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Elevated Hemolysis Index is associated with higher risk of cardiovascular diseases

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Abstract

Objectives: *In vivo* hemolysis is associated with thromboembolism. Although an increased Hemolysis Index (HI) can be due to *in vitro* as well as *in vivo* hemolysis, both reflects a more fragile erythrocyte population. We therefore hypothesized that HI above upper reference limit would be associated with an increased risk of cardiovascular disease (CVD).

Methods: We identified persons with two elevated HI (HI⁺) from blood samples analyzed at a university hospital laboratory from 2012 to 2017. We compared their risk of CVD with the risk in matched comparators with normal HI and from the general population. HI⁺ persons and comparators were followed from start date (date of the second elevated HI) until the first of the main outcome: CVD, emigration, death, or end of observation time on December 31, 2018.

Results: In 43,102 unique HI⁺ persons, the risk of developing CVD was 40% higher compared with the general population and 13% higher compared with the matched blood sample cohort. HI⁺ was associated with a significantly increased cumulative incidence of both arterial and venous CVD compared with the matched blood sample cohort and the general population (respectively 47 and 14% for arterial CVD; 78 and 24% for venous CVD). Moreover, overall mortality risk was significantly higher in patients with HI⁺ than in the two comparator groups.

Conclusions: Elevated HI is associated with increased risk of arterial and venous CVD and with increased mortality.

Our findings imply that HI may contribute as a CVD risk biomarker.

Keywords: cardiovascular disease; hemolysis; Hemolysis Index.

Introduction

Hemolysis, *in vivo* as well as *in vitro*, may affect a wide range of biochemical analyses [1]. Hemolysis Index (HI) is a measurement performed automatically in most laboratories to assure that hemolysis of the sample does not interfere with the requested laboratory analyses [2]. Measurement of HI is an objective and reproducible way to estimate interference from cell-free hemoglobin [3, 4]. Hemolysis and cell-free hemoglobin can occur *in vitro* due to pre-analytical circumstances during blood sampling or transportation. *In vivo*, cell-free hemoglobin can be induced by several conditions and has been related to different clinical outcomes, e.g., cardiovascular disease (CVD) [5].

Atherosclerosis is the underlying pathology of CVD within the arterial vessels, which include myocardial infarction, stroke, and peripheral arterial disease [6]. Complications of atherosclerosis, particularly luminal thrombosis triggered by rupture of atherosclerotic lesions, leads to reduced perfusion and are the most common cause of cardiovascular ischemic events [7, 8]. Plaque neovascularization and intraplaque hemorrhage may cause plaque progression, rupture, and hemolysis within the plaque [9–12].

Inflammation has a fundamental role in atherosclerosis, where it orchestrates all the different stages [8, 13]. Several endogenous molecules, e.g., heme, the prosthetic group of hemoglobin, can activate cellular receptors leading to inflammation. Heme has been demonstrated to trigger LDL oxidation and amplify oxidant-mediated endothelial damage [14]. Also, cell-free hemoglobin, seen in the presence of hemolysis or hemorrhagic episodes, is found associated with inflammation and atherosclerosis, primarily explained by a pro-inflammatory cytokine response by free heme [9].

Along with the involvement in inflammation and atherosclerosis, hemolysis and high levels of cell-free hemoglobin have been shown to induce platelet activation and

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apoptosis in a concentration-dependent way [15]. The effect of cell-free hemoglobin on platelets are mainly explained by the scavenging and redox of nitric oxide (NO), which plays an important role in relaxation of smooth musculature. NO scavenging (e.g., by cell-free hemoglobin) results in systemic vasoconstriction and increased blood stasis, thereby affecting one of the components in Virchow's triad used to describe the etiology of thrombosis, especially of venous origin [5].

We therefore hypothesized that a HI above upper reference limit (as defined in reference [17], reflecting fragility of the erythrocytes due to an undiscovered condition associated with hemolysis, could be associated with the risk of vasoconstriction and arterial CVD, and increased blood stasis and venous CVD. This association should not depend on whether hemolysis happened *in vivo* or *in vitro* as both reflects a more fragile erythrocyte population.

Materials and methods

We identified persons with an elevated HI from all persons with a blood sample analyzed on chemistry or immunochemistry equipment (laboratory-derived population) to examine their risk of CVD compared with the risk in matched persons with normal HI from the same laboratory-derived population and matched persons from the general population. All persons in Denmark are assigned a unique and permanent civil registration number (CRN) [16], used to identify persons with elevated HI and comparators, which allows individual linkage across all public registers.

