8)	199 (44.1)	1048 (46.4)
30–< 35	**131 (27.7)**	569 (24.0)	120 (26.6)	532 (23.5)
≥ 35	**45 (9.5)**	170 (7.2)	41 (9.1)	191 (8.5)
Smoking				
Never	**174 (37.0)**	1099 (46.6)	**212 (47.3)**	1240 (55.3)
Former	**144 (30.6)**	757 (32.1)	**135 (30.1)**	656 (29.3)
Current	**152 (32.3)**	502 (21.3)	**101 (22.5)**	347 (15.5)
Alcohol consumption (g/day)	5.7 (0; 20.9)	6.8 (0; 22.9)	5.7 (0; 18.4)	5.7 (0; 17.0)
Vigorous physical activity	**284 (62.3)**	1602 (70.1)	**259 (60.2)**	1436 (67.2)
Total cholesterol (mg/dL)	**227.0 (198.3; 260.5)**	223.9 (196.8; 250.5)	223.9 (196.4; 255.0)	226.2 (200.0; 256.0)
< 200	**131 (27.5)**	663 (27.9)	128 (28.2)	570 (25.2)
200–< 280	**280 (58.8)**	1495 (62.8)	276 (60.8)	1427 (63.0)
≥ 280	**65 (13.7)**	221 (9.3)	50 (11.0)	269 (11.9)
HDL cholesterol (mg/dL)	**48.3 (41.0; 58.4)**	52.0 (43.7; 62.6)	**53.9 (43.3; 63.0)**	54.5 (45.6; 65.0)
< 40	**110 (23.3)**	346 (14.6)	**71 (15.6)**	267 (11.8)
40–< 80	**350 (74.2)**	1870 (79.0)	**357 (78.6)**	1820 (80.6)
≥ 80	**12 (2.5)**	151 (6.4)	**26 (5.7)**	170 (7.5)
CRP (mg/L)	**2.5 (1.3; 4.5)**	1.7 (0.8; 3.3)	**2.3 (1.1; 4.4)**	1.8 (0.9; 3.5)
≤ 3	**284 (59.7)**	1723 (72.4)	**279 (61.5)**	1579 (69.6)
> 3–≤ 10	**155 (32.6)**	529 (22.2)	**141 (31.1)**	679 (25.2)
> 10	**37 (7.8)**	128 (5.4)	**34 (7.5)**	138 (5.2)
eGFR (mL/min/1.73 m^2)[a]	80.7 (68.5; 93.0)	83.8 (71.5; 93.1)	77.0 (66.1; 88.9)	78.0 (66.9; 89.8)
≥ 60	**420 (88.2)**	2185 (91.8)	383 (84.4)	1978 (87.1)
< 60	**56 (11.8)**	195 (8.2)	71 (15.6)	292 (12.9)
History of hypertension	**329 (70.0)**	1314 (55.4)	**314 (69.3)**	1346 (59.7)
History of diabetes	**114 (24.2)**	331 (14.0)	**95 (21.0)**	347 (15.4)
d-ROMs (Carr U)	**364 (319; 418)**	354 (313; 403)	**377 (329; 426)**	364 (316; 413)
≤ 340	**173 (36.3)**	974 (40.9)	140 (30.8)	840 (37.0)
341–400	**149 (31.3)**	785 (33.0)	152 (33.5)	726 (32.0)
401–500	**126 (26.5)**	543 (22.8)	137 (30.2)	587 (25.9)
> 500	**28 (5.9)**	78 (3.3)	25 (5.5)	117 (5.2)

Table 2 (continued)

Baseline	Myocardial infarction		Stroke	
Characteristics	Cases (n = 476)	Controls (n = 2380)	Cases (n = 454)	Controls (n = 2270)
TTL (μmol/L)	401 (335; 474)	406 (338; 483)	371 (309; 425)	373 (312; 435)

Unless indicated otherwise, the table shows proportions (%) for categorical and medians (25th; 75th percentile) for continuous variables. Numbers shown were drawn from the not imputed data set. Therefore, numbers do not always add up to the total because of missing values

