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Which leukocyte subsets predict cardiovascular mortality? From the LUDwigshafen Risk and Cardiovascular Health (LURIC) Study

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ABSTRACT

Objective: White blood cells are known to predict cardiovascular mortality, but form a highly heterogeneous population. It is therefore possible that specific subtypes disproportionately contribute to the prediction of cardiovascular outcomes. Therefore, we compared leukocyte subsets alone and in conjunction with an established inflammatory marker, C-reactive protein, for predicting death due to cardiovascular disease in a high-risk population.

Methods: Patients, 3316, (mean [SD] age, 62 [10] years) scheduled for coronary angiography were prospectively followed up. Neutrophil, monocyte and lymphocyte counts were determined. Neutrophil and monocyte subsets were further analysed on the basis of surface expression of CD11b, CD18, CD31, CD40 and CD58. Lymphocytes were further subdivided into CD3, CD4, CD8, and CD19 subsets. The association between each marker and subsequent cardiovascular mortality was assessed using multi-variable Cox regression models.

Results: During a median follow-up period of 7.8 years, 745 (22.5%) patients died, of which 484 were due to cardiovascular events. After entering conventional risk factors and removing patients with a current infection, neutrophil count (HR [95% CI] = 1.90 [1.39, 2.60], $P < 0.001$) and the neutrophil/lymphocyte ratio (HR [95% CI] = 1.68 [1.24, 2.27], $P = 0.003$) emerged as independent predictors of cardiovascular mortality. After mutual adjustment, neutrophil count (HR [95% CI] = 1.87 [1.35, 2.50], $P < 0.001$) outperformed C-reactive protein (HR [95% CI] 1.32 [0.99, 1.78], $P = 0.06$) as a predictor of cardiovascular mortality.

Conclusions: Due to its predictive potential and inexpensive determination, assessment of high neutrophil counts may represent an important marker, possibly improving cardiovascular mortality risk prediction.

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1. Introduction

A modest but consistent relationship between the white blood cell (WBC) count and cardiovascular disease (CVD) has been

reported in several epidemiologic studies [1–3], and these observations are in line with the role of leukocytes in atherosclerosis [4,5]. WBCs are however, a very heterogeneous population of cells, and more recent studies have begun to evaluate the predictive utility of specific WBC subsets. For example, in one prospective study of patients with acute myocardial infarction (MI), Dragu et al. [6] reported that, of the three main leukocytes subsets (i.e., neutrophils, monocytes, and lymphocytes), elevated neutrophils best correlated with mortality. Likewise, in a secondary sample of

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patients referred for angiography, Horne et al. [7] confirmed the greatest prediction for coronary artery disease (CAD) was provided by neutrophils and the neutrophil/lymphocyte ratio. The neutrophil/lymphocyte ratio was also found to be a strong predictor of long-term mortality in patients who underwent percutaneous coronary intervention (PCI) and in patients who were admitted for ST segment elevation MI (STEMI) [8,9].

Moving forward, there are further important distinctions between functionally distinct immune cells. Within the lymphocyte compartment, these include T-helper cells (CD4), T-cytotoxic cells (CD8), B cells (CD19) and Natural Killer cells (CD16/CD56). These sub-populations greatly differ in functionality and proportions, in which they are present in the atheromata [10,11] and the same holds true for monocyte subtypes [12]. Nonetheless, very little is known concerning the interplay between these subsets and CVD. Further, the limited data on such subsets have seldom evaluated their predictive utility independent of validated inflammatory markers such as C-reactive protein (CRP) [13].

In light of the preceding comments, the present study was undertaken to investigate the predictive ability of the total WBC count and its differential subsets with CVD mortality. Further, we sought to determine whether these associations, if any, were independent of the well-established inflammatory marker, CRP. Lastly, we examined whether these findings would remain robust after controlling for the potential confounding effects of infection.

