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ORIGINAL RESEARCH - CLINICAL

Circulating Hepatocyte Growth Factor Reflects Activation of Vascular Repair in Response to Stress

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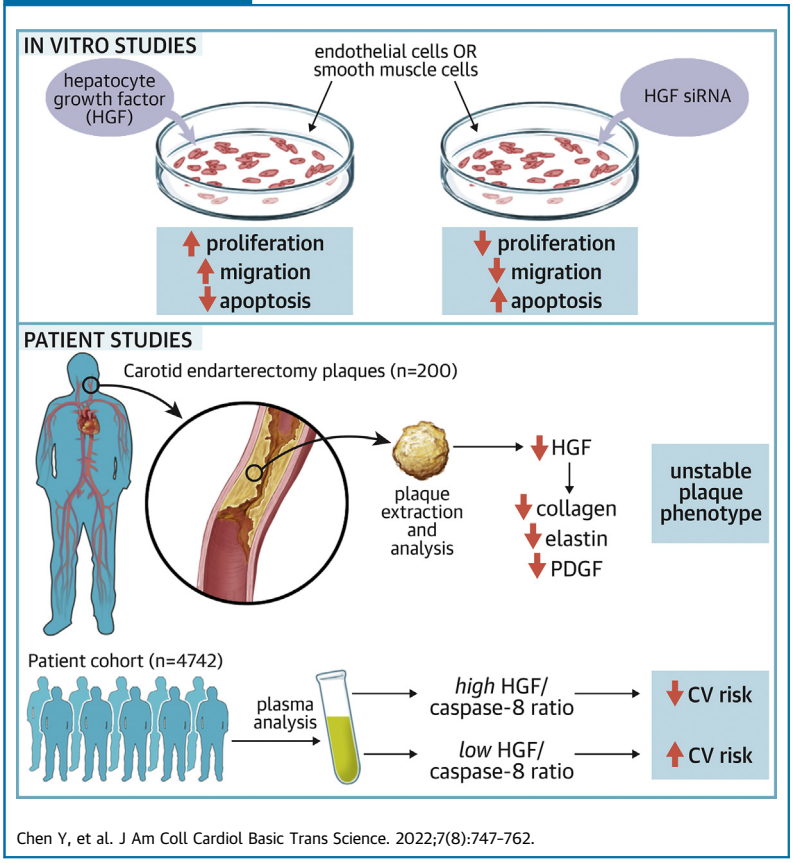
VISUAL ABSTRACT



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HIGHLIGHTS

- **HGF is released by stressed human vascular cells and promotes vascular cell repair responses in autocrine and/or paracrine ways.**
- **Subjects with a low capacity to express HGF in response to systemic stress have an increased cardiovascular risk.**
- **Human atherosclerotic plaques with a low content of HGF have a more unstable phenotype.**
- **The present study shows that subjects with a low ability to express HGF in response to metabolic stress have an increased risk to suffer cardiovascular events.**

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ABBREVIATIONS AND ACRONYMS

AU = arbitrary unit

BrdU = bromodeoxyuridine

CVD = cardiovascular disease

EC = endothelial cell

HCASMC = human coronary artery smooth muscle cell

HDL = high-density lipoprotein

HGF = hepatocyte growth factor

HUVEC = human umbilical cord endothelial cell

ICD-9 = International Classification of Diseases-9th Revision

IL = interleukin

LDL = low-density lipoprotein

MMP = matrix metalloproteinase

mRNA = messenger RNA

PlGF = placental growth factor

s = soluble

si-HGF = hepatocyte growth factor small interfering RNA

siRNA = small interfering RNA

SMC = smooth muscle cell

TGF = transforming growth factor

TNF = tumor necrosis factor

TRAIL = tumor necrosis factor-related apoptosis-inducing ligand

VEGF = vascular endothelial growth factor

SUMMARY

Hepatocyte growth factor (HGF) is released by stressed human vascular cells and promotes vascular cell repair responses in both autocrine and paracrine ways. Subjects with a low capacity to express HGF in response to systemic stress have an increased cardiovascular risk. Human atherosclerotic plaques with a low content of HGF have a more unstable phenotype. The present study shows that subjects with a low ability to express HGF in response to metabolic stress have an increased risk to suffer myocardial infarction and stroke. (J Am Coll Cardiol Basic Trans Science 2022;7:747–762)

Maintaining endothelial integrity is critical for prevention of acute cardiovascular events.[1,2] The most common cause of loss of endothelial integrity is rupture of a thin-cap fibroatheroma.[3] These plaques are characterized by a core of necrotic debris, extracellular lipid deposits and inflammatory cells covered by a thin fibrous cap containing fibrous proteins and smooth muscle cells (SMCs). They arise as a result of a buildup of oxidized lipoprotein-derived lipids in the arterial intima activating an array of different immune and repair responses.[4-6] Inability of immune cells to clear the oxidized lipids increases the risk of plaque destabilization with proteolytic degradation of connective tissue proteins and impaired SMC repair function. Treatment with statins and other cholesterol-lowering drugs reduces lipid-driven plaque inflammation,[7] but other factors such as local low shear stress and autoimmune responses toward modified self-antigens may continue to fuel plaque inflammation and destabilization also in patients who are well-treated.[8] Another common cause of loss of endothelial integrity are endothelial erosions.[3] These may give rise to thrombus formation on the surface of a plaque with an intact fibrous cap.[9] The mechanisms involved in development of endothelial erosions involve a combination of increased endothelial stress and reduced endothelial repair capacity. The imbalance between injury and repair is thus a common feature for thrombus formation caused by both plaque rupture and endothelial erosion. Recent studies using Mendelian randomization and experimental models have shown that placental growth factor lowers cardiovascular risk by promoting vascular repair.[10,11] It is an interesting possibility that stimulating such processes in the future may help to lower cardiovascular risk in patients with insufficient protection from current therapeutic strategies.

Hepatocyte growth factor (HGF) was initially identified as potent mitogen and antiapoptotic factor for liver cells but has subsequently been shown to have important effects on multiple cell types and organs.[12] It is secreted as a single chain proprotein that binds to heparin-containing proteoglycans in the extracellular matrix. HGF mediates its effects through activation of the tyrosine kinase receptor c-Met.[13] The general effects of c-Met activation by HGF include stimulation of cell viability, proliferation, and migration, as well as inhibition of inflammation.[12] Although such effects potentially could be beneficial for stabilization of atherosclerotic plaques, the possible role of HGF in atherosclerosis remains relatively unexplored. However, HGF is also known to possess potent antifibrotic effects by inhibiting transforming growth factor (TGF)-β[14] and stimulating the expression of matrix metalloproteinases (MMPs),[15] which are processes considered to contribute to plaque destabilization. Clinical studies have shown that high levels of HGF are associated with more severe atherosclerosis and risk for development of cardiovascular events.[16-18] However, it remains unclear whether these associations reflect a proatherogenic role of HGF or activation of a protective response to cardiovascular stress. In the present study, we combined experimental investigations of the role of HGF in human vascular cell repair

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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processes with analyses of HGF associations with human atherosclerotic plaque composition, as well as how the relation between HGF and biomarkers of cellular stress reflects the risk for future cardiovascular events to get a better understanding of the role of HGF in cardiovascular disease (CVD).

METHODS

CLINICAL COHORTS. The population cohort consisted of 4,742 subjects participating in the MDC (Malmö Diet and Cancer) study that were followed from baseline examination in 1991-1994 until first event of CVD, emigration from Sweden, or death, until December 31, 2014.[19] Myocardial infarction was defined as a fatal or nonfatal myocardial infarction on the basis of the International Classification of Diseases-9th and 10th revisions (ICD-9 and ICD-10) codes 410 and I21, respectively. Death caused by ischemic heart disease was defined based on codes 412 and 414 (ICD-9) or I22, I23, and I25 (ICD-10). Ischemic stroke was defined as ICD-9 codes 434 or 436 or ICD-10 codes I63 or I64 and hemorrhagic stroke as ICD-9 codes 430 or 431 or ICD-10 codes I60 or I61.