Bold printed: statistical significant ($p < 0.05$) difference among cases and controls assessed by Chi^2 test for categorical and Wilcoxon Ranksum test for continuous variables

BMI body mass index, *CRP* C-reactive protein, *CVD* cardiovascular disease, *d-ROMs* derivatives of reactive oxygen metabolites, *eGFR* estimated glomerular filtration rate, *HDL* high-density lipoprotein, *MI* myocardial infarction, *TTL* total thiol levels

[a]The estimated glomerular filtration rate (eGFR) was calculated with the creatinine based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [43]

Analytical study sample

Because of limited funding, d-ROMs levels and TTL were not measured in all participants in the HAPIEE cohorts; instead, a matched case control design was adopted (Supplementary (Suppl.) Figure 1). Cases were defined as all subjects that died during follow-up (n = 1433) or experienced incident non-fatal myocardial infarction (MI) or stroke (n = 658). Controls (n = 4396) were frequency matched to cases by sex and 5-year age-groups. For the current analysis, subjects that died of other causes than MI or stroke (potential competing risks events) or had a history of MI or stroke were excluded. This left 400 primary MI, 299 primary stroke cases and 3913 eligible controls. The pool of eligible controls was used to individually match exactly 5 controls to each case by cohort, sex and age (± 5 years). As analyses for the outcomes MI and stroke are performed separately, the same control could serve for both outcomes. In the ESTHER cohort, d-ROMs levels and TTL were measured in all available serum samples from the 8-year follow-up. After applying the same exclusion criteria as used in the HAPIEE cohorts, 76 primary MI and 155 primary stroke cases were matched to exactly 5 controls by sex and age (± 5 years) from a pool of 2481 eligible controls (Suppl. Figure 2). Finally, the matched cases and controls from the HAPIEE cohorts and the ESTHER study were pooled in one data set and 476 MI cases and 2380 MI controls as well as 454 stroke cases and 2270 stroke controls were obtained.

Statistical analyses

Differences in baseline characteristics between cases (MI or stroke during follow-up) and controls were assessed with the Chi^2 test for categorical variables and the Wilcoxon rank-sum test for continuous variables. For longitudinal analyses, conditional logistic regression was utilized to estimate odds ratios for an increase in d-ROMs levels by 100 Carr U and TTL by 100 μmol/L. In addition, d-ROMs levels were also modelled as a categorical variable, with manufacturer recommended clinical cut-offs for moderate (341–400 Carr U), high (401–500 Carr U) and very high oxidative stress (> 500 Carr U) with reference to subjects with not increased or low oxidative stress (≤ 340 Carr U). As no such cut-off recommendations are available for TTL, cohort-specific quintiles were used to build a categorical variable (Suppl. Table 1).

We assessed the associations of d-ROMs levels and TTL with six outcomes: total MI, non-fatal MI, fatal MI, total stroke, non-fatal stroke and fatal stroke. Stroke subtypes were not analyzed because this information was not recorded in the cohorts. For each outcome, four statistical models were developed, with an increasing inclusion of established cardiovascular risk factors into the models. Both d-ROMs levels and TTL were always included in the same model because their correlation was low ($r < 0.08$ in each cohort). In model 1, just the age, sex, and cohort—matched data were analysed. Model 2 additionally adjusted for education, body mass index (BMI), smoking, alcohol consumption and physical activity. Model 3 also included diseases that could potentially mediate the association of oxidative stress and MI or stroke (i.e. dyslipidemia, assessed by total and HDL cholesterol, renal impairment, diabetes and hypertension). Finally, model 4 was additionally adjusted for CRP that was strongly correlated with d-ROMs levels (r: 0.34–0.41 in the cohorts) and, less strongly, with TTL (r 0.11–0.17 in the cohorts). Therefore, model 2 is considered to show the main results. Age was modelled as a continuous variable and all other variables were modelled as categorical variables (categories are shown in Table 2). We tested for interactions of d-ROMs/TTL with covariates by adding appropriate interaction terms to model 2. Potential non-linear associations were addressed by modelling the exposure (d-ROMs levels or TTL) with restricted cubic splines [17]. Subgroups to be analysed were chosen a priori (cohort/country, sex and age-groups (45–< 60; 60–< 65; 65–< 70; 70–< 85)).