Study population

Overall, the laboratory-derived population consisted of all persons subjected to blood sampling for chemistry and immunochemistry analyses, including the corresponding HI results, at Department of Clinical Biochemistry, Odense University Hospital, Denmark, during the period February 2012 through December 2017 ($n=3,125,336$, Figure 1). Blood sample analyses were requested both from hospital and community-based doctors. The number of unique persons in this laboratory-derived population was 373,126.

Hemolysis Index measurement

Blood sample analyses were performed using an Architect c8000/c16000 clinical chemistry equipment (Abbott Diagnostics, Chicago, IL). HI, a validated analysis in our laboratory [17], was measured at eight wavelengths, more specifically four specific wavelengths pairs (primary/secondary) covering the whole hemoglobin absorbance spectrum. Further analytical specifications are detailed in the method section in Supplementary Material.

HI⁺ cohort and comparator cohorts

HI⁺ cohort: An elevated HI result was considered as the exposure and all persons with twice elevated HI (HI⁺) within the laboratory-derived cohort was identified. A HI result was considered elevated when above the upper reference limit, namely 0.01–0.16 g/L [17]. We considered a single elevated HI could be due to analytical variation around the upper limit of the reference interval as we did not have any minimum increase of HI above the reference interval as an inclusion criterion. Therefore, we considered that two consecutive elevated HI were more indicative of hemolysis and not only a result of analytical variation. A total of 43,102 unique persons

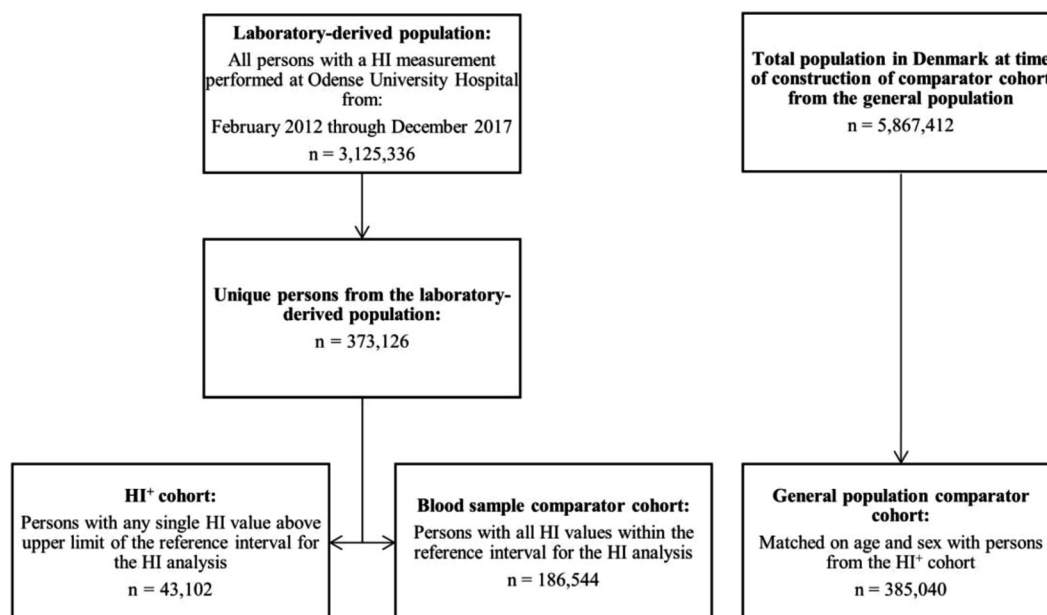


Figure 1: The construction of the three cohorts in a study of cardiovascular disease risk among persons with an elevated Hemolysis Index (HI⁺ cohort) in blood samples analyzed on chemistry equipment and age-sex matched comparators subjected for blood sampling (blood sample comparator cohort) and from the general population (general population comparator cohort).

were identified (Figure 1) and included in the study population at the date of the second elevated HI result. The date of the second elevated HI marked the start of follow up (start date) for HI⁺ persons.

Comparator cohorts: Using the CRN, we constructed the two comparator cohorts: a blood sample comparator cohort and a general population comparator cohort. First, the blood sample comparator cohort was defined by matching each HI⁺ person on the date of inclusion (± 1 month) on age (year of birth) and sex with five persons from the laboratory cohort. Persons were eligible as blood sample comparators if all their measured HI had been normal at the start date. Persons in the blood sample cohort could later enter the HI⁺ cohort if HI became elevated and would then be censored as comparators. Second, a general population comparator cohort was constructed by matching each HI⁺ person on age (year of birth) and sex with 10 persons from the general population identified in the civil registration system (CRS, described below). The general population consists of all residents in Denmark at the time of construction of this comparator cohort ($n=5,867,412$). Comparators from the general population had to be alive and residing in Denmark at the time of matching. Both comparator cohorts were matched with the HI⁺ persons on start date and assigned this date as start of follow-up.

