Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. published online October 30, 2014;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Objective—The enzyme heme oxygenase-1 (HO-1) exerts cytoprotective effects in response to various cellular stressors. A variable number tandem repeat polymorphism in the HO-1 gene promoter region has previously been linked to cardiovascular disease. We examined this association prospectively in the general population.

Approach and Results—Incidence of stroke, myocardial infarction, or vascular death was registered between 1995 and 2010 in 812 participants of the Bruneck Study aged 45 to 84 years (49.4% males). Carotid atherosclerosis progression was quantified by high-resolution ultrasound. HO-1 variable number tandem repeat length was determined by polymerase chain reaction. Subjects with ≥32 tandem repeats on both HO-1 alleles compared with the rest of the population (recessive trait) featured substantially increased cardiovascular disease risk (hazard ratio [95% confidence interval], 5.45 [2.39, 12.42]; $P<0.0001$), enhanced atherosclerosis progression (median difference in atherosclerosis score [interquartile range], 2.1 [0.8, 5.6] versus 0.0 [0.0, 2.2] mm; $P=0.0012$), and a trend toward higher levels of oxidized phospholipids on apolipoprotein B-100 (median oxidized phospholipids/apolipoprotein B level [interquartile range], 11364 [4160, 18330] versus 4844 [3174, 12284] relative light units; $P=0.0554$). Increased cardiovascular disease risk in those homozygous for ≥32 repeats was also detected in a pooled analysis of 7848 participants of the Bruneck, SAPHIR, and KORA prospective studies (hazard ratio [95% confidence interval], 3.26 [1.50, 7.33]; $P=0.0043$).

Conclusions—This study found a strong association between the HO-1 variable number tandem repeat polymorphism and cardiovascular disease risk confined to subjects with a high number of repeats on both HO-1 alleles and provides evidence for accelerated atherogenesis and decreased antioxidant defense in this vascular high-risk group. (***Arterioscler Thromb Vasc Biol*. 2015;35:00-00.)**

Key Words: genetic polymorphism ■ risk factor

Low-grade inflammation, oxidation, and vascular remodeling are cardinal components in the pathophysiology of atherosclerosis.[1] Heme oxygenase-1 (HO-1) is the inducible, rate-limiting enzyme of heme degradation and exerts potent anti-inflammatory, antioxidative, and antiapoptotic effects in response to various stressors.[2,3] Compelling evidence for a protective effect of HO-1 on the vasculature derives from animal studies, demonstrating that HO-1 suppresses the development of atherosclerotic lesions[4–6] and thrombi.[7]Moreover, prominent endothelial damage was observed in rare human HO-1 deficiency,[8] as well as in HO-1 knockout mice.[9]

There is a $(GT)_n$ dinucleotide repeat polymorphism (variable number tandem repeat, VNTR) in the HO-1 gene promoter region, and higher repeat numbers translate into lower enzyme expression.[10–13] A deficiency in HO-1–mediated vascular protection in subjects with greater repeat lengths

Received on: March 28, 2014; final version accepted on: October 14, 2014.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.114.304729**

Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
CVD	cardiovascular disease
GT	guanidine thymidine
HO-1	heme oxygenase-1
OxPL	oxidized phospholipids
VNTR	variable number tandem repeat

was proposed to predispose to atherosclerosis and its clinical sequelae myocardial infarction (MI) and stroke.[14] Studies examining the association between $(GT)_n$ repeat length and cardiovascular disease (CVD) have to date been restricted to selected patient series, mainly subjects admitted for coronary angiography or patients with coronary artery disease (CAD) or peripheral arterial disease, and yielded inconsistent results. A summary of the literature is presented in Table 1. Apart from differences in study design, patient characteristics, and end point definitions, heterogeneous results may arise from the different cut-offs applied to categorize repeat number.

We present here the first prospective study on the potential relationship of the HO-1 $(GT)_n$ polymorphism with CVD conducted in the general community.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

HO-1 genotyping resulted in unambiguous results for 812 of 816 subjects for which DNA samples were available (call rate, 99.5%). Duplicate measurement of 95 random DNA samples yielded 100% concordant findings. The distribution of $(GT)_n$ repeat lengths ranged from 12 to 44 repeats and was trimodal, with peaks at 23, 30, and 37 repeats, constituting 20.5%, 40.9%, and 3.9% of alleles (Figure 1). The most common allele combinations were 30/30 and 23/30, observed in n=145 (17.9%) individuals each.

Categorization of study subjects by VNTR length (S, <23; M, 23–31; L, ≥32) resulted in only 2 subjects homozygous for short alleles (SS genotype), and we therefore merged SS and SM genotype groups to form SS/SM (n=35), MM (n=665), ML (n=101), and LL (n=11) groups. Distributions of baseline characteristics according to these 4 groups are shown in Table 2. Levels of standard risk factors emerged as independent of HO-1 genotype.

Crude incidence rates (95% confidence intervals [CIs]) for CVD were 6.5 (0.0, 15.3), 13.2 (10.8, 15.8), 13.0 (7.1, 19.8), and 65.1 (24.1, 130.4) events per 1000 person-years for SS/SM, MM, ML, and LL groups, respectively. Accordingly, 55% of subjects in the LL group developed hard CVD end points (stroke, MI, or vascular death) in the 15-year follow-up period. End point–specific event counts during the survey period in LL subjects and in other subjects were 4 and 61 for stroke, 2 and 51 for MI, and 0 and 20 for vascular death not caused by stroke or MI.