Multiple imputation was employed to impute the number of missing baseline covariate values. The proportion of missing values was below 5% for all variables with the exceptions alcohol consumption, which had up to 18% of missing values. To the best of our knowledge, data were missing at random, which is the assumption of the multiple imputation. Separately by cohort, case status and sex, 5 complete data sets were imputed with the SAS 9.3 procedure "PROC MI", using the *Markov chain Monte Carlo* method. Variables from model 4 were used for the imputation model. All multivariable analyses were performed in the 5 imputed data sets and results of the individual data sets were combined by the SAS 9.3 procedure "PROC MIANALYZE".

All analyses were performed with *SAS, version 9.3* (Cary, North Carolina, USA) and all statistical tests were two-sided using an alpha level of 0.05.

Results

Baseline characteristics of 476 incident MI and 454 incident stoke cases and age-, sex- and cohort- matched controls are shown in Table 2. Because of the matching, the distribution of age and sex among cases and controls was comparable. Study participants who experienced a stroke during follow-up were on average 2.4 years older than individuals with a primary MI. The proportion of males was higher among MI cases (70.2%) than among stroke cases (52.6%). Incident MI was associated with higher BMI, current smoking, less vigorous physical activity, higher total cholesterol, lower HDL cholesterol, higher CRP levels and a history of hypertension and diabetes. Furthermore, d-ROMs levels were statistically significantly higher among MI cases than controls but not TTL. Incident stroke was associated with current smoking, lower vigorous physical activity, lower HDL cholesterol, higher CRP levels and a history of hypertension and diabetes. Likewise the result for MI, d-ROMs levels but not TTL was significantly higher among stroke cases than controls.

D-ROMs levels were statistically significantly associated with total MI incidence (Table 3). A remarkably strong five-fold increased risk of fatal MI was observed for very high d-ROMs levels (> 500 Carr U) whereas associations with non-fatal MI were weaker and not statistically significant.

TTL was only associated with fatal MI (OR, 95% CI per 100 µmol/L increase: 0.69, 0.51–0.93). D-ROMs levels were also statistically significantly associated with total stroke incidence (OR 95% CI per 100 Carr U increase: 1.17, 1.01–1.35, Table 4) and again associations were stronger with fatal than with non-fatal stroke but results were not statistically significant for fatal stroke due to small case numbers. For TTL, all three top quartiles indicated a decreased risk for stroke when compared to the bottom quartile (Table 4) and when the three top quartiles were combined, the association was statistically significant (OR 95% CI: 0.79, 0.63–0.99, Table 5). Point estimates suggested a stronger association of TTL with fatal than with non-fatal stroke but all associations were not statistically significant. There were some indications for non-linear associations of d-ROMs and TTL with the six

Table 3 Associations of d-ROMs levels and TTL with total, fatal and non-fatal myocardial infarction