Data sources

The civil registration system (CRS) and the Danish national patient registry (DNPR): Data from the CRS [16] and the DNPR [18] were used for this study. The CRS contains personal identification numbers (CRN) of all persons living in Denmark since 1968 as well as information on sex, date of birth, and dates of emigrations and of death [18]. The DNPR has collected routine data such as CRN and dates of admission and discharge from all Danish hospitals since 1977. Diagnoses are registered by physicians as the primary diagnosis that led to hospital contact, and if relevant secondary diagnoses. Diagnoses are categorized based on the International Classification of Disease (ICD) eighth revision (ICD 8) 1977–1993 and tenth revision (ICD 10) thereafter [18]. During 1977–1993, only in-hospital admissions were registered, while from 1994 also out-patient specialist clinic and emergency room visits were included.

Outcomes of CVD and follow-up

HI⁺ persons and comparators were followed from start date until the first of the main outcome: CVD, emigration, death, or end of observation time on December 31, 2018. We identified all defined CVDs in each of the three cohorts in the DNPR and defined CVDs as registrations of acute myocardial infarction (AMI), overall stroke, ischemic stroke, peripheral arterial disease in lower extremities (PAD), venous thromboembolism (VTE), or splanchnic vein thrombosis (SVT). We disregarded all patients with CVD before start date. ICD diagnosis codes for inclusion and exclusion are available in Supplementary Table 1.

Comorbidity

Information on comorbidity as registered in DNPR before and up to start date were aggregated using the Charlson Comorbidity Index. The included comorbid conditions and their ICD diagnosis codes are available in Supplementary Table 2.

Statistical analyses

All data management and statistical analyses were performed using Stata 16.1 [19]. We performed basic description of the HI⁺ cohort and comparator cohorts including proportion of women, mean age at start date (i.e., age at the second elevated HI), numbers of included patients and persons in following periods: 2012–2013, 2014–2015 and 2016–2017, and proportion of comorbidities. Estimates were provided with 95% confidence intervals (CI). The proportion of children under 10 years of age in each cohort is calculated to discuss a potential limitation of many pediatric samples. Raw data of HI readings are presented in box plots for possible members of HI⁺ cohort and the blood sample comparator cohort. The box plots are presented with the median value, interquartile ranges, lowest value, and highest value. To test the concept of fragile erythrocytes as the reason for an elevated HI, we presented data on the time between the first and the second elevated HI for possible members of the HI⁺ cohort. These data are presented with median days between the two measurements, interquartile range and the most frequent time difference between two increased HI measurements.

Cumulative incidence of first event of overall CVD as well as isolated arterial or venous CVD were estimated for HI⁺ persons and comparators in competing risk settings. All cumulative incidences were estimated as cumulative incidence proportions at 1 and 5 years after inclusion. Competing events were death and loss to follow-up by emigration.

Semi-parametric Fine-Gray proportional subhazard ratios (subHR) were estimated for the overall cumulative incidences of CVD, and isolated arterial and venous CVD events using the blood sample comparator cohort as reference. To assist the interpretation of subHR we estimated cause-specific hazard ratios (csHR). Death and emigration were treated as competing events when estimating subHR and censoring events for csHR. All subHR and csHR were estimated unadjusted and subsequently adjusted for inclusion date, sex, age, and comorbidities, see Supplementary Table 2.

Overall survival was assessed with the Kaplan-Meier estimator and unadjusted cox proportional hazard ratios (HR) subsequently adjusted for inclusion date, sex, age, and comorbidities, see Supplementary Table 2.

Results

Description of the HI reading values

Raw data of HI readings for the HI⁺ cohort and the blood sample comparator cohort are shown in box plots in Supplementary Figure 1. For the HI⁺ cohort, the HI values are: Median value 0.3 g/L with interquartile range of 0.2–0.5 g/L. The lowest value of 0.2 g/L, and the highest value of 111.3 g/L. For the blood sample comparator cohort, the median value is 0.1 g/L with interquartile range of 0.0–0.1 g/L. The lowest value of 0.0 g/L, and the highest value of 0.2 g/L. The days between the first and second increased HI value for the possible members of the HI⁺ cohort are shown in Supplementary Figure 2. The median value is 191 days with interquartile range of 29–539 days. The most frequent value is 1 day between the measurement in 3,678 (7.6%) of the cases.