Under adjustment for age and sex, subjects homozygous for the longest repeat lengths (LL) faced a substantially elevated risk for CVD compared with MM subjects (hazard ratio (HR) [95% CI], 5.46 [2.39, 12.50]; $P<0.0001$; Table 3). A recessive model best fitted the data and revealed a HR [95% CI] of 5.45 [2.39, 12.42] ($P<0.0001$) in a comparison of LL to the rest of the study population. Effects remained virtually unchanged under further multivariable adjustment, were similar when excluding 50 subjects with prior CVD (HR [95% CI], 4.44 [1.63, 12.10]; $P=0.0036$), and were highly significant for the extended CVD end point as well ($P<0.0001$). Analyses of individual disease end points yielded a HR [95% CI] of 7.87 [2.84, 21.86] ($P<0.0001$) for stroke and 2.18 [0.52, 8.96] ($P=0.282$) for MI.

In sensitivity analyses, we used penalized cubic splines to examine the precise scale of relationship between VNTR length of each allele and CVD irrespective of predefined cut-offs. This gave significant results for the shorter allele ($P=0.0073$)

Table 1. Summary of the Literature on HO-1 VNTR Polymorphism and Cardiovascular Disease End Points in Humans

Reference	Primary End Point	n (Cases)	Years of FU	Sample Composition	VNTR Cut-Off(s) (≥)	Result*	Effect (Short Allele)†	Effect (Long Allele)†
Exner 2001[15]	Restenosis after femoropopliteal BA	96 (23)	0.5	Caucasian, PAD	25 and 29	p	(D) OR 0.2 (0.06, 0.70)	
Chen 2002[11]	CAD	796 (474)	CC	Asian, CAG	23 and 32	p		(D) OR 4.7 (1.9, 12.0) in diabetics
Kaneda 2002[16]	CAD	577 (298)	CS	Asian, CAG	27	p	(E) S/S vs L/L: OR 0.23 (0.07, 0.72) in subjects with high cholesterol; OR 0.23 (0.08, 0.71) in diabetics; OR 0.40 (0.17, 0.95) in smokers	
Schillinger 2002[17]	AAA, CAD, PAD	271 (210)	CC	Caucasian, vascular risk patients	25	p		(R) more L/L genotype in AAA, $P=0.04$ NS for CAD, PAD

(*Continued*)

Table 1. Continued

Reference	Primary End Point	n (Cases)	Years of FU	Sample Composition	VNTR Cut-Off(s) (≥)	Result*	Effect (Short Allele)†	Effect (Long Allele)†
Chen 2003[18]	Restenosis after coronary stenting, ACE	323 (111)	0.5	Asian, CAD	26	p		(D) OR 3.74 (1.61, 8.70) for stenting (D) OR 3.26 (1.58, 6.72) for ACE
Endler 2004[19]	CAD, MI	649 (438)‡	CC	Caucasian, vascular risk patients	25	n	(D) *P*=0.94	
Funk 2004[20]	Ischemic stroke or TIA	797 (399)	CC	Caucasian, stroke	25	p	(E) S/S vs L/L: OR 0.2 (0.1,0.6)	
Schillinger 2004[21]	Restenosis after femoropopliteal BA	381 (95)	0.5	Caucasian, PAD	25	p		(R) RR 2.33 (1.41, 4.17), NS for stenting
Dick 2005[22]	MI or PCI or CABG	472 (133)	1.75 (M)	Caucasian, PAD	25	p		(R) HR 2.17 (1.15, 4.17), NS for MACE, all-cause mortality, cerebrovascular events
Gulesserian 2005[23]	Restenosis after coronary stenting	199 (102)	0.5–0.75	Caucasian, CAD	30	p		(D) OR 1.9 (1.0, 3.4), stronger effect in smokers
Li 2005[24]	Restenosis after coronary stenting	187 (52)	0.5	Asian, CAD	30 and 38	n	(D) 30.8% restenosis in S carriers, 22.4% in others; *P*=0.22	
Wijpkema 2006[25]	Restenosis after coronary angioplasty	3146 (287)	0.8 (M)	Caucasian, CAD	25	n	S/L vs. S/S: HR 1.14 (0.90, 1.45); L/L vs. S/S: HR 0.87 (0.55, 1.38)	
Tiroch 2007[26]	Restenosis after coronary stenting	1357 (401)	0.5	Caucasian, CAD	25	n	restenosis in 29.2% (S/S), 29.5% (S/L), 29.6% (L/L); *P*=0.99	
Chen 2008[27]	CAD	986 (664)	CS	Asian, CAG	27	p		(R) OR 2.81 (1.22, 6.47) in diabetics; NS with adjustment for ferritin and bilirubin
Lüblinghoff 2009[28]	CAD	3219 (2526)§	7.8 (M)	Caucasian, CAG	26 or 28	n	S/L vs. S/S: OR 0.70 (0.49, 1.01); L/L vs. S/S: OR 0.71 (0.49, 1.02)	
Bai 2010[29]	Ischemic stroke	347 (183)	CC	Asian, stroke patients and hospital controls	27	p		(M) OR 2.07 (1.07–4.01) in subjects with low HDL
Wu 2010[30]	CVD mortality	504 (22)	10.7 (M)	Asian, arsenic exposure	27	p		(R) OR 2.63 (1.11, 6.25)
Chen 2012[13]	CAD	4596 (2298)	CC	Asian, general population	26	p	(E) S/S vs L/L: OR 0.60 (0.44, 0.81) in subjects with high oxidative stress	
Chen 2013[31]	CVD	1080 (307)	4.2 (M)	Asian, hemodialysis	27	p		(R) HR 1.62 (1.28, 2.04)
Gregorek 2013[32]	AAA	234 (117)	CC	Caucasian, AAA patients and hospital controls	25	n	S/L vs. L/L: OR 1.53 (0.90, 3.09); S/S vs. L/L: OR 1.24 (0.87, 1.96)	

