

ORIGINAL RESEARCH

Combined Effects of Clonal Hematopoiesis and Carotid Stenosis on Cardiovascular Mortality



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ABSTRACT

BACKGROUND The expansion of hematopoietic stem cells caused by acquired somatic mutations (clonal hematopoiesis [CH]) is a novel cardiovascular risk factor. The prognostic value of CH in patients with carotid atherosclerosis remains to be evaluated.

OBJECTIVES This study assessed the prognostic significance of CH in patients with atherosclerosis as detected by ultrasound of the carotid artery.

METHODS We applied deep sequencing of selected genomic regions within the genes *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* to screen for CH in 968 prospectively collected patients with asymptomatic carotid atherosclerosis evaluated by duplex sonography.

RESULTS We detected clonal markers at variant allele frequency $\geq 2\%$ in 133 (13.7%) of 968 patients (median age 69.2 years), with increasing prevalence at advanced age. Multivariate analyses including age and established cardiovascular risk factors revealed overall presence of CH to be significantly associated with increased risk of cardiovascular death (HR: 1.50; 95% CI: 1.12-2.00; $P = 0.007$), reflected also at the single gene level. The effect of CH was more pronounced in older patients and independent of the patients' inflammatory status as measured by high-sensitivity C-reactive protein. Simultaneous assessment of CH and degree of carotid stenosis revealed combined effects on cardiovascular mortality, depicted by a superior risk for patients with $>50\%$ stenosis and concomitant CH (adjusted HR: 1.60; 95% CI: 1.08-2.38; $P = 0.020$).

CONCLUSIONS CH status in combination with the extent of carotid atherosclerosis jointly predict long-term mortality. Determination of CH can provide additional prognostic information in patients with asymptomatic carotid atherosclerosis. (J Am Coll Cardiol 2024;83:1717-1727) © 2024 by the American College of Cardiology Foundation.



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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the [Author Center](#).

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ABBREVIATIONS AND ACRONYMS

CH = clonal hematopoiesis

CHIP = clonal hematopoiesis of indeterminate potential

CVD = cardiovascular disease

MI = myocardial infarction

MPN = myeloproliferative neoplasms

NGS = next-generation sequencing

VAF = variant allelic frequency

Clonal hematopoiesis (CH) is defined as clonal expansion of hematopoietic stem cells caused by acquired somatic mutations. For decades, CH was acknowledged as the underlying cause of hematological malignancies, defining the malignant cellular clone, or alternatively representing a premalignant state. With the advent of deep sequencing technologies (ie, next-generation sequencing [NGS]), it became evident that somatic mutations frequently detected and causally implicated in myeloid malignancies are also highly prevalent in the general population in the absence of an obvious disease phenotype.¹⁻³ As a consequence, the term clonal hematopoiesis of indeterminate potential (CHIP) was proposed. CHIP is defined as the presence of CH caused by a somatic mutation in known driver genes with a variant allelic frequency (VAF) (corresponding to the clone size) $\geq 2\%$ in the absence of disease-defining features such as myelodysplasia or blasts in the bone marrow.³ The majority of CHIP-relevant mutations were reported in the genes *DNMT3A*, *TET2*, *ASXL1*, and *JAK2*.^{1,2} The incidence of CHIP strongly correlates with age and the frequency of mutant clones is estimated to be about 5% at 60 years of age, increasing to approximately 20% in individuals aged >90 years. While CHIP was shown to elevate the risk of developing a hematological malignancy approximately 10-fold, progression rates to myeloid malignancies in CHIP carriers are still relatively low (0.5%-1%/y).³ Surprisingly, a large pivotal cohort study of CHIP¹ reported a strongly increased all-cause mortality for individuals with CHIP that could not be explained by the progression to cancer, but in follow-up studies was attributed to the consequences of chronic cardiovascular disease (CVD).^{2,4} CHIP carriers are 2 times more likely to experience coronary artery disease, making it an equally strong risk factor as smoking, hypertension, hyperlipidemia, or diabetes. Notably, gene-level analysis suggested a particularly high risk for mutations in *JAK2* (12.1-fold) compared with *DNMT3A*, *TET2*, and *ASXL1* (1.7- to 2.0-fold increase in risk).² CH is now recognized as a major risk factor for many CVD-related conditions.^{3,4} Although there is a large amount of data on associations between CH and clinical manifestations of symptomatic atherosclerotic CVD, the effect of CH on long-term outcome in patients with documented subclinical atherosclerosis has not yet been addressed. Here, we apply targeted resequencing to screen for CHIP-associated mutations in a clinically well-characterized cohort of 968 patients with asymptomatic carotid atherosclerosis

to assess the prognostic significance of CH in patients with carotid artery disease. Moreover, we examine a possible interplay of CH with predictive parameters such as degree of carotid stenosis with respect to long-term mortality in these patients.

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METHODS

STUDY COHORT. Patients included in this study were prospectively enrolled into the ICARAS (Inflammation and Carotid Artery-Risk for Atherosclerosis Study) clinical trial. The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board of the Medical University of Vienna. All patients provided written informed consent. The study design of ICARAS has been published previously.⁵ Details on the study cohort are described in the [Supplemental Methods](#).

DATA ACQUISITION. Clinical characteristics such as medical history, current medication, biometric data, and family history were collected through a detailed questionnaire. The degree of carotid artery narrowing was assessed through duplex ultrasound examinations as detailed in the [Supplemental Methods](#). To screen for CH, we applied an amplicon-based NGS assay, targeting selected regions in the genes *DNMT3A*, *TET2*, *ASXL1*, and *JAK2*. A description of high-throughput sequencing and subsequent determination of somatic mutations is provided in the [Supplemental Methods](#).