2. Methods

2.1. Participants and setting

Data reported in this paper come from the LUDwigshafen Risk and Cardiovascular Health (LURIC) study, which is an ongoing prospective cohort study designed to investigate environmental and genetic risk factors for CVD [14]. Of the 3358 subjects enrolled in the LURIC study, 18 (0.5%) patients were lost during follow-up. Another 24 (0.7%) study participants were excluded because we did not obtain sufficient data. Hence, the analytic sample included a total of 3316 subjects (2309 men and 1007 women), 18–95 years. Baseline examination was performed between July 1997 and January 2000 in a single tertiary care medical centre in South-West Germany (Herzzentrum, Ludwigshafen). Inclusion criteria were availability of a coronary angiogram, Caucasians of German ancestry to limit genetic heterogeneity, and clinical stability, with the exception of acute coronary syndromes (ACS). ACS presentation included a stable pattern of chest pain (i.e. stable angina pectoris) or an unstable pattern of chest pain, suggesting unstable angina pectoris. Participants with a history of malignancy within the past five years, or any predominant non-cardiac disease were excluded from the study. The LURIC study was approved by the institutional review board of the ethics committee at the “Landesärztekammer Rheinland-Pfalz” (Mainz, Germany) and written informed consent was obtained from all study participants.

2.2. Study endpoint

Information on vital status was obtained from local community registries. The primary endpoint in this study was death due to CVD. Death certificates were reviewed to classify the deceased into those who died from cardiovascular and non-cardiovascular events. Death from cardiovascular causes included sudden cardiac death (SCD), fatal MI, death due to heart failure, death after intervention to treat CAD, stroke and other deaths due to heart disease. Two experienced physicians that were blinded to any data of the study probands except for the information from the death certificates

independently classified the causes of death. In the case of a disagreement concerning the classification, it was discussed and the final decision was made by one of the principal investigators of LURIC (W.M.), who was also blinded to any data except the death certificates.

2.3. Biochemical measurements

Sampling of fasted venous blood occurred in the early morning, and was always performed in the supine position. Haematological laboratory parameters were immediately measured on a daily basis as previously mentioned [14]. Total WBC and differential counts were determined using EDTA whole blood and were later quantified using an automated analyser (Technicon H-1, Bad Vilbel, Germany; until Dec 1998/Advia 120, Bayer Diagnostics, Tarrytown, USA; since Jan 1999). Glucose was determined using the enzymatic hexokinase/glucose 6-phosphate dehydrogenase method (GLU Hitachi 717, Roche Mannheim, Germany). Lipids (i.e., total cholesterol, LDL- and HDL-cholesterol, and triglycerides) were measured with enzymatic reagents from WAKO (Neuss, Germany) and lipoproteins were separated with a combined ultracentrifugation and precipitation method as described [14]. High-sensitivity C-reactive protein (hs-CRP) was measured by immunonephelometry (N LATEX CRP mono from Dade Behring, Marburg, Germany).

2.4. Flow cytometry

Leukocytes were prepared using a whole blood lyse no-wash method, according to manufacturer recommendations (Becton–Dickinson). Preparations were analysed on a four-color flow cytometer (FACSCalibur, Becton–Dickinson) at the Institute of Haematology and Transfusion Medicine of the Ludwigshafen General Hospital using routine methods. In brief, main leukocyte subsets (neutrophils, monocytes, lymphocytes) were identified by forward vs side-scatter; whereby CD14 expression was used to further validate separation of monocytes and neutrophils [15]. Mean fluorescence intensity (MFI) of the neutrophil and monocyte compartments was assessed for the surface markers CD11b, CD18, CD31, CD40 and CD58 on cells. Within the lymphocyte compartment CD3, CD4, CD8, CD16/CD56 and CD19 surface markers were used to identify main lymphocyte subsets (cytotoxic T cells, helper T cells, Natural Killer [NK] cells, and B cells), as described previously [16,17]. All fluorescent-labelled antibodies were purchased from Becton–Dickinson (Heidelberg, Germany), with the exception of CD40, which was purchased from DiaClone (Besacon, France).