Marker	Modelling	Total MI		Fatal MI		Non-fatal MI	
		n_{cases}	OR (95%C I)[a]	n_{cases}	OR (95% CI)[a]	n_{cases}	OR (95% CI)[a]
D-ROMs	≤ 340 Carr U	173	Ref	47	Ref	126	Ref
	341–400 Carr U	149	1.02 (0.79; 1.31)	52	1.33 (0.82; 2.16)	97	0.94 (0.69; 1.27)
	401–500 Carr U	126	1.27 (0.96; 1.68)	36	1.57 (0.91; 2.70)	90	1.25 (0.89; 1.74)
	> 500 Carr U	28	**2.04** (**1.23**; **3.37**)	10	**5.08** (**1.78**; **14.49**)	18	1.54 (0.85; 2.80)
	Increase per 100 Carr U	476	**1.21** (**1.05**; **1.40**)		**1.54** (**1.14**; **2.06**)		1.15 (0.97; 1.35)
TTL	Quartile 1[b]	128	Ref	48	Ref	80	Ref
	Quartile 2[b]	116	0.90 (0.68; 1.19)	40	0.86 (0.51; 1.43)	76	0.91 (0.64; 1.29)
	Quartile 3[b]	129	1.03 (0.78; 1.37)	33	0.70 (0.41; 1.18)	96	1.22 (0.86; 1.71)
	Quartile 4[b]	103	0.82 (0.61; 1.10)	24	0.57 (0.32; 1.01)	79	0.92 (0.64; 1.32)
	Increase per 100 µmol/L	476	0.92 (0.79; 1.07)	145	**0.69** (**0.51**; **0.93**)	331	1.00 (0.84; 1.20)

Bold printed: statistically significant ($p < 0.05$)

CI confidence interval, *d-ROMs* derivatives of reactive oxygen metabolites, *MI* myocardial infarction, n_{cases} incident case numbers, *OR* odds ratio, *Ref* reference category, *TTL* total thiol levels

[a]Cases were individually matched to five controls by cohort, age and sex. In addition, the model was adjusted for education, BMI, smoking, alcohol consumption and vigorous physical activity and the other oxidative stress marker (i.e. TTL or d-ROMs)

[b]Cohort-specific quartiles of TTL (see Suppl. Table 1)

Table 4 Associations of d-ROMs levels and TTL with total, fatal and non-fatal stroke

Marker	Modelling	Total stroke		Fatal stroke		Non-fatal stroke	
		n_{cases}	OR (95% CI)[a]	n_{cases}	OR (95% CI)[a]	n_{cases}	OR (95% CI)[a]
D-ROMs	≤ 340 Carr U	140	Ref	15	Ref	125	Ref
	341–400 Carr U	152	1.27 (0.98; 1.66)	22	1.56 (0.71; 3.45)	130	1.24 (0.93; 1.65)
	401–500 Carr U	137	**1.40** (**1.05**; **1.86**)	18	1.55 (0.64; 3.78)	119	**1.38** (**1.01**; **1.88**)
	> 500 Carr U	25	1.27 (0.76; 2.12)	5	3.56 (0.89; 14.19)	20	1.09 (0.62; 1.92)
	Increase per 100 Carr U	454	**1.17** (**1.01**; **1.35**)	60	1.46 (0.96; 2.22)	394	1.13 (0.97; 1.32)
TTL	Quartile 1[b]	133	Ref	23	Ref	110	Ref
	Quartile 2[b]	113	0.84 (0.64; 1.12)	14	0.66 (0.30; 1.42)	99	0.87 (0.64; 1.18)
	Quartile 3[b]	98	**0.70** (**0.52**; **0.94**)	11	0.54 (0.23; 1.24)	87	**0.73** (**0.53**; **1.00**)
	Quartile 4[b]	110	0.80 (0.60; 1.08)	12	0.58 (0.26; 1.31)	98	0.84 (0.61; 1.15)
	Increase per 100 µmol/L	454	0.88 (0.75; 1.04)	60	0.68 (0.44; 1.05)	394	0.92 (0.77; 1.09)

Bold printed: statistically significant ($p < 0.05$)

CI confidence interval, *d-ROMs* derivatives of reactive oxygen metabolites, *MI* myocardial infarction, n_{cases} incident case numbers, *OR* odds ratio, *Ref* reference category, *TTL* total thiol levels

[a]Cases were individually matched to five controls by cohort, age and sex. In addition, the model was adjusted for education, BMI, smoking, alcohol consumption and vigorous physical activity and the other oxidative stress marker (i.e. TTL or d-ROMs)

[b]Cohort-specific quartiles of TTL (see Suppl. Table 1)

Table 5 Association of dichotomized d-ROMs levels and TTL variables with total myocardial infarction and stroke in the total sample and in subgroups of cohort/country, sex and age