AAA indicates abdominal aortic aneurysm; ACE, adverse coronary events; BA, balloon angioplasty; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAG, coronary angiography; CC, case-control study; CS, cross-sectional study; CVD, cardiovascular disease; FU, follow-up; HDL, high-density lipoprotein cholesterol; HO-1, heme oxygenase-1; HR, hazard ratio; (M), median follow-up in years; MACE, major adverse cardiovascular events; MI, myocardial infarction; NS, not statistically significant; OR, odds ratio; PAD, peripheral arterial disease; PCI, percutaneous coronary intervention; RR, risk ratio; TIA, transient ischemic attack; and VNTR, variable number tandem repeat.

*p, positive study—found significant association of HO-1 VNTR length with primary end point; n, negative study—did not find significant association of HO-1 VNTR length with primary end point.

†(D), dominant effect, ie, applies to allele carriers (eg, pooled S/S and S/L vs L/L); (E), extreme group comparison (eg, S/S vs L/L); (R), recessive effect, ie, applies to those homozygous for the respective allele (eg, S/S vs pooled S/L and L/L); (M), 1 study applied the cut-off to average within-subject allele length, forming L and S genotypes.

‡258 MCI and 180 stable CAD.

§2526 CAD and 1339 MI; 752 death.

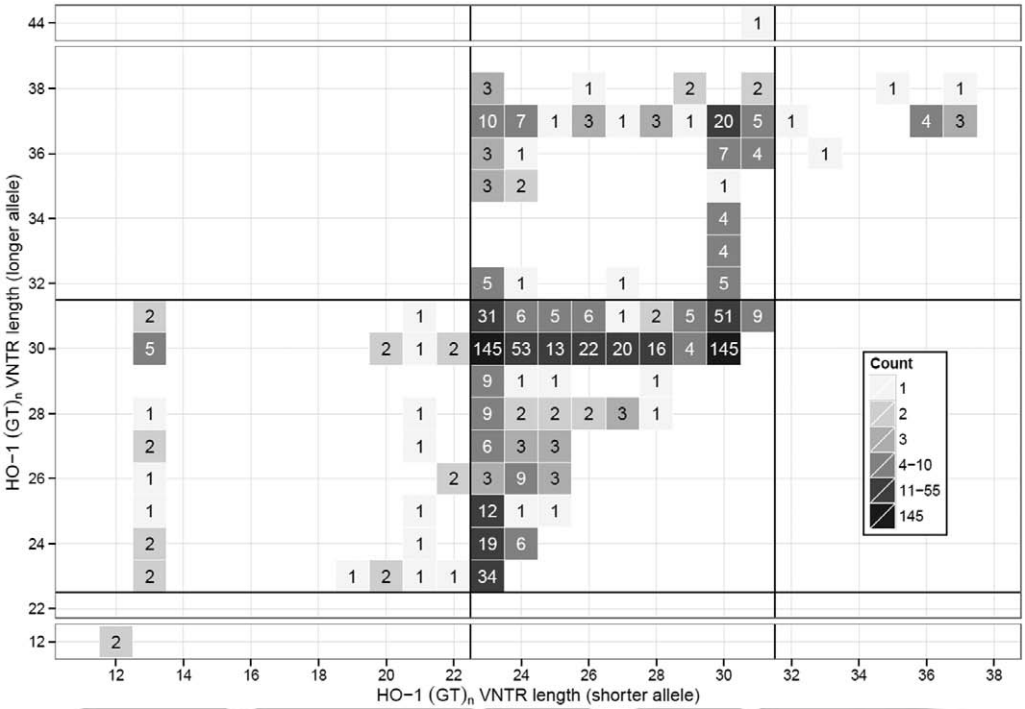

Figure 1. Joint distribution of heme oxygenase-1 (HO-1) variable number tandem repeat (VNTR) length on each allele. Numbers give the count of subjects that had the corresponding combination of allele lengths. Black lines show the cut-offs we applied to form genotype groups.

and provided a post hoc confirmation of our a priorily fixed cut-off of 32 (Figure 2). When applying alternative and mostly lower cut-offs previously used in the literature (Table 1), findings were not significant, underscoring that high risk was confined to subjects homozygous for the longest HO-1 VNTRs.

Finally, subjects in the LL group tended to experience atherosclerosis progression (incidence of new plaques or growth of existing ones) more frequently (82% versus 46%, odds ratio [95% CI], 4.72 [0.91, 36.68]; P=0.089) and showed a significantly larger change in the atherosclerosis score over 5 years (median difference in atherosclerosis score [interquartile range], 2.1 [0.8, 5.6] versus 0.0 [0.0, 2.2] mm; P=0.001), suggesting that the enhanced burden of CVD is at least in part mediated by accelerated atherogenesis. Subjects in the LL

Table 2. Baseline Characteristics of the Study Population According to Heme Oxygenase-1 Genotype