STATISTICAL ANALYSIS. Continuous data are presented as median (Q1-Q3). Discrete data are given as counts and percentages. Mann-Whitney *U* test and the chi-square test were used for comparisons between, as appropriate. The log-rank test was used for comparison between groups. Survival probabilities were estimated using the Kaplan-Meier method. Multivariate Cox proportional hazards models were applied to assess the association between presence of CH (any mutation and mutations in individual genes) and the occurrence of either cardiovascular or all-cause death. The following variables were included as covariates in all multivariate analyses: age (years), sex (male/female), peripheral arterial disease (binary), history of myocardial infarction (MI) (binary), history of stroke (binary), presence of diabetes mellitus (binary), current smokers (binary), statin treatment (binary), body mass index (kg/m²), degree of stenosis at baseline (binary; 0%-49%, 50%-100%), arterial hypertension (binary; patients with repetitively measured resting blood pressure values $>140/90$ mm Hg and patients taking antihypertensive

TABLE 1 Baseline Characteristics of Study Cohort, Overall and Stratified by CH Status

	All Patients	No CH	CH	P Value
Age, y	69.03 (61.22-76.17)	68.84 (61.18-75.81)	72.15 (63.69-78.70)	0.002
Male (binary)	610 (63.0)	530 (63.5)	80 (60.1)	0.461
Previous PAD (binary)	413 (42.7)	355 (42.5)	58 (43.6)	0.813
Previous MI (binary)	234 (24.2)	196 (23.5)	38 (28.6)	0.202
Previous stroke (binary)	164 (16.9)	142 (17.0)	22 (16.5)	0.894
Diabetes mellitus (binary)	218 (22.5)	193 (23.1)	25 (18.8)	0.268
Carotid stenosis >50% (binary)	556 (57.4)	478 (57.2)	78 (58.6)	0.761
Statins (binary)	577 (59.6)	502 (60.1)	75 (56.4)	0.268
BMI (ratio)	26.10 (24.00-28.70)	26.30 (24.20-28.70)	25.60 (23.20-28.00)	0.006
Arterial hypertension (binary)	668 (69.0)	573 (68.6)	95 (71.4)	0.516
Current smokers (binary)	263 (27.2)	227 (27.1)	36 (27.2)	0.977
Serum creatinine, mg/dL	1.06 (0.93-1.23)	1.06 (0.93-1.22)	1.07 (0.94-1.27)	0.377
HbA1c, %	6.00 (5.60-6.60)	6.00 (5.60-6.50)	5.90 (5.60-6.50)	0.941
LDL cholesterol, mg/dL	118.00 (94.00-146.00)	118.00 (95.00-146.00)	118.00 (91.00-148.00)	0.630
HDL cholesterol, mg/dL	50.00 (42.00-60.00)	49.00 (41.00-59.00)	51.00 (40.00-61.00)	0.772
hsCRP, mg/dL	0.29 (0.14-0.64)	0.29 (0.13-0.63)	0.29 (0.15-0.68)	0.508
Survival time, y	11.81 (6.02-12.43)	11.85 (6.60-12.49)	8.79 (4.11-12.45)	0.005

Values are median (Q1-Q3) or n (%). No corrections for multiple testing were applied.
BMI = body mass index; CH = clonal hematopoiesis; HbA1c = glycosylated hemoglobin; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; MI = myocardial infarction; PAD = peripheral arterial disease.

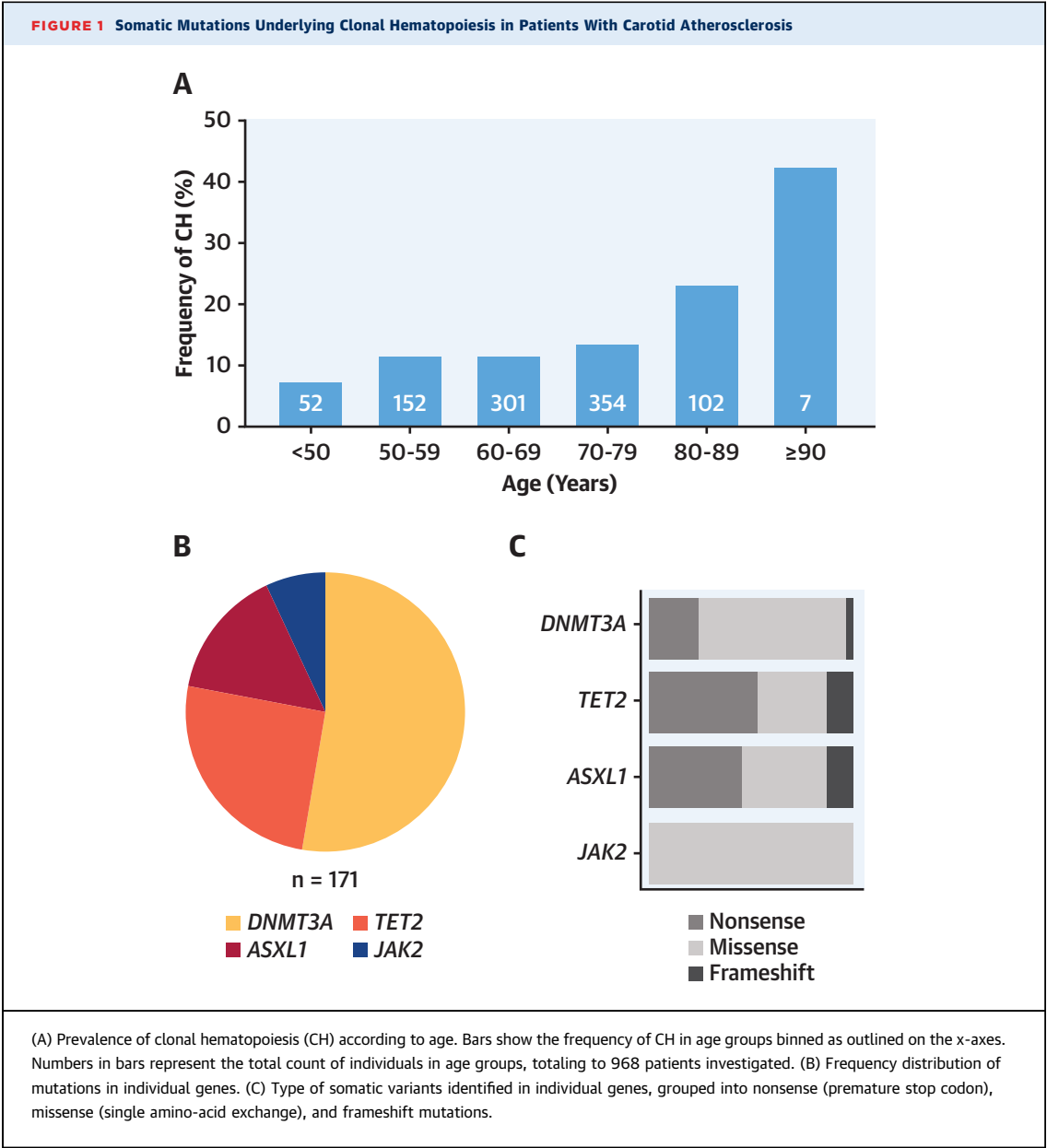
drugs), low-density lipoprotein cholesterol levels (mg/dL), high-density lipoprotein cholesterol levels (mg/dL) and high-sensitivity C-reactive protein (hsCRP) levels (mg/dL). To test for association of CH and covariates with cardiovascular and all-cause mortality, we applied univariate Cox regression analyses in conjunction with a multivariate Cox proportional hazard model using stepwise backward elimination. Variables significant in univariate analysis underwent a multivariate stepwise backward procedure using likelihood-ratio statistics based on conditional parameter estimates. Interactions between presence of CH and degree of carotid stenosis were tested by including interaction terms in a multivariate Cox proportional hazard regression model. Results of the Cox models are presented as HRs in conjunction with the corresponding 95% CI. A 2-sided *P* value <0.05 was considered statistically significant. Statistical outcomes presented were not adjusted for multiplicity, and therefore inferences drawn from these statistics may not be reproducible. All calculations were performed using GraphPad Prism version 10.0 (GraphPad Software), SPSS version 28.0 (SPSS Inc) or the STATA11 software package (Stata Corp).