2.5. Other variables measured

The LURIC baseline examination has been described in detail elsewhere [14]. Briefly, all body measures were obtained and recorded by trained nurses belonging to the LURIC study team. Resting heart rate was obtained during the morning by electrocardiography. Five measures were taken 30 s apart, following a 10 min rest in the supine position, with the average derived from the last two measures. Brachial artery pressure values were measured with an automated oscillometric device (Hamburg, Germany) after the patient had rested in the supine position for 10 min. At least five consecutive measures of systolic and diastolic blood pressures were taken with a minimum interval of 30 s, with the average obtained from the last two. Arterial hypertension was diagnosed if mean systolic or diastolic blood pressures exceeded 140/90 mmHg or if there was a clinically significant history of hypertension. Body mass was measured using a Tanita body composition analyser (Tanita corp., Japan). Dyslipidaemia was defined if total cholesterol was ≥ 6.2 mmol/L, HDL-cholesterol was

<1.03/1.29 mmol/L (male/female), triglycerides were ≥ 1.7 mmol/L or use of lipid-lowering medication. Diabetes mellitus was diagnosed if fasting glucose was > 7.0 mmol/L or the 2 h value from an oral glucose tolerance test was > 11.1 mmol/L. Patients receiving anti-diabetic medication were also classed as being diabetic. Family history of CVD was self-reported when a first-degree relative had suffered either fatal or non-fatal MI or stroke (<55 years in father or other first-degree male relative, or <65 years in mother or other first-degree female relative). The functional capacity for patients with cardiac disease, especially heart failure, was estimated according to a classification developed by the New York Heart Association (NYHA) [14]. Coronary angiography allowed for disease severity to be determined. The angiographic severity of disease was defined as zero-, one-, two- or three-vessel disease based on the number of luminal narrowing's $\geq 50\%$ in the three major coronary arteries. Self-reported data were available on the diagnosis of infections (e.g., "At this time have you been diagnosed with an ongoing infection"? Possible answers were "yes" or "no"), permitting us to remove subjects who reported having an infection and who may have spuriously contributed to the observed inflammatory state, or number of deaths due to CVD mortality [18–20]. Rather than simply exclude patients with a WBC count above the clinically defined normal range, this approach may, in part, eliminate the potential contributions from other infections that may not have caused the WBC count to rise above the normal range.

2.6. Statistical methods

Continuous parameters following a non-normal distribution underwent a natural logarithmic transformation (base e logarithm) before being used in statistical procedures. We also calculated z -values of logarithmically transformed total WBC and differential compartments based on their mean and SD values (formula for z -values; $x - \text{mean}/\text{SD}$). Exploratory analyses also revealed a curvilinear association between lymphocytes and CVD mortality. Upon removal of the CD16/CD56 subset, a normal-linear distribution was restored. For the purpose of this investigation, lymphocyte count excluding the CD16/CD56 sub-population was used during analyses. After standardisation, total and differential WBC compartments were split into quartiles. Categorical data are reported as percentages and depending on their distribution, continuous data are presented as means with SD values (normal distribution) or as geometric means with 95% confidence intervals (95% CI) (skewed distribution). Comparisons between groups were performed by analysis of variance (ANOVA), with P for linear trend for continuous parameters and χ^2 test with P for linear-by-linear test for categorical variables. Kaplan–Meier survival function with Log-rank test for equality was used to evaluate the predictive ability of baseline total and differential WBC counts with cardiovascular mortality. Time-to-event analyses were performed using univariable and multivariable Cox proportional hazard models. Multivariable analyses were adjusted for a range of conventional risk factors (e.g., age, sex, BMI, smoking, type 2 diabetes mellitus, resting heart rate, systolic and diastolic blood pressure, hypertension, dyslipidaemia, and angina pectoris), clinical indication of coronary angiography, symptoms of heart failure, CAD severity, atrial fibrillation, family history of CVD, percutaneous transluminal coronary angioplasty, cardiovascular treatment (i.e., ACE-inhibitors, angiotensin receptor blockers, β -blockers, statins and aspirin) and hs-CRP. Additional sensitivity analysis removed patients with a current infection ($n = 318$) who may have spuriously contributed towards the risk of death due to CVD. All statistical tests were two-tailed, and statistical significance was defined as $P < 0.05$. Calculations were performed using the SPSS software version 18.0 (SPSS Inc., Chicago, IL,

USA) and STATA software version 11.2 (Stata Corp., College Station, TX, USA).