The carotid plaque cohort consisted of 200 patients who underwent carotid endarterectomy between October 1, 2005, and October 31, 2010, at Skåne University Hospital.[20] Indications for carotid endarterectomy have been previously described.[20] The clinical charts of all patients were reviewed to gain information about comorbidities and the past medical history. Plaques were snap frozen in liquid nitrogen immediately after surgery, cut into smaller pieces, and homogenized in a homogenization buffer.[21] Analyses of plaque components by histological staining, immunohistochemistry, enzyme-linked immunosorbent assay, proximity extension assay, and biochemical assays were performed as previously reported.[22,23]

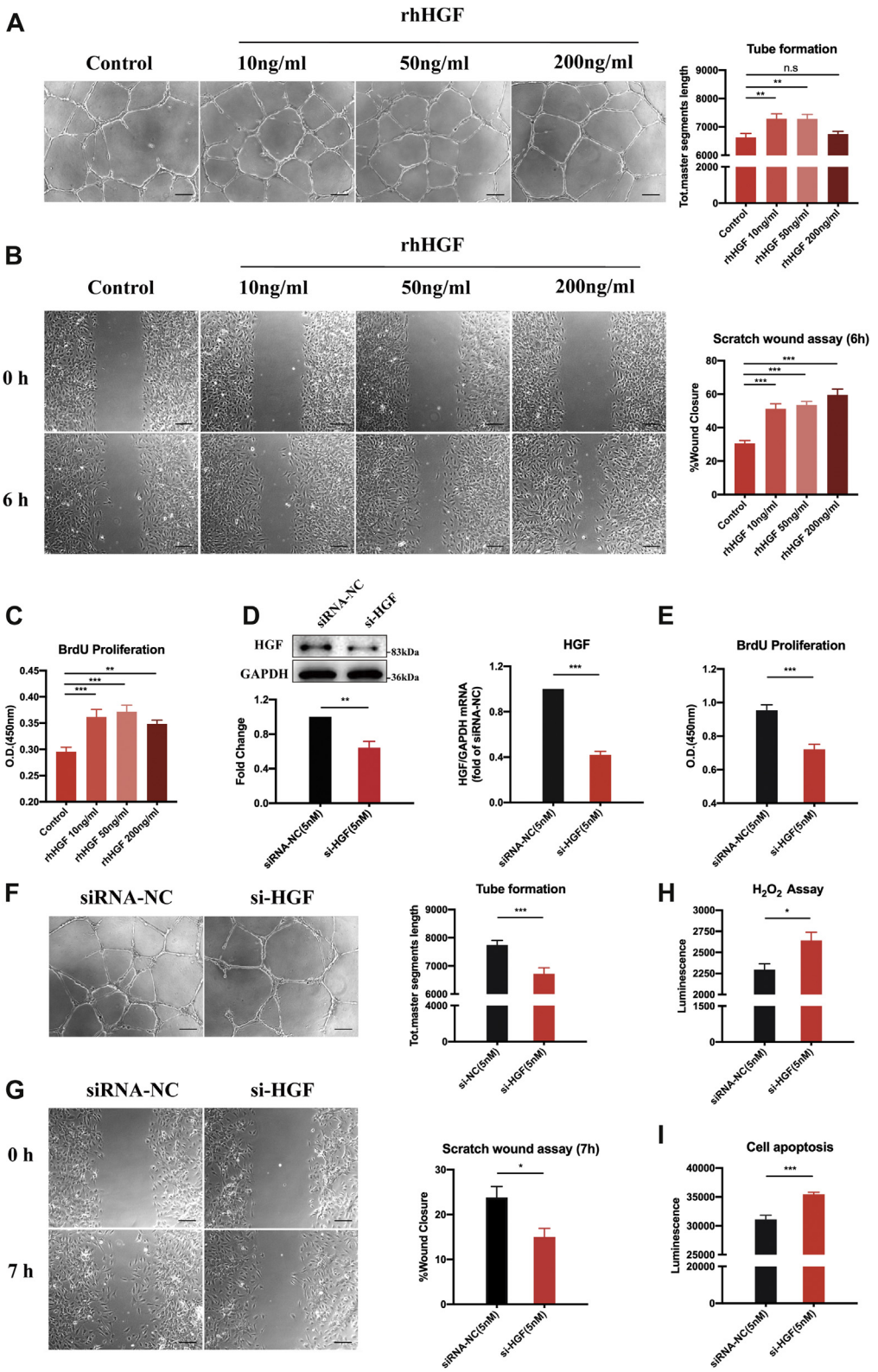
Procedures for patient enrollment, computed tomography coronary artery calcium scanning, and baseline clinical characteristics of the participants of the PROCEED (Progression of Coronary Atherosclerosis in Asymptomatic Diabetic Subjects: Evaluation of the Role of Computed Tomography Coronary Angiography and Novel Biomarkers of Vascular Inflammation and Endothelial Function) trial have previously been reported.[24] Of the original 257 subjects in the study, 43 subjects were excluded because their plasma samples were lost because of accidental thawing, leaving 214 subjects to be analyzed by proximity extension assay in the present study.

All studies were approved by the respective Regional Ethical Review Boards and conducted in accordance with the Helsinki Declaration. All subjects gave written consent. The reporting of the studies is done in accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines.

ANALYSIS OF HGF, TRAIL RECEPTOR-2, AND CASPASE-8. Plasma (EDTA) levels of HGF, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-2, and caspase-8 were analyzed by the proximity extension assay technique using the Proseek Multiplex CVD$^{96\times96}$ reagents kit (Olink Bioscience) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala. The coefficients of variation for intra-assay variation (within-run) and interassay variation (between-run) for HGF, TRAIL receptor-2, and caspase-8 are 8% and 13%, 7% and 10%, and 5% and 10%, respectively, and the analytical ranges were 15.3-31,250 pg/mL, 0.2-7,812 ng/mL, and 3.8-62,500 pg/mL, respectively. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (Multid Analyses). All data are presented as arbitrary units (AUs). General calibrator curves to calculate the approximate concentrations as well as technical information about the assays are available on the Olink homepage.

STATISTICAL ANALYSES. Analysis of skewness and kurtosis were used to test for normality. Continuous data were presented as mean ± SD or SE of mean for data with normal distribution, median (IQR) for skewed data, and percentages for categorical data. Differences between means of normally distributed continuous variables were assessed with independent sample Student's *t*-tests and between skewed variables with the Mann-Whitney *U* test. The chi-square test was used to compare categorical variables. Analysis of variance with Dunnett post hoc test for multiple pairwise comparisons was used to compare more than 2 groups. Correlation coefficients between continuous variables were calculated using the Spearman rank test. Multivariable linear regression models with results presented as regression coefficient (β) with 95% CI were used to adjust for baseline covariates when evaluating the correlations of HGF/soluble (s)TRAIL receptor-2 and HGF/caspase-8 ratios with age, sex, diabetes, current smoking, body mass index, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, fasting glucose, triglycerides, and systolic and diastolic blood pressure included as independent variables. The associations among HGF, HGF/sTRAIL receptor-2, and HGF/caspase-8 tertiles and time to the respective first cardiovascular event during follow-up was assessed using Kaplan-Meier survival curves and the log-rank test Cox proportional hazards regression models

FIGURE 1 **Effect of HGF on EC Function**



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were used to estimate the HR with 95% CI after adjustment for baseline covariates (age, sex, current smoking, diabetes, LDL, HDL, triglycerides, and systolic blood pressure). To correct for the potential effect of mortality (all-cause) on the outcomes, we also performed competing risks regression according to Fine and Gray proportional subhazards model. SubHRs for myocardial infarction, stroke, and CVD death with death from any cause as a competing event were obtained (and adjusted for confounders). Patients were followed from the baseline investigation until the first cardiovascular event, death, or end of follow-up, whichever occurred first. A P value of <0.050 was considered statistically significant. SPSS Statistics (version 22, IBM Corp) and STATA (version 14.2, StataCorp) were used for statistical analysis of clinical data and Prism (version 8; GraphPad Software) for analyses of experimental data.

Materials and methods used in the cell culture studies are described in the Supplemental Appendix.

RESULTS

ROLE OF HGF IN EC FUNCTION. Exposure of human umbilical cord endothelial cells (HUVECs) to HGF resulted in a dose-dependent stimulation of endothelial tube formation (**Figure 1A**), ability to repair an in vitro scratch injury (**Figure 1A**), bromodeoxyuridine (BrdU) uptake (**Figure 1C**), as well as increased messenger RNA (mRNA) expression of neuropilin-1 (a co-receptor to vascular endothelial growth factor [VEGF] receptors), VEGF-A, placental growth factor (PlGF), and VEGF receptor-2, but not the HGF receptor c-Met (Supplemental Figure 1A). There were also enhanced mRNA expressions for tumor necrosis factor (TNF)-α, interleukin (IL)-6, and MMP-3 following stimulation with HGF (Supplemental Figure 1B).