RESULTS

PATIENT CHARACTERISTICS AND DETECTION OF SOMATIC MUTATIONS. A total of 968 patients with neurologically asymptomatic carotid atherosclerosis

prospectively enrolled in the ICARAS trial⁵ underwent targeted resequencing to determine the presence of CH. Median age was 69.2 years (Q1-Q3: 61.3-76.2 years) at the time of inclusion, and 610 (63.0%) patients were men. Demographic and clinical characteristics of included patients are detailed in **Table 1**. An overall survival rate of 47.8% was determined for the median follow-up of 11.8 years (Q1-Q3: 6.1-12.5 years), corresponding to 9,008 person-years overall. A total of 505 (52.2%) deaths were recorded. In detail, 333 (34.4%) patients died of cardiovascular causes and 172 (17.8%) of other causes.

To examine the role of CH in carotid artery disease, we sequenced selected protein-coding regions in *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* genes frequently observed to be mutated in CH. Overall, 76% of somatic mutations reported in the pivotal study of CVD-related CHIP by Jaiswal et al² are located in genomic regions covered by our analysis. In the 968 individuals with carotid atherosclerosis, we detected 171 somatic mutations in 133 (13.7%) patients. The vast majority (80.5%) of patients with CH carried only 1 mutation, followed by 14.3% patients with 2, and 3.8% patients with 3 mutations. Only 2 patients with CH (1.5%) carried >3 mutations (**Supplemental Figure 1A**). Consistent with previous studies on CH, the prevalence of CH in the ICARAS cohort increased with patient age (**Figure 1A**). Clonal markers were detected in 7.7% of patients aged <50 years (4 of 52 patients), 11.8% of patients between ages 50 and 59 years (18 of 152 patients), 11.6% between ages 60 and



69 years (35 of 301 patients), 13.8% between ages 70 and 79 years (49 of 354 patients) and 23.5% between ages 80 and 89 years (24 of 102 patients). In patients aged >90 years ($n = 7$), CH was detected in 3 individuals, representing a CH rate of 42.9% (Figure 1A). Within the 4 genes investigated, more than one-half of all identified somatic mutations were detected in *DNMT3A* (52.6%), followed by *TET2* (25.1%), *ASXL1* (15.2%), and *JAK2* (7.0%) (Figure 1B). Considering that some patients (19.5%) carry >1 mutation, this translates into 51.0%, 24.8%, 16.3%, and 7.8% of CH cases driven by single-gene mutations in *DNMT3*, *TET2*, *ASXL1*, and *JAK2*, respectively. Although missense mutations represent the majority of variants detected in *DNMT3A*, it is nonsense and frameshift variants that are enriched in *TET2* and *ASXL1*, in line with a loss-of-function mechanism frequently observed for tumor suppressor genes. Mutations detected in *JAK2* exclusively code for the *JAK2*-V617F gain-of function single amino-acid substitution (Figure 1C), as expected based on the selection of target regions (Supplemental Table 2). The majority of mutations in all 4 investigated genes had low VAFs between 2% and 10%, corresponding to clonal fractions of 4% to 20% for heterozygous mutations. Although for *DNMT3A*, *TET2*, and *ASXL1* mutations did not exceed

VAFs of 40%, 30% and 20%, respectively, JAK2-V617F was detected also at VAFs >50%, suggesting the presence of homozygous *JAK2* mutations in some patients, most likely as a consequence of acquired uniparental disomy frequently observed subsequent to the clonal acquisition of JAK2-V617F⁶ (Supplemental Figure 1B).

ASSOCIATIONS BETWEEN CLONAL HEMATOPOIESIS AND CLINICAL PARAMETERS. To identify potential associations between CH and clinical parameters available in ICARAS, we applied univariate comparative statistics stratifying the overall study cohort (n = 968) by CH status (Table 1). There were no significant differences except that CH carriers were older, presented with a lower BMI, and most crucially, exhibited decreased survival. Because the association of CH with advanced age is well-described¹ and decreased BMI is a known concomitant of aging,⁷ we subsequently tested whether the impact of CH on mortality is independent of patients' age and other possibly relevant covariates (Table 2, Supplemental Table 1). Multivariate analyses revealed CH to be associated with increased risk of cardiovascular death (HR: 1.50; 95% CI: 1.12-2.00; *P* = 0.007) as well as all-cause mortality (HR: 1.42; 95% CI: 1.11-1.81; *P* = 0.005). Kaplan-Meier analyses demonstrated a significant increase in both cardiovascular death (Figure 2) and all-cause mortality (Supplemental Figure 2) in patients with CH. The cumulative 12-year survival rates were 65% (no CH) vs 53% (CH) for cardiovascular death and 52% (no CH) vs 41% (CH) for death by any cause (log-rank *P* = 0.003 for both) (Figure 2, Supplemental Figure 2). Besides presence of CH, covariates significantly associated with both cardiovascular as well as all-cause death were age, previous PAD, previous MI, presence of diabetes mellitus, and high hsCRP.