Characteristics of the three cohorts

Construction of the three cohorts is depicted in Figure 1. During the study period, 43,102 unique persons with HI⁺ (11.5% of members of the laboratory population), 186,544 blood sample comparators (50% of persons in the laboratory population), and 385,040 general population comparators (6.5% of the total population in Denmark) were included. Results of HI were available for the entire laboratory population and no persons were excluded. Basic description of the three populations is listed in Table 1. The mean ages were: HI⁺ cohort 52.8 years (interquartile range (IQR) 52.5; 53.0 years), blood sample comparators 51.6 years (IQR 51.4; 51.7 years) and general population cohort 51.6 years (IQR 51.6; 51.7 years). The percentage of women in the three groups were 49.5% (95% CI: 49.1; 50.0), 50.3% (95% CI: 50.1; 50.5), and 51.0% (95% CI: 50.8; 51.1), respectively. The proportion

of children under 10 years of age were: Amongst HI⁺ cohort: 10.67% (95% CI: 10.38–10.97), amongst blood sample comparators: 10.65% (95% CI: 10.51–10.79) and amongst the general population comparators: 11.74% (95% CI: 11.63–11.84).

Overall, a larger proportion of the HI⁺ cohort had comorbidity at start date than comparators. Subtyping comorbidity showed that this applied to all diseases except connective tissue disease, renal disease, and metastatic cancer disease, which were more frequent in the matched blood sample cohort (Table 1).

Risk of overall CVD

The cumulative incidence of CVD in the HI⁺ cohort remained higher during the study period than for the two

Table 1: Basic description of the populations at inclusion date in a study of cardiovascular disease risk among persons with an elevated Hemolysis Index (HI⁺) in blood samples analyzed on immunochemistry equipment and age-sex matched comparators subjected to blood sampling and from the general population.

	HI ⁺ cohort n=43,102	Blood sample comparator cohort n=186,544	General population comparator cohort n=385,040
Women, % [95% confidence interval]	49.5 [49.1; 50.0]	50.3 [50.1; 50.5]	51.0 [50.8; 51.1]
Age at time of positive HI, years (mean interquartile range)	52.8 (52.5; 53.0)	51.6 (51.5; 51.7)	51.6 (51.6; 51.7)
Numbers of included			
2012–2013, n/%	13.607/31.6	58.240/31.2	122.321/31.8
2014–2015, n/%	17.421/40.4	75.697/40.6	155.611/40.4
2016–2017, n/%	12.074/28.0	52.607/28.2	107.108/27.8
Comorbidity, % [95% confidence interval]			
Any comorbidity	57.29 [56.82; 57.75]	48.94 [48.72; 49.17]	29.66 [29.52; 29.81]
HIV infection	1.00 [0.91; 1.10]	0.21 [0.19; 0.23]	0.08 [0.07; 0.09]
Antiphospholipid syndrome	0.01 [0.00; 0.03]	0.01 [0.00; 0.01]	0.00 [0.00; 0.00]
Atrial fibrillation	8.92 [8.65; 9.19]	7.30 [7.18; 7.42]	4.64 [4.57; 4.70]
Chronic pulmonary disease	13.00 [12.69; 13.33]	10.69 [10.55; 10.83]	6.69 [6.62; 6.77]
Coagulopathy	0.68 [0.60; 0.76]	0.50 [0.47; 0.54]	0.18 [0.17; 0.19]
Congestive heart failure	5.47 [5.26; 5.69]	4.41 [4.32; 4.50]	2.30 [2.25; 2.35]
Connective tissue disease	5.67 [5.45; 5.89]	6.28 [6.17; 6.39]	2.58 [2.53; 2.63]
Dementia	2.58 [2.43; 2.73]	1.57 [1.52; 1.63]	1.42 [1.39; 1.46]
Diabetes	13.52 [13.20; 13.85]	9.78 [9.64; 9.91]	4.57 [4.51; 4.64]
Dyslipidemia	9.77 [9.49; 10.05]	7.52 [7.40; 7.64]	5.03 [4.96; 5.10]
Hemiplegia	0.91 [0.82; 1.00]	0.65 [0.61; 0.68]	0.29 [0.27; 0.31]
Hypertension	25.73 [25.32; 26.15]	21.78 [21.59; 21.97]	13.45 [13.34; 13.56]
Ischemic heart disease	11.82 [11.51; 12.12]	10.35 [10.21; 10.49]	6.96 [6.88; 7.04]
Liver disease	2.38 [2.24; 2.53]	1.69 [1.63; 1.75]	0.60 [0.58; 0.63]
Renal disease	5.07 [4.87; 5.28]	5.19 [5.09; 5.29]	1.54 [1.51; 1.58]
Obesity	10.95 [10.66; 11.25]	8.48 [8.36; 8.61]	4.37 [4.30; 4.43]
Metastasis	0.28 [0.23; 0.33]	0.31 [0.28; 0.34]	0.07 [0.06; 0.08]