	SS/SM	MM	ML	LL	$P_{\text{any difference}}$	P_{trend}	$P_{\text{LL vs other}}$
n (%)	35 (4.3)	665 (81.9)	101 (12.4)	11 (1.4)			
VNTR range (shorter allele)	12–22	23–31	23–31	32–37			
VNTR range (longer allele)	12–31	23–31	32–44	36–38			
Baseline characteristics							
Age, y	59.8±11.0	62.9±11.1	62.6±11.1	65.3±9.8	0.368	0.361	0.451
Male sex, n (%)	18 (51.4)	337 (50.7)	41 (40.6)	5 (45.5)	0.293	0.116	0.813
Body mass index, kg/m^2	25.2 (23.4, 27.7)	25.3 (23.1, 27.8)	25.7 (23.3, 27.8)	24.5 (22.8, 26.1)	0.694	0.996	0.350
Current smoking, n (%)	8 (22.9)	131 (20.2)	16 (16.0)	1 (9.1)	0.743	0.346	0.458
Diabetes mellitus, n (%)	2 (5.7)	74 (11.1)	10 (9.9)	1 (9.1)	0.850	0.970	0.748
Systolic BP, mm Hg	147.9±21.3	147.9±20.7	150.9±21.3	147.1±15.4	0.650	0.676	0.650
Diastolic BP, mm Hg	87.2±10.1	86.9±9.1	88.2±9.7	87.0±5.3	0.733	0.508	0.927
Total cholesterol, mg/dL	221.7±39.6	229.6±42.9	235.5±42.4	231.6±33.4	0.512	0.187	0.948
HDL cholesterol, mg/dL	59.9±17.5	58.8±16.1	58.1±16.4	56.4±15.4	0.734	0.267	0.567
Ferritin, ng/mL	65 (32, 169)	88 (36, 170)	64 (28, 126)	46 (25, 161)	0.204*	0.194*	0.399*
hsCRP, mg/L	1.9 (0.9, 3.4)	1.6 (0.8, 3.2)	2.0 (1.1, 3.4)	1.8 (1.4, 2.3)	0.098*	0.429*	0.679*

Values are given as n (%), mean±standard deviation, or median (interquartile range); P_{trend} is for linear trend; P values are adjusted for age and sex, except those for age and sex, which are only adjusted for the other; S, <23 tandem repeats; M, 23–31 tandem repeats; L, ≥32 tandem repeats.

BP indicates blood pressure; HDL, high-density lipoprotein; and VNTR, variable number tandem repeat.

*Variables were log-transformed for significance testing.

Table 3. Associations of Heme Oxygenase-1 Genotype With the Primary and Extended Cardiovascular End Points

	Adjustment					
	None		Age and Sex		Multivariable*	
Repeat Length Group	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Primary cardiovascular end point						
SS/SM	0.49 (0.16, 1.55)	0.226	0.62 (0.20, 1.97)	0.420	0.68 (0.21, 2.15)	0.507
MM	1.00 (ref)		1.00 (ref)		1.00 (ref)	
ML	0.99 (0.59, 1.67)	0.971	1.15 (0.68, 1.95)	0.599	1.11 (0.65, 1.88)	0.705
LL	4.78 (2.10, 10.88)	<0.001	5.46 (2.39, 12.50)	<0.0001	6.33 (2.74, 14.64)	<0.0001
LL vs other	4.90 (2.16, 11.13)	<0.001	5.45 (2.39, 12.42)	<0.0001	6.33 (2.75, 14.59)	<0.0001
Extended cardiovascular end point						
SS/SM	0.39 (0.12, 1.23)	0.109	0.47 (0.15, 1.49)	0.202	0.50 (0.16, 1.57)	0.235
MM	1.00 (ref)		1.00 (ref)		1.00 (ref)	
ML	1.02 (0.64, 1.64)	0.925	1.20 (0.75, 1.92)	0.455	1.16 (0.72, 1.87)	0.532
LL	5.07 (2.36, 10.88)	<0.0001	5.88 (2.72, 12.68)	<0.0001	6.55 (3.01, 14.30)	<0.0001
LL vs other	5.21 (2.43, 11.14)	<0.0001	5.87 (2.73, 12.63)	<0.0001	6.56 (3.02, 14.26)	<0.0001

The primary cardiovascular end point included nonfatal stroke, nonfatal myocardial infarction, and vascular death. The extended cardiovascular end point additionally included peripheral vascular disease and revascularization procedures.

*Multivariable adjustment was for age, sex, total and high-density lipoprotein cholesterol, current smoking, diabetes mellitus, systolic blood pressure, and body mass index.

CI indicates confidence interval; and HR, hazard ratio.

group also showed a trend toward elevated baseline levels of oxidized phospholipids (OxPL) on apolipoprotein B (apoB)-100 (median OxPL/apoB levels [interquartile range], 11364 [4160, 18330] versus 4844 [3174, 12284] relative light units; *P*=0.055). Results were similar when the Δatherosclerosis score and OxPL/apoB were log-transformed (*P*=0.014 and *P*=0.073, respectively). Differences between subjects in the LL group and the rest of the sample with regards to incident CVD, Δatherosclerosis score, and OxPL/apoB are summarized in Figure 3.

We gathered data from 3 additional prospective cohorts (KORA F3, KORA F4, and SAPHIR) to corroborate our main result. As is visible in Table 4, these cohorts differed in most baseline characteristics. In particular, the additional 3 cohorts had substantially lower prevalences of the LL genotype and also substantially lower CVD incidence rates (*P*=0.011 for heterogeneity after adjustment for age and sex). As a consequence, we were unable to perform a strict independent replication of our key result. However, when pooling data from all 4 studies, the subjects in the LL group versus other subjects remained at strongly and significantly elevated risk for CVD (HR [95% CI], 3.26 [1.50, 7.33]; *P*=0.004; 326 events in 7848 subjects). Moreover, when pooling data from the Bruneck and the SAPHIR study, for which data on an extended end point additionally including revascularization procedures and peripheral vascular disease were available, the LL group was also strongly associated with this end point (HR [95% CI], 3.98 [1.76, 9.03]; *P*<0.001; 275 events in 2524 subjects). Both of these associations remained similar and significant under extended multivariable adjustment.