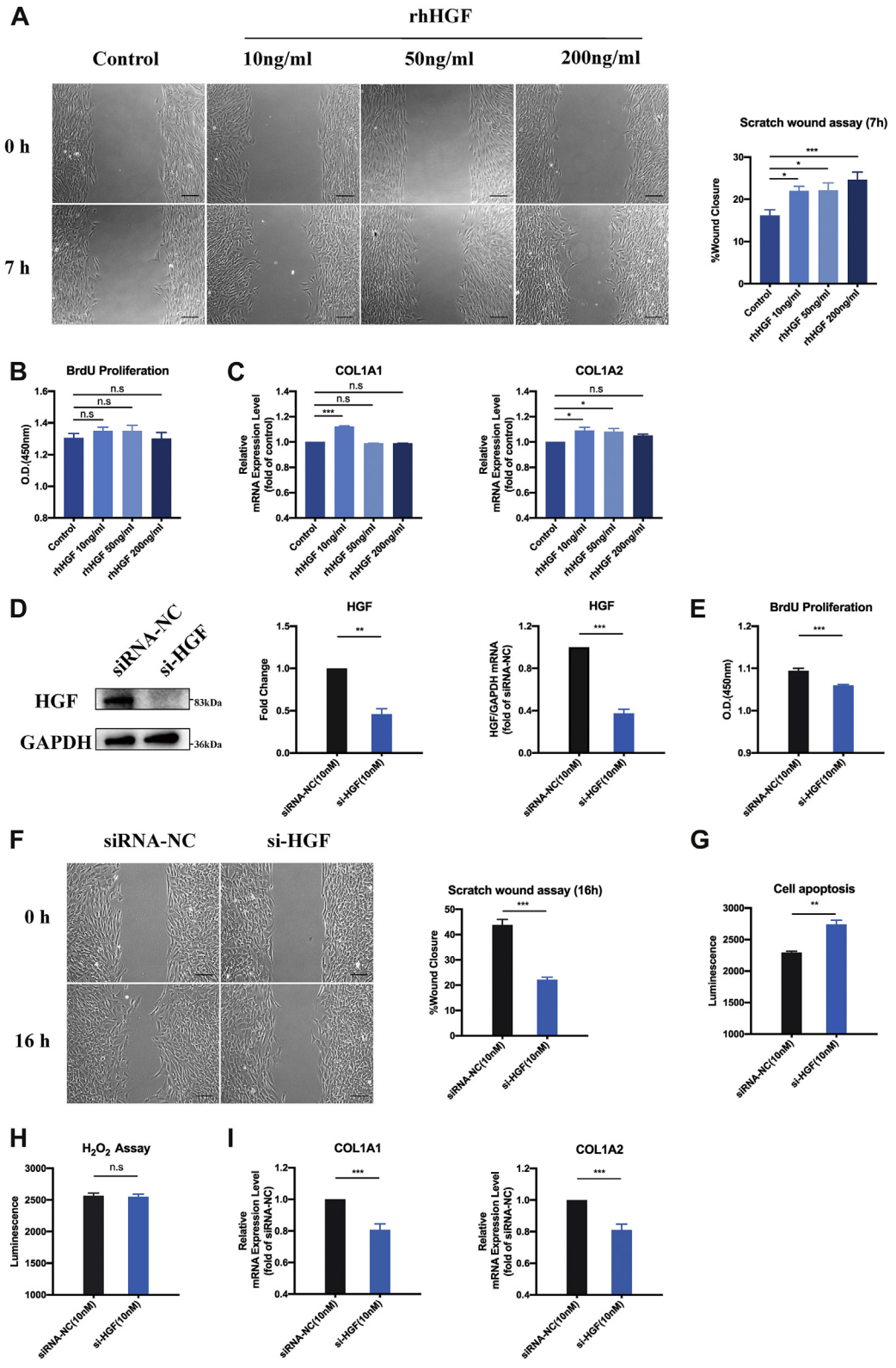
To characterize the possible role of autocrine and/or paracrine HGF in human vascular ECs, we treated cultured HUVECs with small interfering RNA (siRNA). Exposure of HUVECs to 5 nmol/L of HGF siRNA (si-HGF) reduced the expression of HGF mRNA by more than 50% and down-regulated HGF on protein level significantly at the same time (**Figure 1D**). This was associated with a reduced proliferation of HUVECs grown in low serum supplement medium as assessed by the uptake of BrdU (**Figure 1E**), demonstrating that autocrine and/or paracrine stimulation of cell growth by HGF can take place in human ECs. Treatment with si-HGF also impaired the ability of HUVECs to form endothelial tubes (**Figure 1F**) as well as their ability to repair an in vitro scratch injury (**Figure 1G**). Suppression of HGF mRNA levels was further associated with increased oxidative stress and an enhanced rate of apoptosis in HUVECs (**Figures 1H and 1I**). Moreover, it reduced the expression of mRNA for c-Met as well as for several other key factors involved in activation of endothelial growth including VEGF-A, PlGF, and VEGF receptor-2 (Supplemental Figure 1C). The mRNA expressions for TNF-α and IL-6 as well as for MMP-2 and MMP-3 were also reduced following treatment with si-HGF (Supplemental Figure 1D). We next analyzed the effect of adding HGF to HUVECs exposed to si-HGF and found that this dose-dependently reversed the effects on proliferation, apoptosis, tube formation, and repair of in vitro scratch injury (Supplemental Figures 2A to 2D).

ROLE OF HGF IN ARTERIAL SMC FUNCTION. To characterize the role of HGF in SMC, we used cultured human coronary artery smooth muscles (HCASMCs). Stimulating HCASMCs with HGF resulted in a dose-dependent increase in scratch wound closure capacity but had no significant effect on the uptake of BrdU (**Figures 2A and 2B**). There was also an increased expression of the genes encoding collagen type 1 α1 and α2 in HCASMCs exposed to low levels of

FIGURE 1 Continued

The effect of exogenous hepatocyte growth factor (HGF) on endothelial tube formation **(A)** and ability of human umbilical cord endothelial cells (HUVECs) to migrate into an in vitro scratch injury **(B)** was analyzed with ImageJ software (National Institutes of Health). (Representative images shown on the **left**; n = 10-12 per group for **A**; n = 12-20 per group for **B**.) Treatment with recombinant human hepatocyte growth factor (rhHGF) stimulated HUVECs' proliferation **(C)**, which was evaluated with the uptake of bromodeoxyuridine (BrdU) (n = 10-12 per group). **(D)** Western blot (representative blot shown, n = 3) and quantitative real-time polymerase chain reaction (n = 4) confirmed the efficiency of small interfering RNA (siRNA) transfection for 48 hours on HUVECs. Cell proliferation **(E)** of HUVECs treated with nontargeting small interfering RNA negative control (siRNA-NC) or hepatocyte growth factor small interfering RNA (si-HGF) was assessed by BrdU uptake (n = 13). Quantified measurement of tube formation ability **(F)** and wound healing rate **(G)** on HUVECs transfected with siRNA-NC or si-HGF. (Representative pictures shown on the left; n = 17 for **F**; n = 8 for **G**.) The assessment of oxidative stress level **(H)** and cell apoptosis **(I)** between control group and HGF silence group (n = 7 for **H**; n = 8 for **I**) are shown. Bar = 150 μm. Data are presented as mean ± SEM and were acquired from 3-4 independent replicate tests. *P < 0.050, **P < 0.010, and ***P < 0.001 by one-way analysis of variance and Dunnett post hoc test **(A to C)** and Student t-test **(D to I)**. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; M = mol/L; mRNA = messenger RNA; OD = outer diameter; Tot = total.

FIGURE 2 **Effect of HGF on SMC Function**



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HGF, but this effect was attenuated at higher concentrations (Figure 2C). Stimulation with HGF resulted in a marked down-regulation of c-Met mRNA in HCASMCs, but the effect on the mRNA expression for other genes related to the VEGF/VEGF receptor family was relatively modest (Supplemental Figure 3A). Moreover, exposure to HGF reduced mRNA expression for IL-6, MMP-2, and MMP-3, while that of TNF-α was enhanced (Supplemental Figure 3B).