To specifically address the relevance of CH in context of the patients' age, we conducted multivariate Cox regression analyses on subgroups assembled by setting lower age thresholds at 5-year intervals between 50 and 85 years of age. Despite decreasing numbers of patients in subgroups with advanced age, the association of CH with increased cardiovascular mortality remained statistically significant for subgroups up to "age ≥65 years." Interestingly, when restricting the analysis to patients ≥80 years (n = 110; 11.6% of total) multivariate analyses revealed highest risk of cardiovascular death for CH carriers aged ≥80 years (HR: 3.23; 95% CI: 1.64-6.40) (Supplemental Figure 3A). Of note, the previously established risk factor hsCRP⁵ was lacking prognostic significance for patients aged ≥80 years (Supplemental Figure 3B). Thus, in our cohort, hsCRP

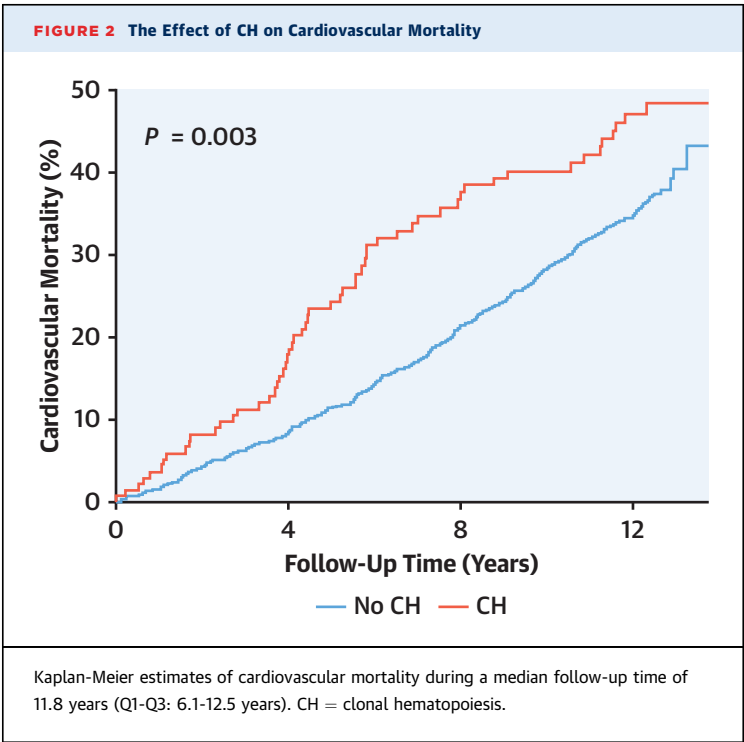
TABLE 2 Cox Proportional Hazard Regression Analyses to Assess the Association Between Clinical Parameters and Cardiovascular Mortality

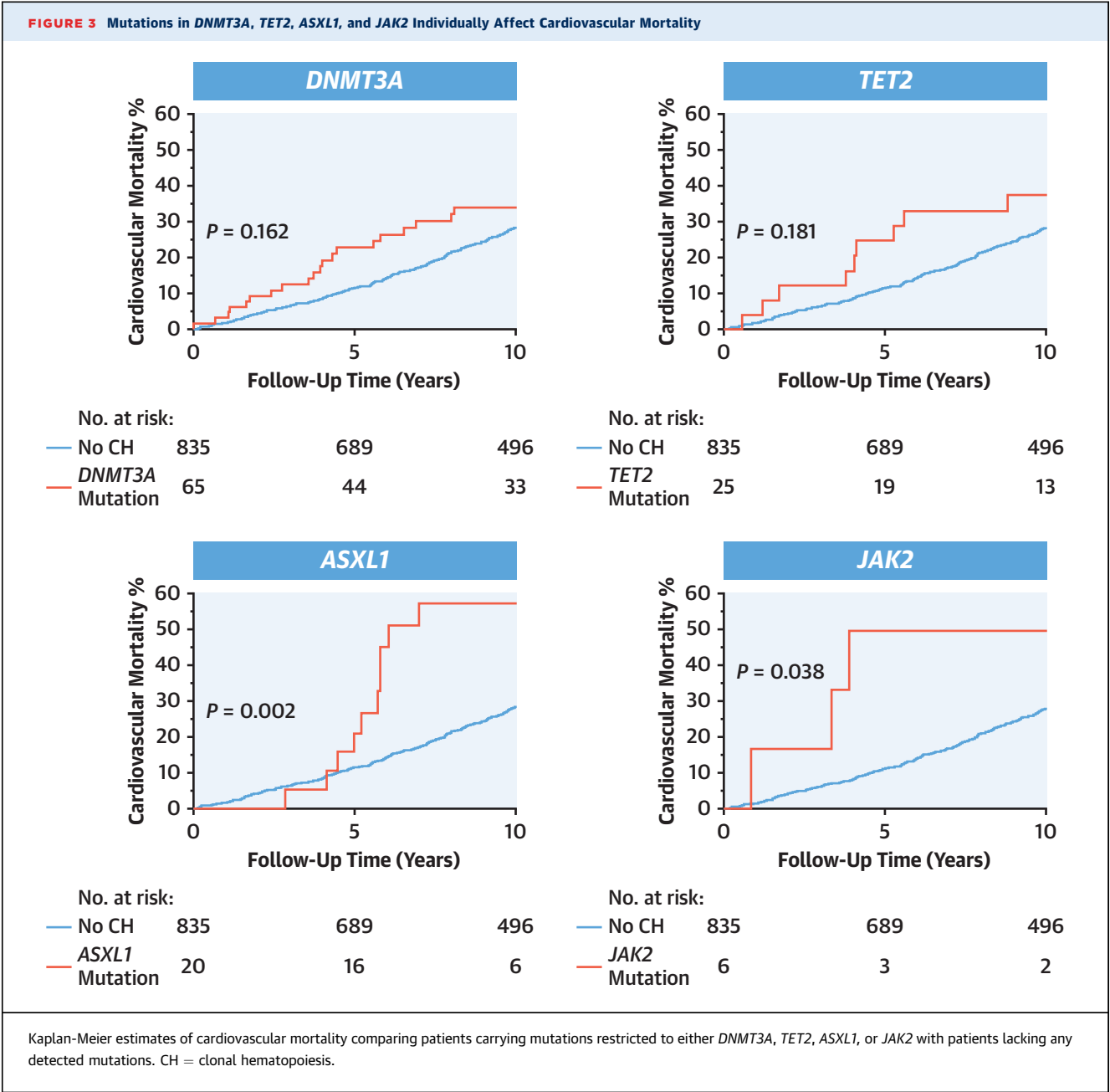
	Univariate		Multivariate	
	HR (95% CI)	<i>P</i> Value	HR (95% CI)	<i>P</i> Value
CH	1.55 (1.16-2.06)	0.003	1.50 (1.12-2.00)	0.007
Age, y	1.07 (1.05-1.08)	<0.001	1.06 (1.05-1.07)	<0.001
Male (binary)	1.20 (0.97-1.48)	0.099	—	—
Previous PAD (binary)	1.65 (1.34-2.03)	<0.001	1.41 (1.13-1.75)	0.003
Previous MI (binary)	2.04 (1.65-2.53)	<0.001	1.67 (1.33-2.10)	<0.001
Previous stroke (binary)	1.49 (1.15-1.91)	0.002	1.06 (0.80-1.41)	0.676
Diabetes mellitus (binary)	2.07 (1.68-2.55)	<0.001	1.62 (1.30-2.03)	<0.001
Current smokers (binary)	0.81 (0.63-1.03)	0.084	—	—
Statins (binary)	1.09 (0.89-1.35)	0.402	—	—
BMI (ratio)	0.98 (0.96-1.01)	0.147	—	—
Carotid stenosis >50% (binary)	1.72 (1.38-2.14)	<0.001	1.07 (0.84-1.35)	0.586
Arterial hypertension (binary)	1.33 (1.06-1.68)	0.016	1.10 (0.84-1.39)	0.586
LDL cholesterol, mg/dL	1.00 (0.99-1.00)	0.004	1.00 (1.00-1.00)	0.128
HDL cholesterol, mg/dL	1.00 (0.99-1.00)	0.26	—	—
hsCRP, mg/dL	1.42 (1.25-1.62)	<0.001	1.38 (1.20-1.60)	<0.001