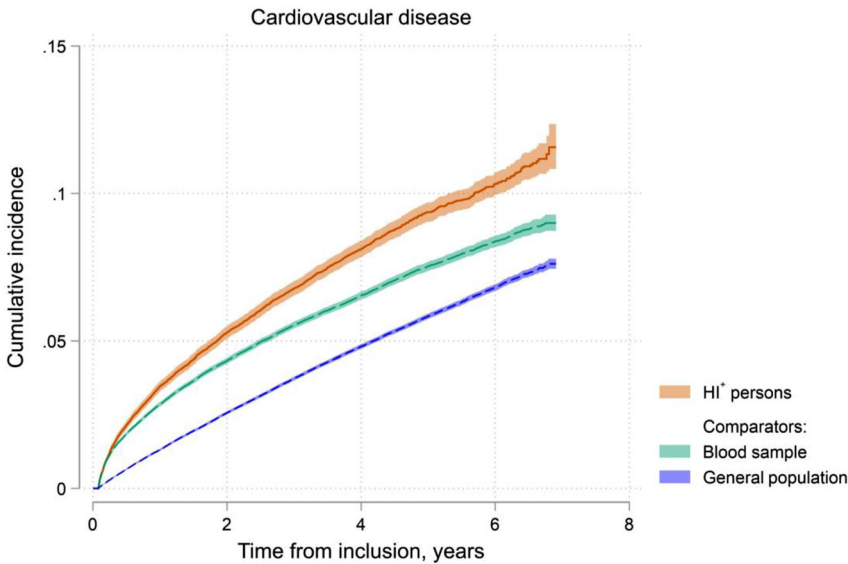


Figure 2: Cumulative incidences of cardiovascular disease in persons with elevated Hemolysis Index (HI⁺) in blood samples analyzed on chemistry equipment and age-sex matched persons from the blood sample comparator cohort and general population comparator cohort.

comparator groups (Figure 2). For the HI⁺ cohort, the cumulative incidence of CVD was 3.5% (95% CI: 3.3; 3.6) after one year and 9.4% (95% CI: 9.1; 9.7) after five years (Table 2). In comparison, the corresponding 1-year and 5-year cumulative incidences were 2.9% (95% CI: 2.8; 2.9) and 7.5% (95% CI: 7.4; 7.7) in the blood sample cohort and 1.3% (95% CI: 1.3; 1.4) and 5.8% (95% CI: 5.8; 6.0) amongst general population comparators (Table 2). The adjusted csHRs for CVD were 1.13 (95% CI: 1.08; 1.17) for HI⁺ cohort and 0.73 (95% CI: 0.70; 0.73) for general population both using the blood sample comparator as reference (Table 2). The adjusted subHRs and associated 95% CIs were 1.06 (1.02; 1.10) for HI⁺ cohort and 0.77 (0.76; 0.79) for general population both using the blood sample comparator as reference (Table 2).

Risk of arterial CVD

The cumulative incidence of arterial CVD in the HI⁺ cohort remained higher during the study period than for the two comparator groups (Supplementary Figure 3). For the HI⁺ cohort, the cumulative incidence of arterial CVD was 2.6% (95% CI: 2.4; 2.7) after one year and 7.1% (95% CI: 6.8; 7.4) after five years (Supplementary Table 3). The corresponding 1- and 5-years cumulative incidences were 2.1% (95% CI: 2.1; 2.2) and 5.6% (95% CI: 5.6; 6.0) among blood sample comparators and 1.0% (95% CI: 1.0; 1.1) and 4.5% (95% CI: 4.5; 4.6) amongst general population comparators. The adjusted csHRs for arterial CVD were: 1.11 (95% CI: 1.06; 1.16) for HI⁺ cohort and 0.75 (0.73; 0.77) for the general population using the blood

Table 2: Risk of cardiovascular disease in a study of cardiovascular disease risk among persons with an elevated Hemolysis Index (HI⁺) in blood samples analyzed on immunochemistry equipment and age-sex matched comparators subjected to blood sampling and from the general population.