3. Results

3.1. Study population

The study population consisted of 3316 patients referred for coronary angiography with a median follow-up time of 7.8 years. Of these patients, 745 (22.5%) had died due to either a non-CVD event ($n = 261$) or a CVD event ($n = 484$) (Table 1). Those who suffered an event were older and predominantly male (Table 1). Compared to the other groups, current infections, hs-CRP, glucose and prevalence of type 2 diabetes mellitus were all higher among patients who had died as a result of CVD (Table 1). Total and HDL-cholesterol levels were lower in those who had died during the follow-up as compared to those who had survived (Table 1). Also, resting heart rate, systolic blood pressure and arterial hypertension were significantly higher among those who had died as compared to survivors (Table 1). Patients who had suffered a CVD event reported more severe symptoms of heart failure as compared to the other categories (Table 1). Likewise, the prevalence in the number of diseased vessels and cardiac arrhythmias was higher among these patients (Table 1).

3.2. Baseline WBC counts

We subsequently examined the predictive value of baseline total and differential WBC counts with CVD mortality using Kaplan–Meier survival function. Log-rank tests revealed a significant association between total and differential WBCs with CVD mortality. For example, patients in the highest quartile for total WBCs, monocytes, or neutrophils had a lower cumulative survival, while patients in the lowest quartile for lymphocytes had a lower cumulative survival ($P < 0.001$, Fig. 1).

3.3. Univariable and multivariable analyses

In univariable analyses, the risk for CVD mortality increased with increasing levels of total WBCs, neutrophils, monocytes, neutrophil/lymphocyte ratio and monocyte/lymphocyte ratio, while mortality decreased with lymphocytes (Table 2). Further analyses of the neutrophil, monocyte and lymphocyte compartments revealed a reduced risk for CVD mortality according to CD3, CD4, and CD19 lymphocytes, CD31 neutrophils and an increased risk for CD58 monocytes (Table 3). Entering conventional risk factors, symptoms of heart failure, CAD severity, family history of CVD and medical therapy partly attenuated the associations found for total WBC, neutrophil, monocyte and lymphocyte counts and neutrophil/lymphocyte and monocyte/lymphocyte ratios (Table 2).

After removing patients with a current infection, we found the test for trend no longer remained significant for total WBC, monocyte, lymphocyte, counts as well as the individual compartments (Tables 2 and 3). Although the HR across each quartile for monocyte/lymphocyte ratio diminished, a significant albeit modest trend remained (Table 2). In contrast, the neutrophil count and neutrophil/lymphocyte ratio were retained as independent predictors of CVD death (Table 2).

To better understand the relationship between neutrophils and CVD mortality, we repeated the latter analyses including only patients with stable CAD ($n = 2232$). After comparing the uppermost quartile to the lowest, the fully adjusted association between the neutrophil count and CVD mortality remained virtually identical, HR (95% CI) = 1.93 (1.39, 2.67), $P < 0.001$. We then performed the analyses including only individuals with ACS

Table 1
Demographic characteristics of patients.