No corrections for multiple testing were applied.
Abbreviations as in Table 1.

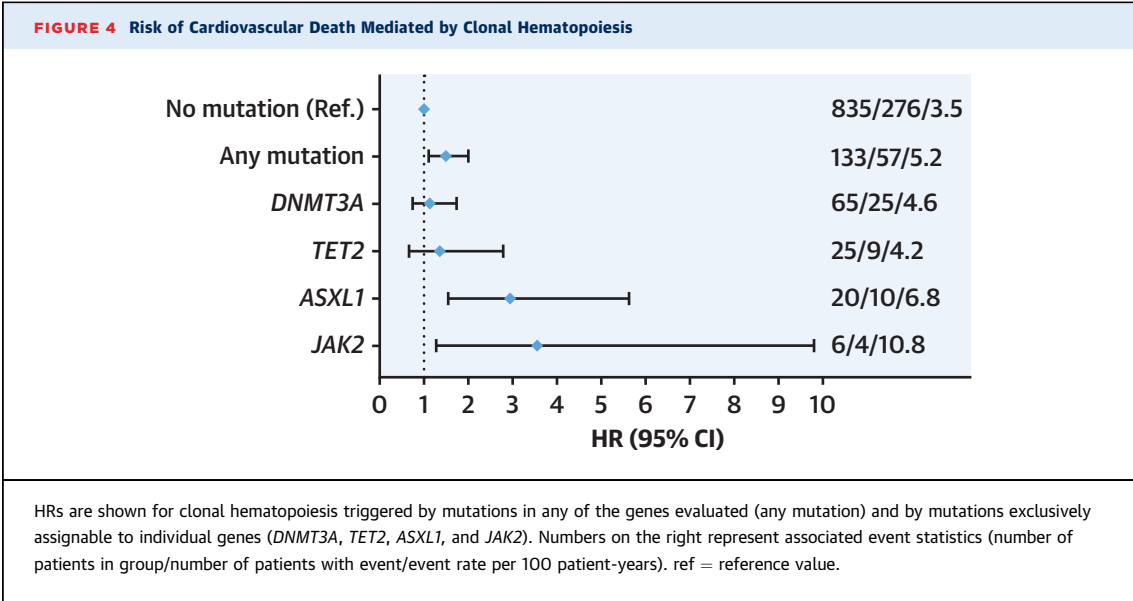
levels do not predict increased cardiovascular mortality in patients aged ≥80 years, whereas presence of CH exhibits its strongest predictive power for patients at advanced age.

PROGNOSTIC SIGNIFICANCE OF SOMATIC MUTATIONS IN INDIVIDUAL GENES. We next evaluated contributions of the individual genes included in our analysis,





excluding patients with mutations detected in >1 gene. Kaplan-Meier analyses for cardiovascular mortality at 10-year follow-up, comparing patients carrying mutations restricted to either *DNMT3A*, *TET2*, *ASXL1*, or *JAK2* with patients lacking any detected mutations, revealed increased cardiovascular death in all mutated subgroups independent of the specific gene involved, reaching statistical significance for *ASXL1*- and *JAK2*-mutated subgroups (Figure 3). When applying multivariate analysis including all covariates, formal statistical significance for the association with cardiovascular mortality was demonstrated for both *ASXL1*-mutated CH (HR: 2.95; 95% CI: 1.55-5.63; adjusted $P = 0.001$) and *JAK2*-mutated CH (HR: 3.56; 95% CI: 1.29-9.79; adjusted $P = 0.014$) (Figure 4, Supplemental Table 3). We further investigated the impact of mutation type (nonsense, missense, frameshift) (Figure 1C) in individual genes on cardiovascular mortality. The analysis was not applicable to *JAK2*, where *JAK2*-V617F represents a single missense mutation. For *TET2* and *ASXL1*, Kaplan-Meier analysis of mutation subtypes did not reveal any clear trend, likely caused by low total numbers. For *DNMT3A*, however, we observed a