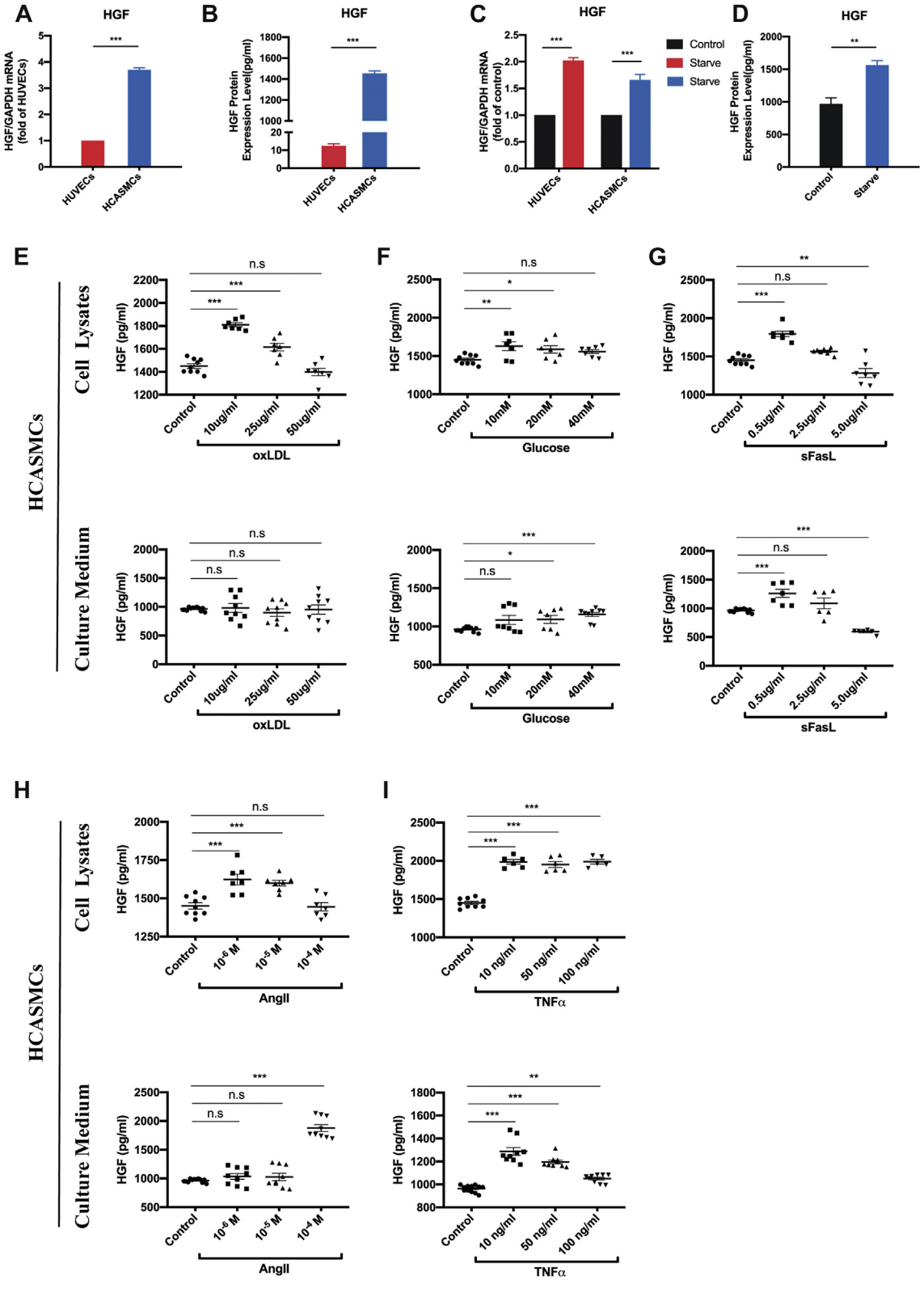
Treatment with 10 nmol/L of si-HGF reduced the HGF expression markedly on both transcript and protein levels in HCASMCs (Figure 2D). This was associated with a modest reduction in cell proliferation as assessed by incorporation of BrdU (Figure 2E), whereas the ability to close an in vitro scratch wound was reduced by more than 50% (Figure 2F). These observations indicate that stimulation of autocrine and/or paracrine HGF is more important for SMC migration than proliferation is. Exposure to si-HGF increased apoptosis of HCASMCs (Figure 2G) but did not affect the level of intracellular oxidative stress (Figure 2H). Inhibition of HGF mRNA resulted in reduced expression of the genes encoding collagen type 1 α1 and α2 (Figure 2I), suggesting that HGF, in contrast to most other tissues, may have a profibrotic role in the vascular wall. HGF mRNA inhibition reduced the expression of several receptors involved in VEGF signaling, but it did not affect the expression of VEGF-A and PlGF (Supplemental Figure 3C). Finally, treatment with si-HGF reduced the mRNA levels of TNF-β, IL-6, and MMP-2 but not MMP-3 in HCASMCs (Supplemental Figure 3D). Adding HGF to HCASMCs exposed to si-HGF dose-dependently reversed the effects on proliferation, apoptosis, and repair of in vitro scratch injury (Supplemental Figures 4A to 4C).

ENDOTHELIAL CELL AND SMC EXPRESSION OF HGF IN RESPONSE TO STRESS. There is strong evidence from studies of liver regeneration and multiple other organs that the expression of HGF is activated by tissue injury and stress and that it plays a key role in maintaining cell viability and stimulating repair processes.[12] To determine whether this is true also for human vascular cells we exposed cultured HUVECs and HCASMCs to various stressful conditions. Both the basal expression of HGF mRNA and the intracellular HGF protein levels were several-fold higher in HCASMCs than in HUVECs (Figures 3A and 3B). Serum-starvation significantly increased HGF mRNA levels in both HUVECs and HCASMCs (Figure 3C) and stimulated the release of HGF from HCASMCs (Figure 3D), whereas no HGF could be detected in the medium of serum-starved HUVECs. Accordingly, we focused our studies of the effects of cellular stress on HGF secretion on HCASMCs. Exposure of HCASMCs to 10 μg/mL of oxidized LDL increased intracellular levels of HGF, but this effect gradually disappeared with increasing concentrations of oxidized LDL (Figure 3E). Addition of oxidized LDL had no effect on the release of HGF from HCASMCs. There was a modest increase in intracellular HGF in HCASMCs cultured in 10 mmol/L glucose and a modest increase in HGF secretion in HCASMCs cultured in 40 mmol/L glucose (Figure 3F). Exposing HCASMCs to Fas ligand to activate the extrinsic pathway of apoptosis resulted in increased levels of HGF at the lowest concentration of Fas ligand, whereas higher concentrations of Fas ligand decreased both intracellular and secreted HGF (Figure 3G). Stimulation of the cells with angiotensin II modestly increased intracellular HGF levels at lower concentrations, whereas it activated marked secretion of HGF from the cells at a concentration of 10 mmol/L (Figure 3H). Exposure of HCASMCs to TNF-β increased both the intracellular and secreted levels of HGF although the latter declined with increasing concentrations of TNF-β (Figure 3I). Culture of cells in presence of TGFβ2 and TGFβ3 markedly reduced both intracellular and secreted levels of HGF

FIGURE 2 Continued

The effect of HGF stimulations with rising dose on cultured human coronary artery smooth muscle cells (HCASMCs) wound healing rate **(A)** and proliferation **(B)** was evaluated with scratch wound assay in vitro and BrdU incorporation (representative pictures shown on the left; n = 10-15 per group for **A**; n = 11 for **B**). quantitative real-time polymerase chain reaction (qRT-PCR) analysis of collagen type 1 α1 (COL1A1) and COL1A2 mRNA expression **(C)** in HGF-stimulated HCASMCs (n = 4). HCASMCs were incubated with siRNA-NC or si-HGF for 48 hours, followed by Western blot (representative blots shown, n = 3) and qRT-PCR (n = 4) analysis **(D)**. The uptake of BrdU **(E)** and scratch wound assay **(F)** were performed to measure cell proliferation and wound healing ability of HCASMCs with siRNA-NC or si-HGF, respectively (n = 10 for **E**; n = 12 for **F**; representative images shown on the left). Cell apoptosis **(G)** and oxidative stress level **(H)** in the control group and si-HGF group were assessed with active caspase-3/-7 and H_2O_2 production (n = 9 for **G**; n = 7 for **H**). The gene expression **(I)** of COL1A1 and COL1A2 induced by si-HGF was detected with quantitative real-time polymerase chain reaction (n = 6-8). Bar = 150 μm. Data are presented as mean ± SEM and were acquired from 3-4 independent replicate tests. *$P < 0.050$, **$P < 0.010$, and ***$P < 0.001$ by one-way analysis of variance and Dunnett post hoc test **(A to C)** and Student *t*-test **(D to I)**. Abbreviations as in Figure 1.

FIGURE 3 Effect of Cellular Stress on the Expression of HGF in Cultured ECs and SMCs



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(Supplemental Figures 5A and 5B). Taken together, these observations suggest that vascular cells increase intracellular HGF levels in response to stress, but with limited extracellular secretion from ECs.