	HI ⁺ cohort n=43,102	Blood sample comparator cohort n=186,544	General population comparator cohort n=385,040
Numbers with cardiovascular disease	3,479	10,517	18,833
Cumulative incidence, %			
One year	3.46 [3.29; 3.64]	2.86 [2.78; 2.94]	1.31 [1.27; 1.35]
Five years	9.37 [9.05; 9.69]	7.54 [7.38; 7.69]	5.84 [5.75; 5.93]
Cause-specific hazard ratio and sub hazard ratio			
csHR (95% CI)			
Unadjusted/adjusted	1.27 [1.22; 1.32]/1.13 [1.08; 1.17]	1.00 [.; .]/1.00 [.; .]	0.69 [0.67; 0.70]/0.73 [0.71; 0.74]
subHR (95% CI)			
Unadjusted/adjusted	1.22 [1.18; 1.27]/1.06 [1.02; 1.10]	1.00 [.; .]/1.00 [.; .]	0.72 [0.70; 0.73]/0.77 [0.76; 0.79]

csHR, cause-specific hazard ratio; subHR, subdistribution hazard ratio.

Table 3: Survival rates and risk of death in a study of cardiovascular disease risk among persons with an elevated Hemolysis Index (HI⁺) in blood samples analyzed on immunochemistry equipment and age-sex matched comparators subjected to blood sampling and from the general population.

	HI ⁺ cohort n=43,102	Blood sample comparator cohort n=186,544	General population comparator cohort n=385,040	p-Values
Survival rates, %				
One year	91.49 [91.22; 91.75]	94.20 [94.09; 94.31]	98.24 [98.20; 98.29]	<0.001
Five years	79.54 [79.09; 79.99]	85.93 [85.72; 86.13]	91.18 [91.07; 91.29]	<0.001
Hazard ratio of death [95% confidence interval]				
Unadjusted/adjusted	2.67 [2.61; 2.74]/2.24 [2.18; 2.29]	1.83 [1.80; 1.86]/1.81 [1.78; 1.84]	1.00 [.; .]/1.00 [.; .]	

sample comparator cohort as reference (Supplementary Table 3). With blood sample comparators as reference the adjusted subHRs and associated 95% CIs were 1.04 (1.00; 1.09) for HI⁺ cohort and 0.81 (0.78; 0.83) for the general population (Supplementary Table 3).

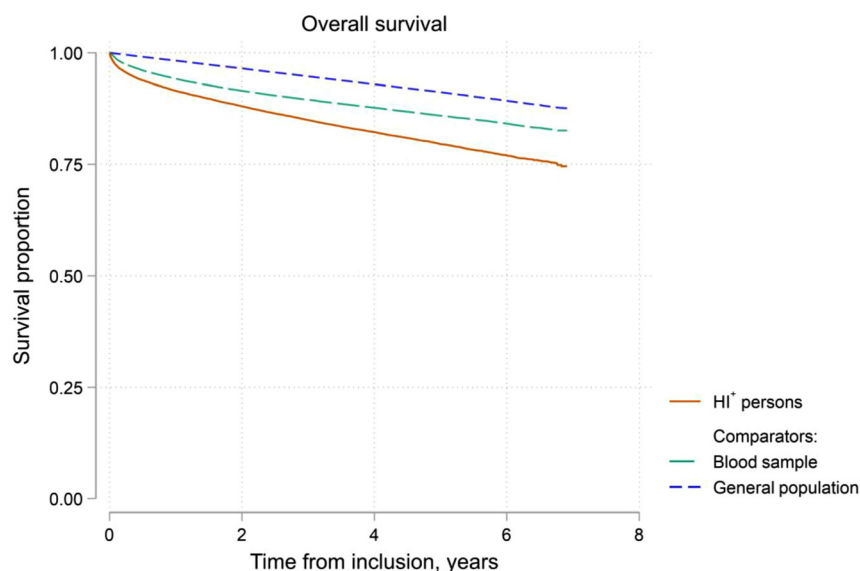
Risk of venous CVD

The cumulative incidence of venous CVD in the HI⁺ cohort remained higher during the study period than for the two comparator groups (Supplementary Figure 4). For HI⁺ cohort, the 1- and 5-years cumulative incidence of venous CVD was 1.0% (95% CI: 0.9; 1.0) and 2.6% (95% CI: 2.4; 2.8), respectively (Supplementary Table 4). The corresponding 1- and 5-years cumulative incidences were 0.8% (95% CI: 0.7; 0.9) and 2.1% (95% CI: 2.0; 2.2) among blood sample comparators and 0.3% (95% CI: 0.3; 0.3) and 1.5% (95% CI: 1.5; 1.5) amongst general population comparators. The adjusted cSHRs for venous CVD were: 1.15 (95% CI: 1.07; 1.24) for HI⁺

cohort and 0.65 (0.62; 0.68) for the general population, using the blood sample comparators as reference (Supplementary Table 4). The adjusted subHRs (95% CIs) were 1.09 (1.01; 1.17) for HI⁺ cohort and 0.69 (0.66; 0.72) for the general population using blood sample comparators as reference (Supplementary Table 4).