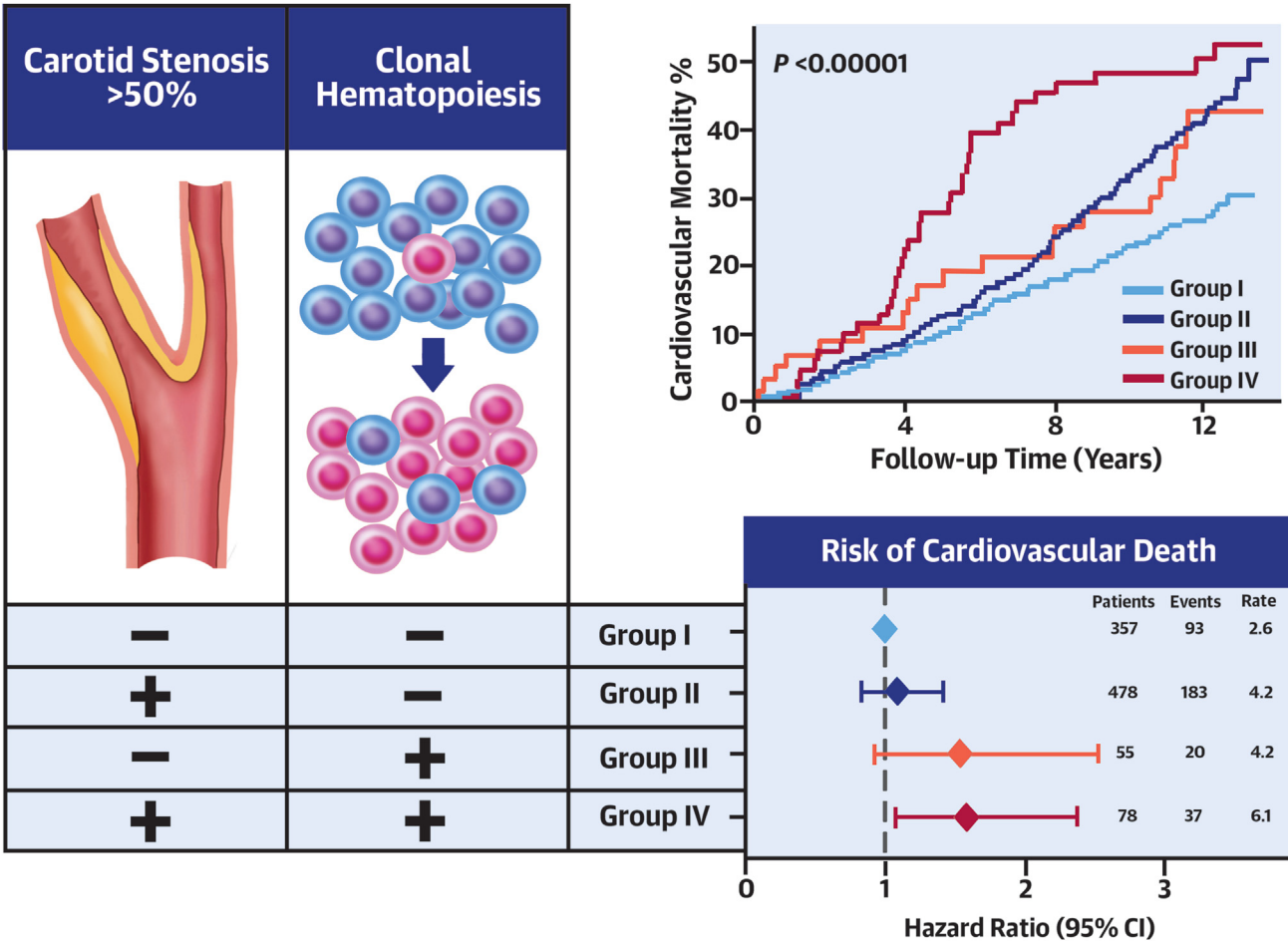


statistically nonsignificant trend for increased cardiovascular death in carriers of nonsense mutations compared with missense mutations, in line with high penetrance of nonsense-mediated loss-of-function in tumor suppressors (Supplemental Figure 4). Furthermore, we addressed a potential impact of clone sizes as reflected by VAFs on cardiovascular mortality. For this, we focused on patients who died from cardiovascular events during 10-year follow-up. Applying simple linear regression did not reveal any correlations between survival time and VAFs in individual genes, nor for all mutations together (data not shown). Similarly, when analyzing VAFs in patient groups stratified by survival time, we did not detect any dependencies of cardiovascular mortality on VAF (Supplemental Figure 5).

COMBINED EFFECT OF CLONAL HEMATOPOIESIS AND DEGREE OF CAROTID STENOSIS. We have previously made use of the ICARAS cohort to show associations of the inflammatory marker hsCRP with morphological features of carotid atherosclerosis and progression of carotid narrowing.⁵ We have further reported that the degree of carotid narrowing and levels of hsCRP jointly contribute to cardiovascular risk in ICARAS patients.⁸ Functional studies involving mouse models have linked the effect of CH to proinflammatory effects.^{9,10} Nevertheless, multivariate analysis in the ICARAS cohort suggests an effect of CH on cardiovascular risk independent of hsCRP levels (Table 2, Supplemental Figure 3). Thus, we hypothesized that similarly to but independently from hsCRP, CH may act as a risk factor additive to “degree of carotid narrowing,” both factors jointly contributing to cardiovascular risk. To assess the combined effects of