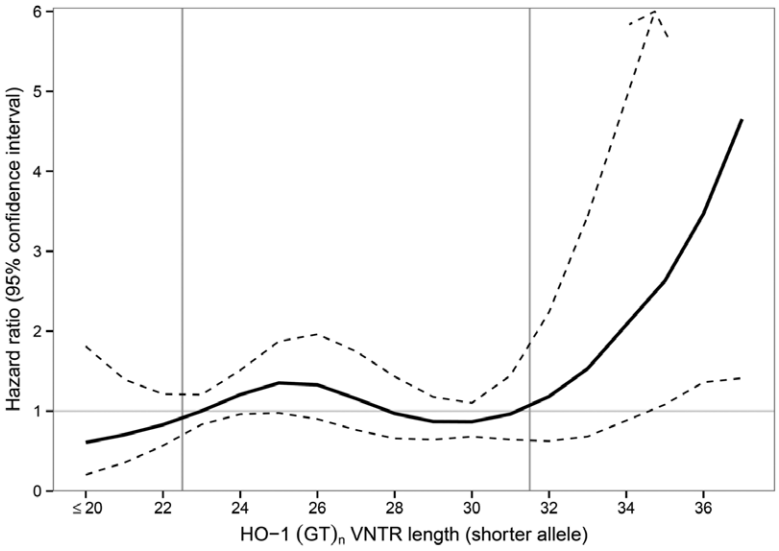

Figure 2. Penalized cubic spline fit of the association of variable number tandem repeat (VNTR) length on the shorter heme oxygenase-1 (HO-1) allele with the compound cardiovascular disease end point. Grey lines show the cut-offs we applied.

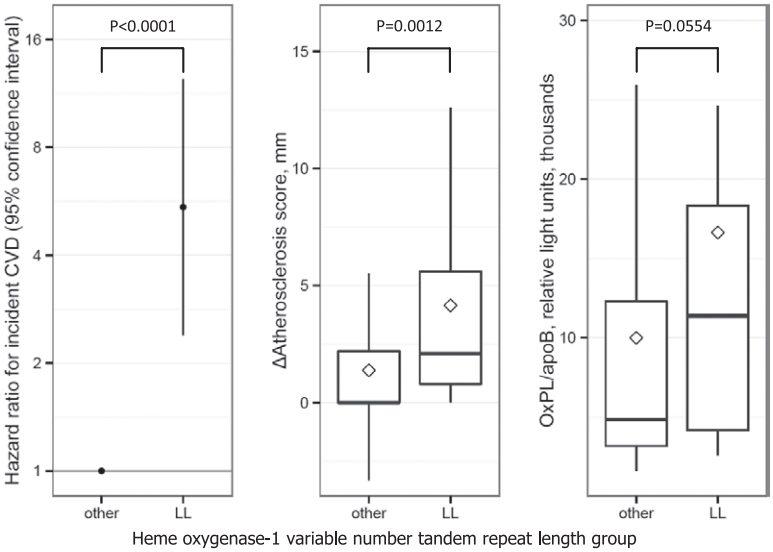

Figure 3. Differences between subjects with the LL genotype and subjects with other genotypes regarding incident cardiovascular disease (CVD; 1995–2010), changes in the carotid atherosclerosis score (1995–2000), and oxidized phospholipids (OxPL)/apolipoprotien B (apoB) levels (measured in 1995). Tests were adjusted for age and sex.

Discussion

In a prospective cohort study, we observed a substantially increased risk of CVD (hazard ratio [95% confidence interval], 5.45 (2.39, 12.42); *P*<0.0001) in subjects homozygous for long HO-1 VNTRs, indicating a recessive gene effect. This recessive nature of association is in line with experimental data, suggesting the shorter allele to be decisive for HO-1 upregulation in human umbilical vein endothelial cells.[10] Excess risk in our study was restricted to a small segment of the population (LL genotype, 1.4%).

This is the first prospective study on the relationship of the HO-1 VNTR with CVD conducted in the general population. To the best of our knowledge, the previous studies were conducted in high-risk populations, such as patients with preexisting CVD, coronary stenting, or hemodialysis (Table 1). One Chinese study was population-based but cross-sectional in design.[13] Many of the previous reports on this matter used lower VNTR cut-offs, most commonly 25 to 27. Of these, 3 large studies,[25–26] including 1800 to 3000 patients, found no relationship between HO-1 VNTR repeat length and their primary end points restenosis[25,26] or CAD,[28] but a large number of smaller studies did. Putting these data in perspective with our study, it should be considered that HO-1 induction occurs in response to stress conditions,[10,11,33] and a more severe deficit in HO-1 might be necessary in the general (low-risk) population to evoke deleterious effects, whereas a less severe deficit could suffice in higher-risk patients. This interpretation is consistent with several reports that found an