Stratum	Myocardial infarction			Stroke		
	n_{cases}	d-ROMs[a] OR (95% CI)[c]	TTL[b] OR (95% CI)[c]	n_{cases}	d-ROMs[a] OR (95% CI)[c]	TTL[b] OR (95% CI)[c]
Total sample	476	**1.32** (**1.04**; **1.67**)	0.91 (0.72; 1.15)	454	1.20 (0.95; 1.51)	**0.79** (**0.63**; **0.99**)
Stratified by cohort/country						
HAPIEE PL	146	1.35 (0.86; 2.13)	0.77 (0.51; 1.18)	65	1.55 (0.81; 3.00)	0.58 (0.32; 1.05)
HAPIEE CZ	152	1.42 (0.95; 2.10)	1.23 (0.79; 1.92)	140	0.97 (0.65; 1.46)	0.79 (0.51; 1.20)
HAPIEE LT	102	1.10 (0.64; 1.88)	1.09 (0.64; 1.85)	94	**2.14** (**1.28**; **3.58**)	0.71 (0.41; 1.24)
ESTHER (Ger)	76	1.29 (0.69; 2.41)	**0.54** (**0.31**; **0.95**)	155	0.92 (0.59; 1.43)	0.96 (0.63; 1.45)
Stratified by age						
45–< 60 years	153	1.44 (0.93; 2.24)	1.40 (0.83; 2.35)	99	1.07 (0.64; 1.81)	**0.50** (**0.29**; **0.85**)
60–< 65 years	107	1.15 (0.69; 1.91)	0.78 (0,47; 1.29)	99	1.05 (0.62; 1.78)	1.15 (0.67; 1.96)
65–< 70 years	147	1.50 (0.98; 2.30)	0.76 (0.51; 1.14)	132	**1.68** (**1.10**; **2.58**)	0.82 (0.53; 1.25)
70–< 85 years	69	1.03 (0.55; 1.93)	0.71 (0.39; 1.28)	124	0.97 (0.61; 1.54)	0.81 (0.52; 1.25)
Stratified by sex						
Women	142	1.12 (0.76; 1.65)	0.77 (0.52; 1.15)	215	1.04 (0.77; 1.42)	0.79 (0.57; 1.10)
Men	334	**1.45** (**1.07**; **1.95**)	1.00 (0.74; 1.34)	239	**1.45** (**1.02**; **2.07**)	0.77 (0.55; 1.07)

Bold printed: statistically significant ($p < 0.05$)

CI confidence interval, *d-ROMs* derivatives of reactive oxygen metabolites, *MI* myocardial infarction; n_{cases}, incident case numbers; OR, odds ratio; TTL, total thiol levels

[a]Modelled dichotomously as d-ROMs > 400 versus ≤ 400 Carr U

[b]Modelled dichotomously as cohort-specific top TTL top quartiles 2–4 versus bottom quartile (see Suppl. Table 1)

[c]Cases were individually matched to five controls by cohort, age and sex. In addition, the model was adjusted for education, BMI, smoking, alcohol consumption and vigorous physical activity and the other oxidative stress marker (i.e. TTL or d-ROMs)

outcomes but confidence interval bands were wide and linear associations could not be excluded (Suppl. Figure 3–8). Adding of chronic diseases to the main model attenuated the observed ORs but most ORs remained statistically significant (Suppl. Table 2–3). Additional adjustment for CRP led to further attenuations and all observed associations lost statistical significance.

For subgroup analyses, d-ROMs levels and TTL were dichotomized with a cut-off at 400 Carr U for d-ROMs (high or very high oxidative stress) and the cut-off point of the cohort-specific bottom TTL quartile (low or very low anti-oxidative capacity) (Table 5). The most important findings are that the associations of d-ROMs levels with MI and stroke were restricted to men and that the association of TTL with stroke was only statistically significant for stroke events in mid-life (45–< 60 years).