carotid stenosis and CH on long-term outcome, the patient cohort was stratified into 4 groups according to degree of carotid narrowing and presence of CH. Because carotid narrowing <50% as assessed by sonography is considered hemodynamically insignificant,⁸ group I was defined by carotid narrowing of <50% and no CH detected. Group II represented patients with carotid narrowing >50% but no CH detected. Group III included patients with carotid narrowing <50% and CH present, whereas group IV patients exhibited both carotid stenosis >50% and CH. The cumulative 12-year survival rates for cardiovascular death were 73% in the first, 59% in the second, 58% in the third, and 50% in the fourth group, as shown in a Kaplan-Meier analysis (log-rank $P < 0.0001$) (Central Illustration). Pairwise comparisons using log-rank statistics resulted in statistical significance for the observed effects in groups II, III, and IV compared with group I (Supplemental Table 4). Most notably, Kaplan-Meier estimates between groups II (>50% stenosis, no CH) and IV (>50% stenosis, CH) differ significantly ($P = 0.022$, log-rank test) (Central Illustration, Supplemental Table 4), suggesting additional prognostic power when adding “presence of CH” to “degree of stenosis.” Compared with the first group (<50% stenosis, no CH), adjusted HRs for the risk of cardiovascular death in groups II, III, and IV were 1.10 (95% CI: 0.84-1.43; $P = 0.504$), 1.53 (95% CI: 0.93-2.53; $P = 0.097$), and 1.60 (95% CI: 1.08-2.38; $P = 0.020$), respectively, underpinning the role of CH in driving cardiovascular mortality in the ICARAS cohort (Central Illustration). Statistical significance restricted to the latter comparison indicates the necessity for the inclusion of both “degree of

CENTRAL ILLUSTRATION Combined Assessment of Stenosis and Clonal Hematopoiesis Predicts Cardiovascular Mortality



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Patients were stratified into 4 groups (I: stenosis <50%, no CH; II: stenosis >50%, no CH; III: stenosis <50%, CH; IV: stenosis >50%, CH). Adjusted HRs and corresponding 95% CIs for risk of cardiovascular death as derived from multivariate analysis are depicted in a forest plot, where numbers represent associated event statistics (number of patients in group; number of patients with event; event rate per 100 patient-years). Kaplan-Meier estimates depict cardiovascular mortality during a median follow-up time of 11.8 years (Q1-Q3: 6.1-12.5 years), the P value reflecting the significance of the comparison pooled over strata (log-rank test). CH = clonal hematopoiesis.

stenosis” and “presence of CH” when adjusting for confounding factors. Furthermore, combined assessment of degree of carotid stenosis and presence of CH resulted in a significant improvement in the continuous net reclassification improvement, with 14.9% for cardiovascular mortality ($P = 0.013$) compared with degree of carotid stenosis alone.

DISCUSSION

Our study demonstrates that somatic mutations driving clonal hematopoiesis are frequent in patients

with initially asymptomatic carotid atherosclerosis measured by ultrasound sonography. Moreover, mutations in CH driver genes *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* are associated with cardiovascular mortality. Previous studies have documented a significant association of CH with a spectrum of cardiovascular conditions.⁴ Jaiswal et al¹ were the first to report an association of CH with incident coronary artery disease and ischemic stroke. More recent studies assessed the effect of CH on stroke,¹¹⁻¹³ other incident atherosclerotic diseases across multiple vascular beds, as well as PAD,¹⁴ altogether confirming and

refining the prevalent association of CH with adverse outcomes. Our study now shows that CH is associated with profoundly increased mortality in patients with neurologically asymptomatic, documented carotid artery disease. Moreover, our data demonstrate combined effects of subclinical carotid atherosclerosis as detected by widely available ultrasound examinations and CH in predicting cardiovascular mortality. Associations of CH with increased mortality were statistically significant in multivariate analyses for both cardiovascular death and all-cause death (both $P < 0.01$), with a slightly stronger effect on cardiovascular mortality, which represents the predominant cause of death (65.9% of patients) in our cohort. As previously reported in ICARAS,^{5,8} several of the covariates included in the multivariate analysis, specifically age, previous PAD, previous MI, presence of diabetes mellitus, and high hsCRP levels, proved to be significantly associated with both cardiovascular as well as all-cause death (Table 2, Supplemental Table 2).

MUTATIONS IN *DNMT3A*, *TET2*, *ASXL1*, AND *JAK2* INDIVIDUALLY AFFECT CARDIOVASCULAR MORTALITY.

Consistent with previous reports on CH in different populations and patient cohorts,^{2,3} the prevalence of CH increased with patient age also in our cohort (Figure 1A). Although vast differences related to size and sensitivity of NGS-based assays impede direct comparisons between different studies of CH, the frequency of CH identified in our patients with subclinical atherosclerosis was higher in most age groups compared with the cohorts (Bioimage, MDC, ATVB and PROMIS) screened in the pivotal study by Jaiswal et al.² Specifically, we detected CH in 7.7% of patients aged <50 years, whereas Jaiswal et al.² reported CHIP in 1.3% and 1.7% in cohorts with median age 40.5 years (ATVB) and 46.4 years (PROMIS), respectively. The frequency of CH in ICARAS patients between ages 50 and 70 years was 11.6%, compared with 5.2% reported in the MDC cohort (median age 60 years). Overall, we found CH in 13.7% of ICARAS patients (median age 69.2 years), allowing for comparison with the Bioimage cohort (median age 70 years) analyzed by Jaiswal et al.² where CH was reported in 11.9%. Our 4-gene-assay identified the highest number of somatic mutations in the *DNMT3A* gene, followed by *TET2* and *ASXL1*, and last, *JAK2* (Figure 1B), reflecting a distribution previously reported in other cohorts.^{1,2} Although *DNMT3A*, *TET2*, and *ASXL1* have different or even divergent cellular functions,³ they all act as critical epigenetic regulators of gene expression. Analysis of *JAK2* in our assay was restricted to a single amplicon comprising exon 14,

known to harbor the mutation underlying *JAK2*-V617F, a gain-of-function mutation that represents one of the main drivers of myeloproliferative neoplasms (MPN).⁶ In meta-analyses estimating the risk of developing incident coronary heart disease, HRs for mutations in single genes were calculated to be 1.7, 1.9, 2.0, and 12.0 for *DNMT3A*, *TET2*, *ASXL1*, and *JAK2*, respectively.² Similarly, HRs estimating risk for cardiovascular mortality in our study cohort suggested the lowest effect for *DNMT3A*, followed by *TET2* and *ASXL1*, and finally, the highest risk for *JAK2*-V617F carriers (Figure 4, Supplemental Table 3). Although formal statistical significance was restricted to *ASXL1* and *JAK2*, there is a trend for increased cardiovascular mortality also in carriers of mutations in *DNMT3A* and *TET2* (Figure 3). Interestingly, the survival curve for *ASXL1* mutated patients suggests an onset of increased mortality only after 5 years of follow-up (Figure 3). However, it remains to be investigated in additional studies whether this represents a robust phenomenon.