Table 4. Comparison of Prospective Cohorts

Study	Bruneck	KORA F3	KORA F4	SAPHIR	$P_{\text{any difference}}$
N	812	2584	2740	1712	
Demographic variables					
Age, y	62.73±11.10	56.16±12.53	55.15±12.99	51.38±6.00	<0.0001
Female sex, n (%)	411 (50.6)	1348 (52.2)	1451 (53.0)	635 (37.1)	<0.0001
Metabolic and lifestyle variables					
Diabetes mellitus, n (%)	87 (10.7)	170 (6.6)	163 (5.9)	54 (3.2)	<0.0001
HDL cholesterol, mg/dL	58.71±16.15	59.08±17.05	56.18±14.42	59.69±15.69	<0.0001
Total cholesterol, mg/dL	230.00±42.56	219.38±39.59	216.28±39.09	228.80±39.97	<0.0001
Systolic blood pressure, mm Hg	148.27±20.74	130.16±19.84	121.82±18.32	138.84±17.86	<0.0001
Current smoking, n (%)	156 (19.6)	481 (18.7)	489 (17.8)	332 (19.4)	0.511
Body mass index, kg/m^2	25.64±3.84	27.54±4.55	27.44±4.74	26.79±4.12	<0.0001
HO-1 genotype frequencies					
S/SML	35 (4.3)	83 (3.2)	65 (2.4)	39 (2.3)	0.001
MM	665 (81.9)	2195 (84.9)	2345 (85.6)	1459 (85.2)	
ML	101 (12.4)	298 (11.5)	316 (11.5)	207 (12.1)	
LL	11 (1.4)	8 (0.3)	14 (0.5)	7 (0.4)	
Incident CVD events, n (%)	132 (16.3)	90 (3.5)	34 (1.2)	70 (4.1)	<0.0001

Values are given as n (%) or as mean±standard deviation. The S/SML genotype group subsumed subjects whose shorter allele had <23 tandem repeats.

CVD indicates cardiovascular disease; HDL, high-density lipoprotein; and HO-1, heme oxygenase-1.

association between HO-1 VNTR length and vascular end points only in high-risk sub groups, such as diabetic subjects or smokers.[11,13,16,29]

The dependency of HO-1 protein expression on HO-1 VNTR length has to date been investigated primarily in cell lines. It was found that baseline as well as oxidative stress-induced HO-1 protein levels decreased approximately monotonically parallel to increasing length of the shorter HO-1 allele.[10] This extends earlier findings of reduced HO-1 transcriptional activity with increasing VNTR length.[11,12] One study found lower increase of HO-1 protein in response to oxidative stress but higher HO-1 baseline expression in cells with long alleles,[34] whereas another found higher HO-1 protein expression associated with short alleles only under conditions of oxidative stress.[13] There is to date no direct study of this dependency in humans. However, it has been reported that diabetic subjects homozygous for long alleles had increased CAD risk, reduced bilirubin levels, and increased serum ferritin levels and that the association with CAD risk disappeared with multivariable adjustment for bilirubin and ferritin.[27] These findings are consistent with reduced HO-1 activity in subjects with long alleles and also with reduced HO-1 activity, potentially mediating the effect on CAD risk.

Several lines of evidence suggest that the key finding of our study is valid: (1) the association between HO-1 VNTR and CVD was of particular strength (HR, 5.45; lower confidence bound, 2.39) and highly significant (P=5.51×10^{-5}). It would even retain significance in an exploratory setting, testing for all previously used VNTR cut-off values and adjusting for these multiple comparisons (Bonferroni corrected P=4.95×10^{-4}). (2) The elevated CVD risk observed in the LL HO-1 group was robust in several sensitivity analyses (Table 3). (3) The LL group was at elevated CVD risk also in a pooled analysis of 7848 subjects. (4) Vascular protection conferred by HO-1[2,3] is impressively demonstrated by the prominent vascular damage observed in human HO-1 deficiency.[8] (5) The deficit in HO-1 upregulation in response to cell stress with higher HO-1VNTR number rests on solid experimental evidence.[10–13](6) Subjects with the LL HO-1 genotype in our study had higher levels of OxPL/apoB (P=0.055), which is consistent with decreased HO-1 activity. (7) Finally, we observed a high risk of atherosclerosis progression in the LL HO-1 group, providing a pathophysiological explanation for the elevated CVD risk.

Strengths of our study include its prospective design with long-term high-quality follow-up and representativeness for the general population. Among its weaknesses is the limited number of subjects in extreme repeat length groups, a weakness that extends to the additional population-based cohorts that we used, which precluded subgroup analyses.

In conclusion, subjects with ≥32 tandem repeats on both HO-1 alleles represent a hitherto neglected vascular high-risk group featured by a substantial burden of CVD, amplified progression of atherosclerosis, and impaired antioxidant defense.

Sources of Funding

J. Willeit, S. Kiechl, and G. Weiss are supported by the FWF (Fonds zur Förderung der wissenschaftlichen Forschung; TRP 188). The Bruneck Study is supported by the Pustertaler Verein zur Prävention von Herz- und Hirngefässerkrankungen, the Gesundheitsbezirk Bruneck, and the Assessorat für Gesundheit und Sozialwesen, Bolzano, Italy. J.L. Witztum and S. Tsimikas are supported by the National Institutes of Health [HL 088093]. K. Willeit is supported by a Translational-Research-Program grant funded by the Land Tirol. The KORA research platform (KORA, Cooperative Research in the Region of Augsburg) was initiated and financed by the Helmholtz Zentrum München – German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research and by the State of Bavaria. Furthermore, KORA research was supported within the Munich Center of Health Sciences (MC Health), Ludwig-Maximilians-Universität, as part of LMUinnovativ.

Disclosures

None.