Discussion

In this pooled analysis of four population-based cohort studies from Central and Eastern Europe, the serum oxidative stress marker d-ROMs levels were associated with MI and stroke incidence, whereas TTL was only associated with incident stroke. All addressed associations were stronger with fatal endpoints and d-ROMs and TTL were significantly associated with fatal MI risk. Subgroup analyses showed that the associations of d-ROMs levels with MI and stroke were only present in men and that the association of TTL with stroke was restricted to stroke events in mid-life (45–< 60 years).

Until now, only few groups have assessed the predictive values of d-ROMs and TTL for CVD events. Masaki et al. investigated the prognostic value of the d-ROMs test for CVD events in 265 patients with diagnosed CVDs [12]. They reported that d-ROMs levels > 395 Carr U were associated with a composite outcome of CVD events. Vassalle et al. conducted a retrospective cohort analysis in 93 patients with coronary artery disease (CAD) and observed an increased risk for a composite endpoint of major CVD events and all-cause mortality at d-ROMs levels > 481 Carr U [21]. Moreover, Hirata et al. demonstrated the prognostic value of the d-ROMs test for CVD events in 395 CAD patients and in addition in 324 patients with chronic kidney disease [22, 23]. Previously, our group explored the associations of d-ROMs levels and TTL with mortality endpoints using data from the same cohort studies, and found that both d-ROMs levels and TTL were independently and strongly associated with CVD mortality [9]. In a further analysis of our group with ESTHER study participants, we recently showed that two other oxidative stress biomarkers that were measured in urine samples, 8-isoprostane levels, a marker of lipid peroxidation and oxidized guanine/guanosine concentrations, a marker of oxidative DNA damage, were associated with CVD mortality [24]. Moreover, oxidized guanine/guanosine concentrations were associated with stroke incidence and 8-isoprostane levels with fatal stroke. Both urinary biomarkers were associated with incident MI in obese subjects. In addition, cross-sectional case–control studies showed elevated 8-iso-prostaglandin F2α levels, a marker of lipid peroxidation, in patients with CAD [25, 26] or acute MI [27]. In summary, previous studies were often cross-sectional, restricted to participants with prevalent CVD and/or used composite CVD outcomes. Although not directly comparable, there seems to be broad agreement of an association of oxidative stress biomarkers with CVD prevalence and incidence. To the best of our knowledge, our study is the first to address the associations of d-ROMs levels and TTL with MI and stroke risk in a sample from the general older population. Additionally, the large numbers of cases allowed us to conduct analyses distinct for fatal and non-fatal MI and stroke events.

Interestingly, we found that the associations of d-ROMs and TTL were much stronger with fatal than non-fatal MI and stroke events. The same pattern was observed in our other research project with urinary 8-isoprostane levels and oxidized guanine/guanosine concentrations [24]. Interestingly and maybe explaining this phenomenon, Vassalle et al. [26] observed that elevated levels of lipid peroxidation products and reduced antioxidant capacity are associated with the extent and the severity of CAD. Accumulating evidence suggests that oxidative stress could modulate a sequence of pathological events crucial in the onset and progression of atherosclerosis, including oxidation of LDL, reduction of nitric oxide (NO) bioavailability, vascular inflammation, vascular lesion formation and plaque rupture [2, 28–31].

Whereas the role of oxidative stress in atherosclerosis may explain observed association with MI and ischemic stroke, it should be noted that oxidative stress has also been ascribed a role in hemorrhagic stroke [32, 33]. The brain is a target for ROS-induced damage because of high concentrations of peroxidisable lipids, low levels of protective antioxidants, high oxygen consumption, and high levels of iron that can act as a pro-oxidant under pathological conditions [33, 34]. ROS is involved in secondary brain injury following intracerebral hemorrhage through mechanisms including endoplasmic reticulum stress, neuronal apoptosis and necrosis, inflammation, and autophagy [35]. One of our subgroup analyses showed that TTL was only statistically significantly associated with stroke events in mid-life (45–< 60 years). We think that this was a finding by chance because anti-oxidative capacities decrease with age [9, 36].