ASSOCIATIONS OF HGF WITH FACTORS DETERMINING THE STABILITY OF HUMAN ATHEROSCLEROTIC PLAQUES. Because the findings described suggest that HGF is of importance for maintaining the viability and repair capacity of human vascular cells, we analyzed how plasma and plaque levels of HGF were related to atherosclerotic plaque structure. Carotid plaques from 200 patients undergoing endarterectomy were snap frozen in liquid nitrogen and analyzed as previously described.[22] The clinical characteristics of the carotid plaque cohort are shown in Supplemental Table 1. There were 106 patients who had suffered a recent cerebrovascular event that could be related to the removed plaque. HGF levels were analyzed in both plaque homogenates and in plasma samples taken the day before surgery. The median concentration of HGF was higher in plaque tissue than in plasma (17.9 [IQR: 12.8-24.5] $\times$ 106 AU/g plaque wet weigh vs 147 [IQR: 122-180] AU/mL plasma). There was no significant association between HGF levels in plaque tissue and plasma ($r = 0.01$). HGF was primarily present in the fibrous cap but could also be detected in the core region of the plaque (Supplemental Figures 6A to 6F). The HGF receptor c-Met was expressed by ECs and SMCs in intraplaque vessels as well as by plaque macrophages. There was also a weak staining for c-Met in SMCs located in the shoulder region of the plaque (Supplemental Figures 7A to 7H). The median HGF content was lower in plaques from male patients (17.1 [IQR: 12.0-22.3] vs 19.0 [IQR: 12.2-23.3] $\times$ 10^6 AU/mg plaque wet weigh in female patients; $P = 0.02$) and in plaques associated with cerebrovascular symptoms (16.3 [IQR: 12.2-23.3] vs 19.5 [IQR: 13.1-29.6] $\times$ 10^6 AU/mg plaque wet weigh in asymptomatic plaques; $P = 0.04$). There were no significant differences in the plasma levels of HGF between these groups (data not shown). Also, there were no significant differences in plaque HGF content between patients with or without statin, beta-blocker or renin-angiotensin-aldosterone-system inhibitor treatment (data not shown).

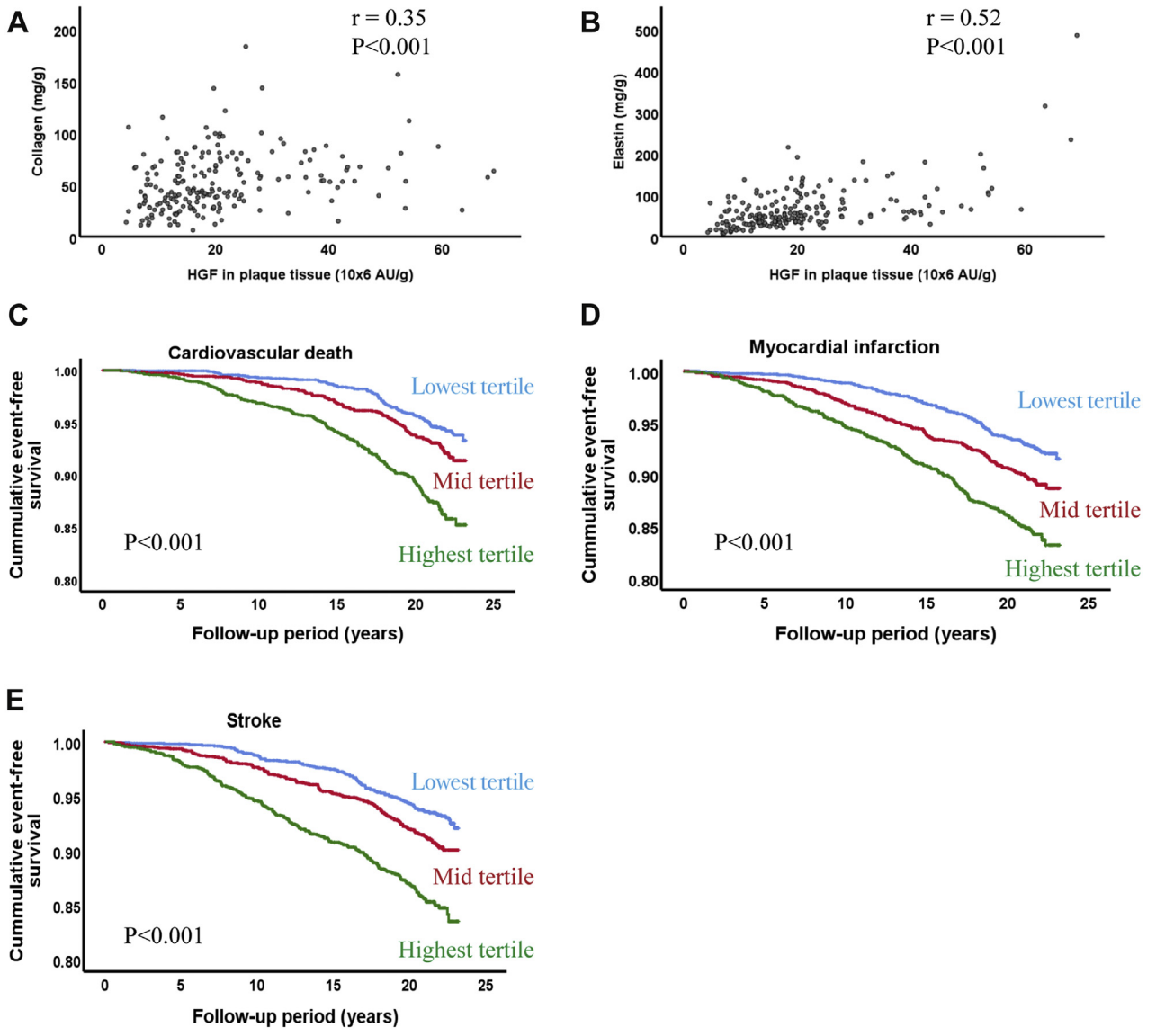
Plaques with a high HGF expression were more fibrotic than those with plasma HGF as assessed by their collagen and elastin contents (**Figures 4A and 4B, Table 1**). Plaque HGF correlated significantly with the plaque content of several factors stimulating fibrosis such as platelet growth factor and tissue inhibitor of metalloproteinases-2, but also with the contents of matrix proteases such as MMP-2 and MMP-3 (**Table 1**). Except for an association with TNF-α, there were no significant associations between HGF and the plaque content of proinflammatory cytokines. There was also no significant association between plaque HGF levels and the plaque content of the erythrocyte marker glycophorin A (**Table 1**).

A LOW HGF RESPONSE TO CELLULAR STRESS IS ASSOCIATED WITH AN INCREASED CARDIOVASCULAR RISK. The observations presented demonstrate that HGF is expressed by vascular cells in response to stress and that is of importance for maintaining cell viability and repair functions. Against this background, we set out to test the hypothesis that a reduced ability to express HGF in response to cellular stress is associated with an increased risk of cardiovascular events. The study cohort consisted of 4,742 subjects participating in the prospective MDC (Malmö Diet and Cancer) study with a mean follow-up period of 19.4 $\pm$ 5.0 years. sTRAIL receptor-2 and caspase-8 were used as biomarkers of cellular stress. TRAIL receptor-2 is a cell surface so-called death receptor that mediates extrinsic activation of apoptosis.[25] Activation of this pathway results in release of sTRAIL receptor-2,[26] suggesting that the circulating level of sTRAIL receptor-2 is a biomarker of apoptosis. Plasma levels of sTRAIL receptor-2 correlate with presence of cardiovascular risk factors and risk of cardiovascular disease.[26] Caspase-8 is activated by TRAIL receptor-2 and functions as a key protease in apoptosis induced through the extrinsic

FIGURE 3 Continued

HUVECs and HCASMCs were grown in their respective growth medium with supplements and the **(A)** level of HGF mRNA expression was assessed by qRT-PCR **(B)** and the release of HGF into the cell culture medium was measured by enzyme-linked immunosorbent assay (ELISA). **(C)** The HGF mRNA expression of HUVECs and HCASMCs serum-starved in basic medium with 0.5% and 0.1% supplement, respectively, for 24 hours was detected by qRT-PCR. **(D)** Effect of serum-starvation on release of HGF into the cell culture medium was measured by ELISA. Cultured HCASMCs were exposed to increasing concentrations of oxidized low-density lipoprotein (oxLDL) **(E)**, glucose **(F)**, soluble Fas ligand (sFasL) **(G)**, angiotensin II (Ang II) **(H)**, and tumor necrosis factor-α (TNF-α) **(I)** for 24 hours and the amount of HGF in cell lysates and culture medium was analyzed by ELISA (n = 5-12 per group). Data are presented as mean $\pm$ SEM and acquired from 3 independent replicate tests. *$P < 0.050$, **$P < 0.010$, and ***$P < 0.001$ by Student t-test **(A to D)** and one-way analysis of variance, and Dunnett post hoc test **(E to I)**. Abbreviations as in **Figures 1 and 2**.