Survival

Survival was lowest among HI⁺ cohort compared with the other two groups with one-year survival in the HI⁺ cohort of 91%, compared to 94 and 98% for blood sample and general population comparator cohorts, respectively. The same overall trend was seen for five-years survival, where survival proportions were 80 vs. 86% and 91% (Table 3 and Figure 3), $p < 0.001$ (Table 3). The adjusted HRs for death were: 2.24 (95% CI: 2.18; 2.29) for HI⁺ cohort members and 1.81 (95% CI: 1.78; 1.84) for the blood sample cohort members using general population comparators as reference (Table 3).

**Figure 3:** Overall survival for persons with elevated Hemolysis Index (HI⁺) in blood samples analyzed on chemistry equipment and age-sex matched persons from the blood sample comparator cohort and general population comparator cohort.

Discussion

We here show that a widely used quality assurance parameter, the Hemolysis Index (HI), may also serve as a biomarker for CVD risk. In the present study, elevated HI levels were associated with increased cumulative incidence of CVD overall when comparing with both comparator groups. The risk of developing CVD was 40% higher in individuals with an elevated HI level compared with a general population, and 13% higher compared with the matched blood sample cohort. Elevated HI levels were also associated with isolated increased cumulative incidences of arterial and venous CVD. In addition, overall mortality risk was higher amongst persons with an elevated HI level than in the two comparator groups.

Several characteristics of HI as an analyte makes its use as a predictive CVD biomarker promising. This includes a high analytical performance [17, 20–24], existing standard material for quality control [25, 26] and a well-established reference interval [17]. Furthermore, the HI is easy accessible in modern laboratories, can be measured in the same sample used for many other analyses – and at a very low cost. Also, many studies have shown that the HI reliably reflects the concentration of cell-free hemoglobin in plasma or serum and measurement of cell-free hemoglobin is considered the most reliable marker of erythrocyte injury and breakdown, both *in vitro* and *in vivo* [17, 27, 28].

The mechanisms behind the association of an elevated HI and increased risk of CVD are unknown. However, it is conceivable that an elevated HI may be associated with cell-free hemoglobin in the blood stream which may promote a prothrombotic state through several pathophysiological pathways:

It has been shown that cell-free hemoglobin, seen in the presence of hemolysis or hemorrhagic episodes, are often associated with inflammation and atherosclerosis [8]. Luminal thrombosis triggered by rupture of atherosclerotic plaques, plaque neovascularization, and intraplaque hemorrhage has been linked to plaque progression, vulnerability, and rupture [9, 10]. It has been demonstrated, microscopically, that intraplaque hemorrhages consist of intact and destroyed erythrocytes and cell-free hemoglobin [8–10]. Outside the protective environment of the erythrocyte, hemoglobin is prone to oxidation and when the hemoglobin molecule is oxidized, the capacity to carry oxygen is lost and oxidation leads to formation of methemoglobin and oxyhemoglobin [12, 13]. In addition, methemoglobin and oxyhemoglobin accumulate within atherosclerotic lesions due to intraplaque hemorrhage, hemolysis, and presence of cell-free hemoglobin [14]. Also, the hemoglobin molecule has a direct effect on NO, as cell-free hemoglobin reacts with NO. Diminishment of NO, in the presence of hemolysis, is thought

to be an additional mechanism behind the chronic vascular damage that is linked to intravascular hemolysis [5, 15].

An important pathogenic component in atherosclerosis is inflammation orchestrating all stages of atherosclerosis [5, 7]. Several endogenous molecules can activate cellular receptors leading to inflammation. Heme, the prosthetic group of hemoglobin and present in plasma in hemolytic conditions, can activate cellular receptors leading to inflammation [14]. Therefore, this heme-triggered event has a pathogenic role in vascular damage, inclusive atherosclerosis [14]. Also, deficiency of the heme-catabolizing enzyme heme oxygenase-1 in humans is associated with elevated plasma heme levels, endothelial damage, and accelerated atherosclerosis [29].