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Significance

Heme oxygenase-1 is a key antioxidant and cytoprotective enzyme, and a repeat length polymorphism in its gene promoter region impacts its expression. We found that this polymorphism is associated with cardiovascular risk such that subjects with high repeat lengths on both heme oxygenase-1 alleles suffer a substantially elevated risk. Moreover, we found evidence that oxidative stress and atherosclerosis at least partly mediate this risk elevation. The prospective population-based framework of the Bruneck Study with its high-quality data assessment allowed, for the first time, an investigation of this association both longitudinally and in the general population. This work may delimit a previously underappreciated cardiovascular high-risk group that merits particular preventive attention.

Materials and Methods

Study population and data collection

The Bruneck Study is a prospective, population-based survey on the epidemiology and pathogenesis of atherosclerosis and CVD[1–4]. At baseline in 1990 the study population comprised an age- and sex-stratified random sample of all inhabitants of Bruneck (125 men and 125 women from each of the fifth through eighth decades of age, all of Western European descent; 93.4% participated). In 1995, 826 subjects participated in the first quinquennial re-examination and DNA samples for HO-1 genotyping were available in 816 individuals. During follow-up from 1995 to 2010, detailed information about fatal and nonfatal new-onset CVD was carefully collected for all of these 816 subjects (follow-up rate, 100%). The study protocol was approved by the ethics committees of Bolzano and Verona and conforms to the Declaration of Helsinki. All study subjects provided written informed consent. Risk factors were assessed by means of validated standard procedures as described previously[1–5].

Additional prospective cohorts

To corroborate the validity of our main finding, we used data from three additional prospective cohorts: KORA F3, KORA F4, and SAPHIR. The protocols of each study were approved by the appropriate local ethics committee.

KORA F3[6] and *KORA F4*[7] are population-based follow-up studies recruited from the KORA S3 and S4 surveys and representative for the general population in Augsburg, Southern Germany, and its two adjacent counties[8,9]. KORA F3 was carried out as a 10-year follow-up of KORA S3 between 2004 and 2005 and a total of 3184 subjects participated. KORA F4 was conducted as a 7-year follow-up of KORA S4 between 2006 and 2008 and a total of 3080 individuals were finally included. The present study included 2584 subjects from KORA F3 and 2740 subjects from KORA F4. The endpoint used in this study was incidence of nonfatal or fatal MI and stroke. MIs were identified through the population-based MONICA/KORA Augsburg coronary event registry which monitors the occurrence of all in- and out-of-hospital fatal and nonfatal MIs among the 25 to 74-year-old inhabitants of the study region. The incidence of stroke was assessed using follow-up questionnaires mailed to the participants in 1997/1998, in 2002/2003, and in 2009/2010. Each time participants were asked whether they had a stroke and if they answered ‘yes’, the date of the event was assessed. Cases with self-reported incident stroke were validated by a questionnaire mailed to the treating physician and/or by medical chart review. Mortality was ascertained by regularly checking the vital status of all sampled persons of the MONICA surveys through the population registries inside and outside the study area; this procedure guaranteed that the vital status of cohort members who had moved out of the study area could also be assessed. Death certificates were obtained from local health departments and coded for the underlying cause of death by a single trained person using the ninth revision of the International Classification of Diseases (ICD-9).

The *SAPHIR* (Salzburg Atherosclerosis Prevention Program in subjects at High Individual Risk) Study[10] is an observational study conducted in the years 1999–2002 involving 1770 healthy unrelated subjects (663 females and 1107 males). In short,

unrelated healthy subjects of the greater Salzburg region who responded to invitations by their workplace or family physicians were included. Subjects with established CHD, cerebrovascular or peripheral arterial disease, congestive heart failure, valvular heart disease, chronic alcohol (more than 3 drinks aday) or drug abuse, or morbid obesity (body mass index BMI > 40 kg/m2) were excluded to reduce possible confounding resulting from therapeutic interventions. This study included 1712 subjects from SAPHIR. The endpoint used in SAPHIR data comprised stroke, myocardial infarction, and vascular death.

Genotype assessment of the HO-1 $(GT)_n$ promoter polymorphism

Genotyping of the HO-1 $(GT)_n$ promoter polymorphism was performed for each study population in the Sequencing & Genotyping Core Facility of Innsbruck Medical University. The 5' flanking region of the HO-1 gene on chromosome 22q13.1 containing a $(GT)_n$ length polymorphism was amplified by PCR using a 5'FAM labeled sense primer (5'-AGAGCCTGCAGCTTCTCAGA-3') and a non-labeled antisense primer (5'ACAAAGTCTGGCCATAGGAC-3')[11]. The fragment length depending on the number of GT-repeats was determined with capillary electrophoresis (3130xl Genetic Analyzer; Applied Biosystems, Foster City, CA, USA; in the following designated AB). The PCR mixture (10µl) contained 5 µl of Type-it Mastermix and 1 µl of Q-Solution (both included in the Type-it Microsatellite PCR Kit; Qiagen, Hilden, Germany), 0.2 µl of each primer (10µM) and 40 ng of DNA. PCR cycling was performed on a BioRad DNA-Engine thermocycler (BioRad, Vienna, Austria). PCR conditions were: Initial denaturation at 95°C for 5', followed by 35 cycles of 95°C for 30'', 55°C for 1'30'' and 72°C for 30''. Final extension was performed at 60°C for 30'. Amplified DNA was diluted 1:20 (v/v) with water, 1 µl of this dilution was mixed with 0.2 µl GeneScanTM 500 LIZTM Size Standard (AB) and denatured with 8.8 µl Hi-Di™ Formamide (AB) for 5' at 95°C. Data were analyzed with GeneMapper (AB). Genotypes of several different homozygous samples were confirmed by sequencing (PCR as above; cycle sequencing with BigDye version 1.1, 3130xl Genetic Analyzer). Each plate contained two non-template controls and 95 samples were randomly selected and analyzed twice for quality control.