FIGURE 4 HGF in Relation to Plaque Fibrous Proteins and Risk of Cardiovascular Events



Scatter plots demonstrating the correlation between **(A)** the plaque contents of hepatocyte growth factor (HGF) and collagen and **(B)** the plaque contents of HGF and elastin. Correlations were calculated using the Spearman rho test. Kaplan-Meier plots show the association between tertiles of HGF and **(C)** cardiovascular death, **(D)** myocardial infarction, and **(E)** stroke during a mean follow-up period of 19.4 ± 5.0 years. Log-rank test was performed to calculate *P* values. AU = arbitrary unit(s).

pathway. However, in contrast to sTRAIL receptor-2, it is not released from living cells as part of death receptor activation and when present in the circulation it will thus function as a biomarker of more advanced stages of cell damage. HGF, sTRAIL receptor-2, and caspase-8 were analyzed in baseline plasma samples. HGF correlated strongly with the plasma levels of both sTRAIL receptor-2 and caspase-8 ($r = 0.65$; $P < 0.001$ and $r = 0.47$; $P < 0.001$, respectively) (Supplemental Figure 8), suggesting that they reflect similar processes. HGF/sTRAIL receptor-2 and HGF/caspase-8 ratios were calculated as measures of the ability to respond with increased HGF expression in response to cellular stress, for example, a low ratio should reflect an impaired HGF response. To validate the biological relevance of analyzing the balance between HGF and biomarkers of cellular stress, we first confirmed that TRAIL induces apoptosis in both HUVECs and HCASMCs (Supplemental Figures 9A and 9B). We subsequently

TABLE 1 Spearman Correlations Between HGF and Human Atherosclerotic Plaque Components

	Plaque HGF	Plasma HGF
Fibrosis		
α-actin, % stained	0.17[a]	0.01
Collagen, mg/g	0.35[b]	0.17[a]
Elastin, mg/g	0.52[b]	0.13
PDGF, pg/g	0.30[b]	0.17[a]
Proteases/inhibitors		
MMP-2, AU/g	0.28[b]	0.14[a]
MMP-3, AU/g	0.22[c]	0.22[c]
TIMP-2, pg/g	0.54[b]	−0.19[a]
Inflammation		
IL-1β, pg/g	−0.05	0.00
IL-6, pg/g	0.00	0.11
MCP-1, pg/g	−0.11	0.05
TNF-α, pg/g	0.36[b]	0.17[a]
RANTES, pg/g	−0.20[a]	0.07
EC and hemorrhage		
Glycophorin A, % stained	−0.11	0.03

Threshold for significance following Bonferroni correction for multiple comparisons is 0.001. [a]$P < 0.050$; [b]$P < 0.001$; [c]$P < 0.005$.

AU = arbitrary unit(s); EC = endothelial cell; HGF = hepatocyte growth factor; IL = interleukin; MCP = monocyte chemoattractant protein; MMP = matrix metallopeptidase; PDGF = platelet growth factor; RANTES = regulated on activation, normal T expressed, and secreted; TIMP = tissue inhibitor of metalloproteinases; TNF = tumor necrosis factor.

TABLE 2 Spearman Correlations Between HGF and Cardiovascular Risk Factors in the MDC Cohort

	HGF	HGF/TRAIL Receptor-2 Ratio	HGF/Caspase-8 Ratio
Age	0.23[a]	−0.22[a]	−0.11[a]
BMI	0.26[a]	−0.08[a]	−0.06[a]
Fasting glucose	0.26[a]	−0.09[a]	−0.06[a]
LDL	0.12[a]	−0.04[b]	−0.07[a]
HDL	−0.26[a]	0.15[a]	0.009[a]
Triglycerides	0.30[a]	−0.15[a]	−0.08[a]
Systolic BP	0.23[a]	−0.09[a]	−0.10[a]
Diastolic BP	0.15[a]	−0.03	−0.09[a]
hsCRP	0.29[a]	−0.18[a]	−0.09[a]

Threshold for significance following Bonferroni correction for multiple comparisons is 0.0016. [a]$P < 0.001$, [b]$P < 0.005$.

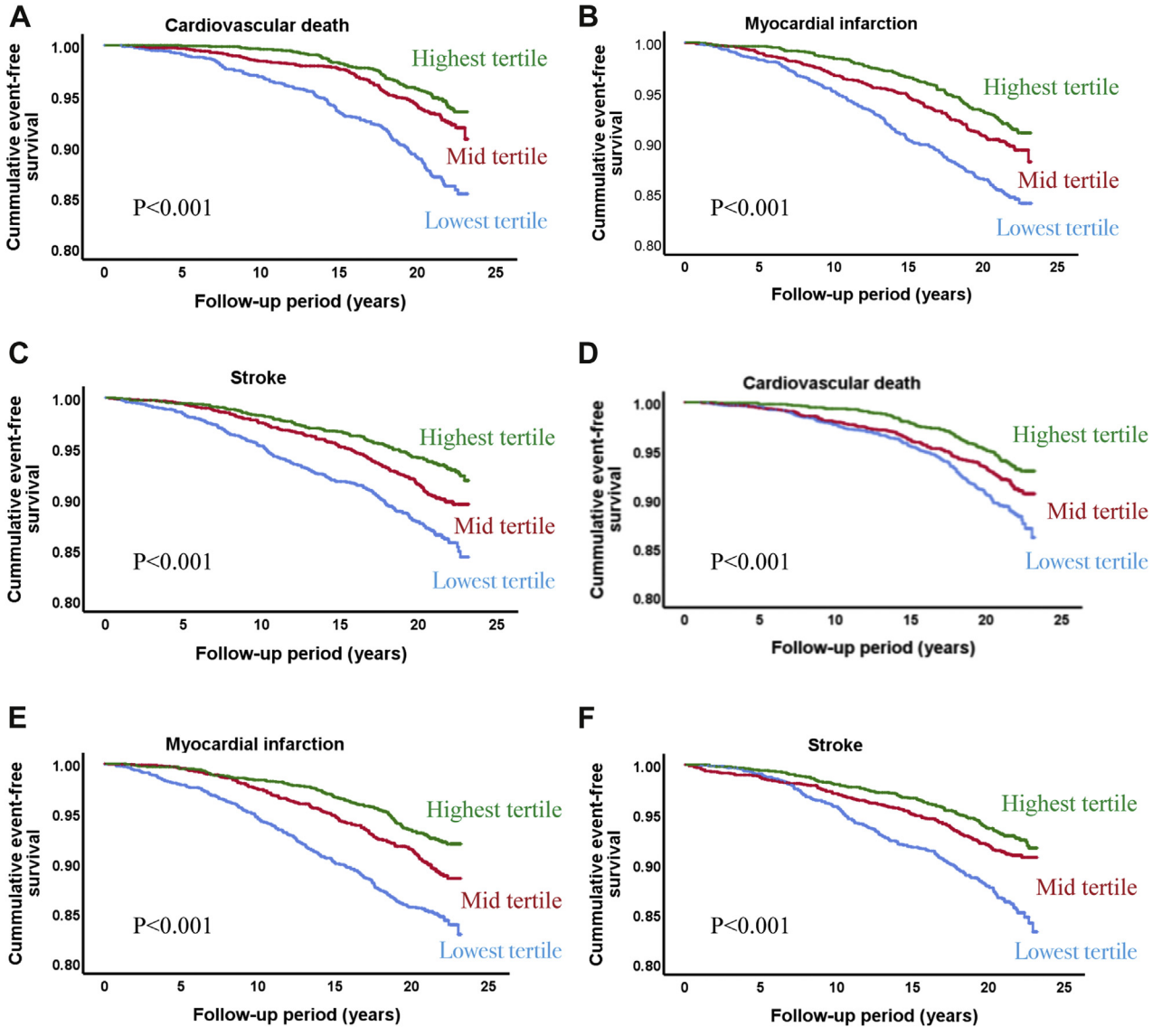
BMI = body mass index; BP = blood pressure; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand.

showed that HGF rescues both cell types from TRAIL-induced apoptosis in a dose-dependent manner (Supplemental Figures 9C and 9D).

The baseline characteristics of the MDC cohort are shown in Supplemental Table 2. All major cardiovascular risk factors were significantly over-represented in the group that died of cardiovascular disease during follow-up (n = 378), and this group had also significantly higher plasma levels of HGF. HGF correlated significantly with age, metabolic risk factors, blood pressure and high-sensitivity C-reactive protein (**Table 2**). HGF levels were also higher in male patients (n = 1,895; median 75.2 [IQR: 61.0-91.4] AU vs 69.3 [IQR: 57.2-84.1] AU in women; $P < 0.001$), current smokers (n = 1,020; median 78.2 [IQR: 64.1-97.1] AU vs 69.6 [IQR: 57.4-85.0] AU in nonsmokers; $P < 0.001$), and in subjects with diabetes (n = 363; median 88.6 [IQR: 71.2-109.6] AU vs 70.2 [IQR: 57.8-85.5] AU in subjects without diabetes; $P < 0.001$). The HGF/sTRAIL receptor-2 and HGF/caspase-8 ratios, in contrast, showed significant inverse associations with cardiovascular risk factors (Supplemental Table 3). To further analyze the factors associated with HGF/sTRAIL receptor-2 and HGF/caspase-8 ratios, we used linear regression models with age, sex, diabetes, current smoking, body mass index, LDL and HDL cholesterol, fasting glucose, triglycerides, and systolic and diastolic blood pressure as independent variables. A low HGF/sTRAIL receptor-2 ratio demonstrated independent association with age (β coefficient = −0.044; $P = 0.006$), male sex (β coefficient = −0.058; $P = 0.003$), and smoking (β coefficient = −0.035; $P = 0.018$). A low HGF/caspase-8 ratio was independently associated with age (β coefficient = −0.06; $P < 0.001$), fasting glucose (β coefficient = −0.049; $P = 0.013$), and systolic blood pressure (β coefficient = −0.047; $P = 0.034$).