Furthermore, we found that the associations of d-ROMs levels with MI and stroke were only statistically significant in male participants. In our previous analysis with mortality outcomes, d-ROMs also had a tendency towards slightly stronger results in men than in women but the sex difference was not as strong as for MI and stroke [9]. These results are surprising because post-menopausal women have higher oxidative stress than men because of lower amounts of estrogen, which usually acts as an antioxidant

in females [9, 37]. One explanation may be the higher NADPH-oxidase activity levels in males than in females, which could cause overproduction of superoxide molecules through an angiotensin II-mediated mechanism [38, 39]. Another explanation may be an interaction of oxidative stress with proinflammatory cytokines (e.g., TNF-α, IL-1, and IL-6), which are produced to a larger extent in male than in female adipose tissue [40]. These cytokines are potent stimulators for the production of reactive oxygen and nitrogen by macrophages and monocytes and both inflammation and oxidative stress play important roles in atherosclerosis [41, 42]. Thus, the combined effects of inflammation and oxidative stress may put male subjects with high oxidative stress at a higher risk for MI and stroke than females.

In our analyses, all observed associations for d-ROMs levels and TTL lost statistical significance after adjusting for diseases and systemic sub-clinical inflammation measured with CRP. However, we believe that this would have been an overadjustment because these factors could be on the pathway to MI and stroke. As described above, inflammation and oxidative stress are closely linked to each other and it is difficult to distinguish their individual contributions to the MI and stroke events. In addition, it should be noted, that odds ratios were not attenuated to null effects (OR 1) after adjustment for CRP [e.g. OR (95% CI) for association of d-ROMs $>$ 500 Carr U vs. $\leq$ 340 Carr U with total MI 1.59, 0.92; 2.74] and meta-analyses with required future studies may show statistical significant results for these weaker effect estimates. Nevertheless, the large sample size with 476 MI and 454 stroke cases, also allowing to address fatal and non-fatal outcomes separately, is a strength of this pooled analysis of four cohort studies from the general Central and Eastern European population.

The ESTHER cohort is not a random sample of the source population (German adults, aged 50–75 years) because study participants were recruited during a routine health check-up in the Saarland region that is offered to every German citizen, aged 35 years and older, free of charge every 2 years. However, the distribution of major sociodemographic characteristics and the prevalences of major diseases in the ESTHER study population closely resembled the characteristics of the study population with the corresponding age range of the German National Health Survey of 1998, which is a true random sample of the German population. Therefore, we are able to generalize results from the ESTHER study 8-year follow-up to German adults, aged 56–85 years. Furthermore, results of the HAPIEE study can be generalized to adults aged 45–69 years living in PL, CZ or LT.

The harmonized data, almost complete registry-based follow-ups and the availability of all major risk factors for MI and stroke for a comprehensive correction for confounding resemble further strengths of this pooled data set. However, a few limitations of our study should also be considered. No data on stroke subtypes were available. Future studies are required to corroborate our findings and to elucidate the associations of d-ROMs levels and TTL with stroke subtypes. Because of the observational nature of this study, residual confounding cannot be totally excluded. Furthermore, common chronic diseases were based on self-reported information only in the HAPIEE cohorts, which could cause information bias. As this potential misclassification affected variables used for adjustment, some overestimation of the findings for d-ROMs and TTL cannot be excluded due to the limited accuracy for control of confounding. However, information bias for exposure to d-ROMs levels and TTL is also possible because they were not repeatedly measured during follow-up. Information bias for the exposure variables can lead to an underestimation of effects. Therefore, information bias for exposure variables and covariates could have balanced out each other in this study.

Conclusion

This pooled analysis of four cohort studies observed associations of both high d-ROMs levels and low TTL with MI and stroke (TTL only with fatal MI). The results suggest an important contribution of an imbalanced redox system to the etiology of mainly fatal MI and stroke events. Further studies are needed to corroborate our findings and to assess the value of oxidative stress markers for cardiovascular risk prediction.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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