The key role of inflammation in the initiation and development of atherosclerosis and CVD encourages research after new inflammatory biomarkers. In a recent American College of Cardiology/American Heart Association Guideline on the Primary Prevention of Cardiovascular Disease, measurement of C-reactive protein (CRP) is recommended [30]. Previously, it has been shown that pooled odds ratio for CVD comparing persons with CRP concentration in the top tertile with the bottom tertile was 1.58 [31]. An elevated HI as a biomarker of CVD provides a result comparable to CRP levels since we found a cause-specific hazard ratio of 1.55 in the population of patients with elevated HI.

Regarding venous CVD, routine blood tests have been investigated as predictive markers of venous CVD [32]. The predictive value of coagulation factors for VTE was evaluated in patients aged 18–70 years. Increased concentration of coagulation factor VIII (CF VIII) predicted recurrent deep venous thrombosis with a hazard ratio of 1.7 in the lowest concentration for CF VIII above the reference interval compared with normal concentrations of CF VIII [33].

D-dimer is a global biomarker of activation of the coagulation and fibrinolytic systems. D-dimer can be used in the risk assessment of VTE recurrence, but there are only few studies on the association between d-dimer and risk of a first lifetime VTE event [34]. In a prospective study of the association between d-dimer levels and the risk of VTE, a hazard ratio of 1.5 was found for the lowest increase of d-dimer and VTE compared with normal levels of d-dimer [34]. Elevated HI in our study was associated with VTE with a hazard ratio of 1.77 compared to the background population. We did however not perform a dose-response analysis of increasing HI levels and corresponding HR as was evident in the association between elevated d-dimer and VTE [34].

Our study does have some limitations: The association of HI and arterial CVD was not adjusted for traditional or non-traditional risk factors for arterial CVD meaning that we cannot conclude whether other factors could modify the association. The same applies for the association of HI and

venous CVD, where we only partly adjust for known acquired or inherited risk factors for venous CVD. In terms of arterial CVD, we did not perform the analysis of free hemoglobin in plasma as an add-on risk factor to the existing risk assessment. Our laboratory data were based on routine registrations, and we did not include any additional biochemical analyses. Therefore, we cannot conclude whether elevated HI modifies clinical risk assessment for arterial CVD independently. To minimize confounding, we constructed two cohorts from the laboratory cohort to adjust for an expected elevated risk of CVD due to e.g., hypercholesterolemia, renal failure etc. among persons subjected to blood sampling. Despite this procedure, we cannot exclude residual confounding i.e., that HI⁺ persons are disproportionally selected for other factors associated with CVD risk. To improve the results of our study, further studies with simultaneous registration and adjustment for other biomarkers and known risk factors are needed to conclude whether HI has an independent predictive value.

One finding could disprove our hypothesis of an elevated HI as an expression of a fragile erythrocyte population; Most (7.6%) of the repeated elevated HI were found in two consecutive days. We consider 7.6% as an insignificant low number. The median value was 191 days which indicates that many of the repeated elevated HI were far apart from each other.

There was no significant difference in the analytical performance in terms of imprecision of HI results in the HI⁺ and blood sample comparator cohort. The inter-serial imprecision of the HI analysis was assessed in a verification study by Gils et al. [35]. At the level of 0.2 g/L, corresponding to the lowest value in the range of HI results in the HI⁺ cohort, the intra-serial imprecision was 0.3%. We did not assess the analytical performance at the levels of the reference interval, which was used for the blood sample comparator cohort. This was however assessed by Lippi et al. who investigated the intra-assay imprecision of HI by measuring in-house prepared control material. They found an imprecision of 1.6% at the level of 0.16 g/L, corresponding to the upper limit of the reference interval [36].

More capillary samples in the HI⁺ cohort is a potential limitation in our study as haemolysis is more frequent in capillary blood sampling. The same limitation applies for more pediatric blood samples in the HI⁺ cohort as capillary blood sampling is an essential method of blood collection in pediatric patients [37]. We are not able to gain information of the sample matrix from our laboratory information system but the proportion of children under 10 years of age did not differ between the three populations. Actually, the lowest proportion was found in the HI⁺ cohort. Numerous studies have shown that haemolysed blood samples are more frequent from emergency departments as well as from pediatrics [37, 38]. We elucidated that the proportion of smaller children did not differ between the study groups. However, we are not able to identify if more

blood samples in the HI⁺ cohort are requested from specific departments, e.g. the emergency department. Therefore, it is unknown whether the differences in the risk estimates are affected by the composition of the patient population.

To our knowledge, this is the first study to evaluate the use and value of an increased HI as a predictor of CVD. We conclude that an elevated HI may be associated with arterial and venous CVD and increased mortality. However, more detailed studies, including known biomarkers and risk factors, are needed to elucidate potential mechanisms behind the association.

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