We next divided HGF and the HGF/TRAIL receptor-2 and HGF/caspase-8 ratios into tertiles and plotted Kaplan-Meier curves. Subjects in the highest (third) tertile of plasma HGF had a 2.56-fold higher risk of cardiovascular death ($P < 0.001$), 2.28-fold higher risk of myocardial infarction ($P < 0.001$), and a 2.43-fold higher risk of stroke ($P < 0.001$) as compared to those in the lowest tertile (**Figures 4C to 4E, Table 3**). However, when adjusting for age, sex, current smoking, diabetes, LDL, HDL, triglycerides, and systolic blood pressure in Cox proportional hazard regression models only the association with stroke remained statistically significant (**Table 3**). Taking into account the competing risk of all-cause death, these results remained largely unchanged (Supplemental Table 4). In contrast, subjects in the highest tertiles of the HGF/TRAIL receptor-2 (**Figures 5A to 5C**) and HGF/caspase-8 ratios (**Figures 5D to 5F**) had lower risks of cardiovascular death (HR: 0.39 and HR: 0.54, respectively; $P_{trend} < 0.001$ for both), myocardial infarction (HR: 0.01 and HR: 0.45, respectively; $P_{trend} < 0.001$ for both), and stroke (HR: 0.47 and HR: 0.49, respectively; $P < 0.001$ for both) (**Figure 5, Table 3**). When adjusting for the factors listed, the HGF/TRAIL receptor-2 ratio remained significantly associated with a lower risk of cardiovascular death

FIGURE 5 Associations of Baseline Plasma HGF/TRAIL Receptor-2 and HGF/Caspase-8 Ratios With Risk of Cardiovascular Events



Kaplan-Meier plots show the association between tertiles of hepatocyte growth factor (HGF)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-2 ratio and **(A)** cardiovascular death, **(B)** myocardial infarction, and **(C)** stroke, and the association between tertiles of HGF/caspase-8 ratio and **(D)** cardiovascular death, **(E)** myocardial infarction, and **(F)** stroke during a mean follow-up period of 19.4 ± 5.0 years. Log-rank test was performed to calculate *P* values.

(P_{trend} = 0.012), myocardial infarction (P_{trend} = 0.017), and stroke (P_{trend} = 0.027); however, when considering the competing risk of all-cause death, sub-HRs were significant for only cardiovascular death (P_{trend} = 0.037) (Supplemental Table 4). HGF/caspase-8 ratio was associated with a lower risk of myocardial infarction and stroke (P_{trend} <0.001 for both) (**Table 3**); however, when running competing risks analyses, sub-HRs were also significant for cardiovascular death (P_{trend} = 0.037) while remaining significant for myocardial infarction and stroke (Supplemental Table 4).

ASSOCIATIONS OF HGF, HGF/TRAIL RECEPTOR-2, AND HGF/caspase-8 RATIOS WITH SEVERITY OF CORONARY AND CAROTID ARTERY DISEASE. To investigate how HGF reflects subclinical coronary disease, we analyzed baseline plasma samples from the PROCEED study.[24] The clinical characteristics of the PROCEED cohort, which included type 2 diabetes

patients free of clinical manifestations of coronary artery disease, are shown in Supplemental Table 5. Plasma HGF correlated with the total coronary calcium score and total number of plaques (Supplemental Figures 10A and 10B), whereas the HGF/TRAIL receptor-2 ratio showed inverse correlation to both angiographic variables (Supplemental Table 6). There were no significant correlations between the HGF/caspase-8 ratio and the angiographic variables. The associations between HGF and the total coronary calcium score and total number of plaques remained significant when adjusting for age, sex, body mass index, current smoking, glycosylated hemoglobin (or HbA_{1c}), LDL and HDL cholesterol, triglycerides, and systolic blood pressure in partial correlation analyses ($r = 0.18$ and 0.17; $P < 0.05$ for all). There was also a correlation between plasma HGF and the pericardial fat volume ($r = 0.41$; $P < 0.001$) (Supplemental Figure 10C), which remained significant ($r = 0.15$; $P < 0.05$) when adjusting for the same factors as discussed earlier in partial correlation analysis. Finally, we determined the associations of HGF, HGF/TRAIL receptor-2, and HGF/caspase-8 ratios with the severity of carotid artery disease as assessed by the carotid intima-media thickness at the MDC baseline investigation. HGF showed a positive correlation with the intima-media thickness in both common carotid artery and the bulb, while the HGF/TRAIL receptor-2 and HGF/caspase-8 ratios correlated negatively (Supplemental Table 6). When adjusting for the same factors as previously discussed in partial correlation analysis, only the associations of HGF with common carotid artery mean and maximum intima-media thickness remained significant ($r = 0.07$; $P < 0.001$; and r = 0.050; $P = 0.002$, respectively).

TABLE 3 Soluble Death Receptor Tertiles and Risk for Cardiovascular Mortality, MI, and Ischemic Stroke

	HR by Tertiles			
	1st	2nd	3rd	P_{trend}
Cardiovascular Death				
HGF				
Model 1, n = 378	1	1.38 (1.04-1.84)	2.56 (1.98-3.32)	<0.001
Model 2, n = 378	1	1.13 (0.85-1.51)	1.66 (1,28-2.16)	<0.001
Model 3, n = 375	1	0.97 (0.73-1.30)	1.12 (0.85-1.49)	0.354
HGF/TRAILR-2				
Model 1, n = 378	1	0.51 (0.41-0.65)	0.39 (0.30-0.51)	<0.001
Model 2, n = 378	1	0.64 (0.51-0.82)	0.60 (0.46-0.78)	<0.001
Model 3, n = 375	1	0.73 (0.57-0.94)	0.73 (0.56-0.96)	0.012
HGF/caspase-8				
Model 1, n = 373	1	0.74 (0.58-0.93)	0.54 (0.42-0.70)	<0.001
Model 2, n = 373	1	0.89 (0.70-1.12)	0.74 (0.57-0.95)	0.021
Model 3, n = 370	1	0.90 (0.71-1.15)	0.79 (0.61-1.02)	0.069
Myocardial Infarction				
HGF				
Model 1, n = 480	1	1.47 (1.15-1.88)	2.28 (1.81-2.87)	<0.001
Model 2, n = 480	1	1.30 (1.01-1.66)	1.65 (1.31-2.09)	<0.001
Model 3, n = 477	1	1.12 (0.88-1.44)	1.15 (0.90-1.48)	0.284
HGF/TRAILR-2				
Model 1, n = 480	1	0.64 (0.52-0.79)	0.50 (0.40-0.63)	<0.001
Model 2, n = 480	1	0.73 (0.59-0.90)	0.66 (0.52-0.83)	0.001
Model 3, n = 477	1	0.80 (0.65-1.00)	0.76 (0.60-0.96)	0.017
HGF/caspase-8				
Model 1, n = 476	1	0.66 (0.53-0.81)	0.45 (0.36-0.57)	<0.001
Model 2, n = 476	1	0.75 (0.61-0.92)	0.58 (0.46-0.73)	<0.001
Model 3, n = 473	1	0.75 (0.61-0.93)	0.61 (0.48-0.77)	<0.001
Stroke				
HGF				
Model 1, n = 440	1	1.41 (1.09-1.82)	2.43 (1.91-3.08)	<0.001
Model 2, n = 440	1	1.22 (0.94-1.59)	1.79 (1,40-2.28)	<0.001
Model 3, n = 437	1	1.07 (0.83-1.40)	1.34 (1.04-1.74)	0.018
HGF/TRAILR-2				
Model 1, n = 440	1	0.68 (0.55-0.85)	0.47 (0.37-0.59)	<0.001
Model 2, n = 440	1	0.79 (0.64-0.99)	0.63 (0.49-0.80)	0.001
Model 3, n = 437	1	0.88 (0.71-1.10)	0.76 (0.59-0.97)	0.027
HGF/caspase-8				
Model 1, n = 434	1	0.61 (0.48-0.76)	0.49 (0.39-0.62)	<0.001
Model 2, n = 434	1	0.68 (0.54-0.85)	0.60 (0.47-0.76)	<0.001
Model 3, n = 431	1	0.69 (0.55-0.87)	0.64 (0.50-0,81)	<0.001

Values are HR (95% CI) unless otherwise indicated. Soluble death receptor tertile HRs and 95% CIs for incident cardiovascular mortality, MI, and ischemic stroke were calculated using Cox regression models. Model 1 was unadjusted; model 2 was adjusted for age and sex; and model 3 was adjusted for age, sex, current smoking, diabetes, LDL, HDL, triglycerides, and systolic BP.