CH AFFECTS CARDIOVASCULAR MORTALITY INDEPENDENTLY OF hsCRP LEVELS.

The central role of inflammation in atherosclerotic diseases provides the basis for the use of circulatory inflammatory biomarkers for cardiovascular risk prediction. C-reactive protein is a highly sensitive marker of inflammation and tissue damage and one of the most frequently measured blood parameters. Multiple studies demonstrated that the degree of inflammation as measured by hsCRP correlates with disease severity and outcome in atherosclerotic CVD.¹⁵ Analyses of the PROVE-IT (Pravastatin or Atorvastatin Evaluation and Infection Therapy) trial have shown that low CRP levels in post-MI secondary prevention correlated with better clinical outcomes, independent of low-density lipoprotein levels.¹⁶ Accordingly, we previously identified a significant association between high hsCRP levels and mortality in the ICARAS cohort.⁸ The causal role of CH in atherosclerosis development has been established only in mice,^{2,9,10} where robust evidence supports a crucial pathogenic role of proinflammatory clonal leukocytes. In line with that, population studies demonstrated that genetically reduced interleukin-6 signaling decreases CVD risk in individuals with CH.¹⁷ Furthermore, results from the CANTOS trial suggested that patients with somatic variants in *TET2* better respond to interleukin-1 β inhibition compared with those without CH.¹⁸ Although published data on potential correlations of CH status with hsCRP levels as a downstream marker of inflammation are ambiguous,^{13,18,19} in our cohort hsCRP levels did not differ

between CH carriers and noncarriers (Table 1). Moreover, we found the effect of CH on cardiovascular mortality to be independent from hsCRP levels (Table 2). Thus, the effects of CH described herein possibly involve other inflammatory pathways. In that regard, a recent study²⁰ provides evidence for a bidirectional interplay between CH and atherosclerosis, where CH can be driven by inflammatory processes inherent to atherosclerosis, while in turn enhancing atherosclerosis. Of note, in our analyses, CH exhibited its strongest effect in patients aged ≥ 80 years, an age group where hsCRP was lacking predictive power (Supplemental Figure 3). Although this observation remains to be reproduced in further studies, it might be suggestive for a potential clinical relevance of CH screening in older patients with subclinical carotid atherosclerosis.

CH AND DEGREE OF STENOSIS JOINTLY PREDICT CARDIOVASCULAR MORTALITY. Most importantly, our prospective cohort study revealed that patients with both carotid stenosis and CH are at highest risk of cardiovascular death. In patient groups stratified based on level of carotid stenosis at a threshold of 50%, determination of CH provided additional prognostic information for both low degree ($<50\%$) and high degree ($\geq 50\%$) of carotid narrowing. Overall, more than one-half of the patients enrolled in this study with carotid stenosis $>50\%$ and presence of CH died within 12 years of follow-up, compared with only 27% of patients with carotid narrowing $<50\%$ and no CH detected (Central Illustration). Therefore, combined determination of presence of CH with degree of stenosis provides a substantial increase in prognostic power compared with assessment of stenosis alone, the latter representing an established risk factor for patients with carotid atherosclerosis.⁸ Altogether, our findings suggest that patients who undergo ultrasound examination of the external carotid arteries at a single time point may profit from an assessment of CH status.

STUDY LIMITATIONS. First, genomic regions included in our study are targeting only selected regions within *DNMT3A*, *TET2*, *ASXL1*, and *JAK2*, covering 76% of somatic mutation sites reported in the pivotal study of CVD-related CHIP.² The present study may therefore underestimate the true frequency of CH in the ICARAS cohort. Nevertheless, our data are well-aligned with published data on CH-associated cardiovascular risk. Second, the *JAK2*-V617F mutation, found at a higher frequency (1.2%) in our cohort compared with other studies,^{2,12,19,21} represents one of the main drivers of MPN.⁶ A recent study on the role of CH in a cohort of young patients with stroke reported a

~ 20 -fold increased prevalence of MPN in the stroke cohort, including previously undiagnosed MPN cases.¹² Considering the study design of ICARAS, which is based on a consecutive enrollment of patients with indications for sonographic carotid examinations,⁵ we cannot rule out that some patients may evolve to *JAK2*-V617F-positive MPN. In a recent population-scale study, low allelic burden *JAK2*-V617 was found in a substantial fraction of the general population, exhibiting low penetrance to evolve to MPN.²² In our cohort, VAFs $>50\%$ indicating homozygosity for *JAK2*-V617F in 2 individuals provide evidence for presence of MPN in some patients.

CONCLUSIONS

Our results demonstrate that CH mediated by somatic mutations in the most prevalent CH-associated genes *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* significantly contributes to cardiovascular mortality in patients with asymptomatic carotid atherosclerosis. Our data further indicate that the individual CH status in combination with the extent of carotid atherosclerosis jointly predict long-term mortality. Thus, in patient groups classified by degree of carotid stenosis, determination of CH can provide additional prognostic information. Overall, our study demonstrates the potential clinical value of targeted screening for CH in patients with subclinical carotid atherosclerosis.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: CH assessed by deep sequencing in combination with measurement of carotid artery stenosis by duplex ultrasound predicts mortality in asymptomatic patients.

TRANSLATIONAL OUTLOOK: Better understanding of the mechanisms underlying the prognostic link between stenosis severity and CH is necessary to develop improved strategies for prevention of ischemic events in patients with carotid atherosclerosis.

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APPENDIX For an expanded Methods section as well as supplemental figures and tables, please see the online version of this paper.