Clinical endpoint definition in the participants of the Bruneck Study

The primary composite CVD endpoint comprised incident non-fatal myocardial infarction, non-fatal ischemic but not hemorrhagic stroke, and vascular death (n=132) between 1995 and 2010. A secondary, extended endpoint was used for sensitivity analyses, which additionally included revascularization procedures and peripheral vascular disease (n=162). There were 50 subjects who had experienced CVD before baseline, including 28 prior strokes and 23 prior myocardial infarctions. Presence of MI was assessed by World Health Organization criteria (definite disease status)[12], while stroke (including hemispheric TIA) was classified according to the criteria of the National Survey of Stroke[13]. The diagnosis of symptomatic peripheral arterial disease required a positive response to the Rose questionnaire (typical claudication), with the vascular nature of complaints confirmed by standard diagnostic procedures (ankle-brachial pressure index or angiography), or an acute peripheral artery occlusion requiring revascularization. All other revascularization procedures (angioplasty and surgery) were carefully recorded. Events were ascertained by a detailed review of

medical records provided by general practitioners, death certificates and all Bruneck Hospital files. A major advantage of the Bruneck Study is that virtually all inhabitants of Bruneck are referred to one local hospital that cooperates closely with the general practitioners. This allowed retrieval of the complete medical information ever assessed on study subjects.

Ultrasound endpoint definitionin the participants of the Bruneck Study

Progression of carotid atherosclerosis between 1995 and 2000 was used as an intermediary disease endpoint. The methodology employed has been described in detail previously[14]. Briefly, internal and common carotid arteries were scanned and atherosclerotic lesions defined by (1) wall surface (protrusion into the lumen or roughness of the arterial boundary) and (2) wall texture (echogenicity). The maximum axial diameter of plaques was measured in millimetres in each of the 16 vessel segments, and an atherosclerosis score was calculated by addition of all diameters. Intra- and inter-observer coefficients of variation (CVs) for this procedure were 13.5 and 15%, respectively[15]. Scanning was performed in 1995 and 2000 by the same experienced sonographer using the same ultrasound equipment for all scans. The sonographer was unaware of the subjects' clinical and laboratory characteristics. Five-year changes in the atherosclerosis score, which constitute the change in sums of axial diameters of plaques over 16 vessel segments, were used as an index of the progression of atherosclerosis. Incident atherosclerosis was defined by the occurrence of new plaques in previously normal sections of the vessels, and growth of pre-existing atherosclerosis was defined by a relative increase in the plaque diameter exceeding twice the measurement error of the method (CVs 10% and 15% for the common and internal carotid arteries, respectively[16]). Both were combined to an alternative measure of atherosclerosis progression. Carotid imaging was performed in 794 subjects in 1995, and in 666 of these follow-up scanning was performed in 2000, which is a proportion of >90% of survivors.

Determination of levels of oxidized phospholipid (oxPL) on apolipoprotein B-100 in the participants of the Bruneck Study

OxPL/apoB levels were measured, as previously described, by chemiluminescent enzyme-linked immunosorbent assay using the murine monoclonal antibody E06, which binds to the phosphocholine head group of oxidized but not native phospholipids[17,18]. When the OxPL/apoB levels in Bruneck were first published[18], they were reported in two ways: as relative light units (RLUs) and as a ratio of E06 (OxPL RLUs) binding to presence of apoB on the plate, measured by monoclonal antibody MB47 (apoB RLUs) (i.e., OxPL/apoB ratio), as previously described[17,18]. It was demonstrated that these measurements provided nearly identical results and were essentially interchangeable, thus subsequent studies reported OxPL/apoB as RLUs due to the simpler methodology of their determination. The intra- and interassay coefficients of variation for OxPL/apoB varied from 6% to 10%.

Statistical analysis

Variables are presented as mean ± standard deviation, median (interquartile range), or count (percentage). Differences in baseline characteristics were tested by linear, binary logistic, and multinomial logistic regression, adjusting for age and sex. Equality of variance was tested by Bartlett's test and was not refuted. Normality was investigated by histogram inspection, and ferritin and C-reactive protein were log-transformed towards normality.

We categorized $(GT)_n$ repeat number using cut-off values of 23 and 32, following the pioneer study on HO-1 VNTR length and CVD by Chen and colleagues[19], who substantiated their findings experimentally. Sensitivity analyses used alternative cut-offs derived from the literature, considering all publications that examined the association of VNTR length with cardiovascular endpoints in humans (listed in **Table 1**).

Associations with incident CVD (1995-2010) were assessed by Cox proportional hazards regression. The proportional hazards assumption was tested by computing the correlation coefficient of survival time with scaled Schoenfeld residuals, and was met. Progressive multivariable adjustment was performed for age, sex, total cholesterol, high-density lipoprotein cholesterol, current smoking, diabetes mellitus, systolic blood pressure, and body mass index. Smooth relationships of repeat length with the primary endpoint were examined by penalized cubic splines[20] with flexibility of the fit determined by Akaike Information Criterion (AIC).

Associations of HO-1 VNTR length with atherosclerosis progression (1995-2000) were examined by logistic regression, adjusting for baseline atherosclerosis. Associations with changes in atherosclerosis score (1995-2000) and with OxPL/apoB levels were examined by generalized linear models. All tests were two-sided and P-values smaller than 0.05 were considered significant. Analyses were performed using the R statistical package, version 3.1.0[21].

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