TRAILR-2 = tumor necrosis factor-related apoptosis-inducing ligand receptor-2; other abbreviations as in Tables 1 and 2.

DISCUSSION

In the present study we show the following. 1) Autocrine and/or paracrine HGF stimulation plays an important role in protecting human vascular ECs and SMCs from apoptotic cell death. Autocrine and/or paracrine HGF stimulation is also important for the ability of these cells to activate repair responses including cell migration and proliferation. Interestingly, HUVECs demonstrated a clearer dose response to HGF in the migration assay (in vitro scratch injury) than in assays also reflecting cell proliferation, possibly indicating the involvement of different signal pathways. 2) The ability of SMCs to secrete HGF is substantially greater than that of ECs but both cell types can respond to autocrine and/or paracrine HGF stimulation. 3) Autocrine and/or paracrine HGF stimulation is important for the ability of ECs to express other endothelial mitogens such as VEGF-A and PlGF, suggesting that HGF, autocrine and/or paracrine, is a key regulator of endothelial repair. 4) HGF activates the expression of collagen genes in human arterial SMCs, and human plaques with a high HGF content are enriched in collagen and elastin. The HGF content in plaques that have caused an acute

cerebrovascular event is lower than in plaques that have remained asymptomatic. 5) Both ECs and SMCs respond with increased HGF expression in response to stress. These observations imply that HGF expressed by stressed vascular cells plays a role in protection against cell death, promotion of tissue repair processes, and maintaining plaque stability. The ability of HGF to stimulate growth and migration of ECs and SMCs has been shown before, but the observation that in human ECs and SMCs this largely is dependent on autocrine and/or paracrine stimulation is novel.

An important question is whether the association between high levels of HGF in circulation and an increased risk of cardiovascular events can be consistent with a cardiovascular protective role of HGF. One possible explanation to this apparent paradox could be that elevation of circulating HGF levels occurs because of release of HGF from stressed vascular cells activating repair responses. In this case, the association between HGF and cardiovascular risk would be explained by the factors that cause vascular damage rather than by a pathogenic role of HGF. The observations that plasma HGF correlates with presence of cardiovascular risk factors known to cause vascular injury and that most of the associations of HGF with risk of myocardial infarction, stroke, and cardiovascular death are lost when controlling for these risk factors is well in line with this possibility. Moreover, plasma HGF correlated strongly with the plasma levels of sTRAIL receptor -2 and caspase-8. The former is released from cells that have received a signal to initiate apoptotic cell death through activation of so-called death receptors on the cell surface.[26] Caspase-8 is an intracellular mediator of death receptor–activated cells, but it is not released from the cells until they start to disintegrate. Both sTRAIL receptor-2 and caspase-8 thus serve as markers of cellular stress, but caspase-8 is likely to represent a marker of a more advanced stage of cell injury than the sTRAIL receptor-2 is. Against this background, we postulated that a weak ability to respond to cellular stress with increased expression of HGF results in an impaired tissue repair response and that in the vasculature this may lead to an increased risk of acute cardiovascular events. In the present study we found that subjects with a low HGF/caspace-8 ratio had increased risk of myocardial infarction, stroke, and cardiovascular death. Moreover, these associations remained statistically significant when controlling for major cardiovascular risk factors and death by other causes. A low ability to respond to cellular stress with increased HGF expression as assessed by the HGF/sTRAIL receptor-2 was also associated with an increased risk of cardiovascular events, but this association only remained significant for cardiovascular death when controlling for major cardiovascular risk factors and death by other causes. It was also associated with more severe coronary and carotid artery disease. Collectively, these observations suggest that a low ability to respond with increased expression of HGF during conditions with systemic cellular stress is associated with an increased risk for development of atherosclerosis, plaque vulnerability, and acute cardiovascular events. It will be important to identify the factors responsible for a decreased ability to express HGF in response to cellular stress. The present observations suggest that it may be the result of high age and exposure to cardiovascular risk factors causing an exhaustion of vascular repair responses.

The idea that HGF has a protective function in the cardiovascular system is not new, but the focus has been on protection of ischemic myocardium and angiogenesis in ischemic tissues rather than on arterial repair.[27] Cardiomyocytes express HGF in response to ischemia/reperfusion,[28,29] and HGF becomes elevated within a few hours in patients with acute myocardial infarction.[30] HGF protects cardiomyocytes from apoptosis in an autocrine or paracrine manner by blocking apoptotic signal pathways[31] as well as the excessive autophagy that can be activated by oxygen radicals.[32] The angiogenic properties of HGF are also considered to be of importance for the formation of new vessels in ischemic tissues.[27,33] The question of whether the protective effects of HGF on the cardiovascular system also extend to reducing the risk for development of myocardial infarction and stroke has gained less attention. Using a similar in vitro scratch injury as applied in our study, McKinnon et al[34] showed that rat SMCs that migrated into the wound began to express c-Met and responded to HGF with increased migration. HGF was also shown to promote carotid re-endothelization after a superficial injury to the endothelial layer of rat carotids.[34] Although these findings suggested a role of HGF in arterial repair, this notion was questioned by epidemiological studies reporting that high circulating levels of HGF are associated with more severe atherosclerosis and an increased risk of ischemic cardiovascular events.[17,18,35,36] This association was confirmed also in our study, but we also present evidence that this association represents activation of a repair response to systemic organ stress and that a weak ability to mount such a response increases the risk of cardiovascular events. It could also be argued that the antifibrotic effects of HGF, which are mediated by inhibition of TGF-β[14] and stimulation of MMP expression,[15] could contribute to

plaque vulnerability. However, HGF in atherosclerotic plaques correlated strongly with the plaque content of both collagen and elastin contents. Plaque HGF also showed a stronger correlation to the plaque content of MMP inhibitor tissue inhibitor of metalloproteinases 2, than to MMP-2 and MMP-3. Moreover, HGF was found to stimulate the expression of collagen genes in HCASMCs in an autocrine manner and/or a paracrine manner. These observations do not support an antifibrotic function of HGF in human atherosclerotic plaques, but rather they are in line with a profibrotic plaque-stabilizing effect. Another possibility could be that the angiogenic effects of HGF could contribute to in-growth of vessels from the adventitia increasing the risk for intraplaque hemorrhage. However, as there was no correlation between the plaque levels of HGF and the red blood cell marker glycophorin A this seems less likely.

STUDY LIMITATIONS. The analyses of biomarkers in the MDC study were based on baseline levels only, and we have no information on variation in levels during the follow-up period. The clinical biomarker data only provide information about associations and do not provide evidence of causality. However, a strength of the present study is the use of experimental models that can help to understand the pathophysiological processes behind these associations. The clinical validity of the biomarkers used to assess cellular stress (eg, circulating sTRAIL receptor-2 and caspase-8) remains to be confirmed. Cell culture studies have shown that they are released from cells following activation of apoptosis, but it remains unknown whether other processes may also be involved in their release into the circulation. We did not observe any association between treatment with different medications and the plaque content of HGF in our study. Because statin treatment is known to increase the viability carotid plaque cells,[7] it could be anticipated that statins would be associated with less vascular cell stress and hence a lower protective HGF response. However, because 87.5% of the patients in our cohort were treated with statins it is likely that the present study did not have sufficient power to identify such an effect if one exists.

CONCLUSIONS

The present study provides evidence for a protective role of HGF in CVD and shows that subjects with a low ability to express HGF in response to cellular stress have an increased risk for development of cardiovascular events. Therapies that mimic HGF or stimulate the expression of HGF may have the potential to provide additional protection to subjects with residual risk on current preventive treatments.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Most acute ischemic cardiovascular events are caused by destabilization of atherosclerotic plaques. The integrity of these plaques depends on the balance between the injury caused by risk factors and the ability of the vascular wall to repair this injury. Patients receiving state-of-the-art risk factor intervention remain at an increased risk of recurrent events. The present study shows that this residual risk may be caused by a reduced ability of the vascular wall to express repair factors such as HGF.

TRANSITIONAL OUTLOOK: Identification of novel targets for intervention is needed to further reduce the risk of recurrent events in subjects with ischemic CVD. One possibility that has gained considerable attention during recent years is anti-inflammatory therapy, but increased risk of infections remains a limitation with this approach. The present observations suggest that stimulating the expression of and/or mimicking the effects of vascular repair factors such as HGF represent an alternative approach.

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APPENDIX For supplemental Methods, figures, and tables, please see the online version of this paper.