Characteristic	No event <i>n</i> = 2571	Non-CVD event <i>n</i> = 261	CVD event <i>n</i> = 484	<i>P</i> value ^a
Age	61 ± 11	69 ± 9*	68 ± 9*	<0.001
Male (%)	68.3	74.7	74.2	0.003
BMI (kg/m ²)	27.5 ± 4.0	26.9 ± 4.6	27.3 ± 4.3	0.06
Current smoker (%)	20.6	18.8	15.7	0.64
Current infection (%)	9.2	8.8	12.4	0.04
High-sensitivity C-reactive protein (mg/L)	7.87 (7.22, 8.51)	12.69 (10.03, 15.35)*	13.21 (11.19, 15.23)*	<0.001
Glucose (mmol/L)	5.24 (5.12, 5.30)	5.58 (5.38, 5.80)*	5.93 (5.76, 6.10)*	0.003
Type 2 diabetes mellitus (%)	13.1	24.9	35.3	<0.001
Blood lipids (mmol/L)				
Triglycerides	1.70 (1.67, 1.74)	1.71 (1.61, 1.81)	1.69 (1.62, 1.77)	0.94
Total cholesterol	4.90 (4.86, 4.94)	4.76 (4.65, 4.88)	4.76 (4.67, 4.85)*	0.003
LDL-cholesterol	2.89 (2.85, 2.92)	2.80 (2.69, 2.91)	2.83 (2.75, 2.91)	0.15
HDL-cholesterol	0.97 (0.96, 0.98)	0.94 (0.91, 0.97)	0.90 (0.88, 0.93)*	<0.001
Dyslipidaemia (%)	68.7	69.3	70.2	0.49
Resting heart rate (bpm)	68 ± 11	70 ± 12	71 ± 13	<0.001
Blood pressure (mmHg)				
Systolic	139 (138, 140)	146 (143, 149)*	144 (142, 147)*	<0.001
Diastolic	81 (80, 81)	80 (79, 81)	80 (79, 81)	0.33
Arterial hypertension (%)	51.1	62.8	59.1	<0.001
New York Heart Association functional class (%)				
1	56.2	37.5	37.0	<0.001
2	28.9	31.4	29.5	
3	12.8	25.7	27.1	
4	2.1	5.4	6.4	
Diseased vessels (%)				
0	35.1	24.3	17.5	<0.001
1	19.1	18.5	18.4	
2	19.1	19.3	18.8	
3	26.7	37.8	45.4	
Coronary angiography (%)	98.6	99.2	97.9	0.45
Unstable angina pectoris (%)	32.5	33.6	24.6	<0.001
Atrial fibrillation (%)	10.3	12.1	21.9	<0.001
Family history of CVD (%)	54.1	44.1	46.3	<0.001
Percutaneous transluminal coronary angioplasty (%)	26.3	29.5	24.6	0.31
Cardiovascular medication use (%)				
ACE-inhibitors	49.9	62.1	67.1	<0.001
ARBs	4.2	4.2	6.4	0.03
β-blockers	65.7	58.6	53.1	<0.001
Statins	47.7	43.7	44.2	0.10
Aspirin	71.3	73.2	71.4	0.39

Continuous data are shown as means ± SD or geometric mean (95% confidence intervals) and categorical data are shown as percentages.

^a ANOVA and χ^2 test were used. **P* < 0.05 when compared to non-event; †*P* < 0.05 when compared to non-CVD event. CVD = cardiovascular disease; BMI = body mass index; LDL = low density lipoprotein; HDL = high density lipoprotein; ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker.

presentation (*n* = 1036), and observed a slight increase in the risk for CVD mortality according to the highest quartile of the neutrophil count, HR (95% CI) = 2.32 (1.32, 4.06), *P* = 0.003.

We compared the predictive value of a high neutrophil count with an established inflammatory marker (hs-CRP). Following mutual adjustment, we found both were independently associated with death due to CVD (Fig. 2). After entering conventional risk factors, neutrophils remained essentially unchanged whereas hs-CRP attenuated by almost 60% (Fig. 2). Lastly, after excluding those who reported a current infection, the neutrophil count remained a strong independent predictor of CVD mortality, while the reliability of hs-CRP was largely weakened (Fig. 2).

3.4. Additional analysis

We employed a multivariable Cox proportional hazard model using competing risks methods as described by Jason and Gray [21] to obtain more accurate and reliable estimates for the risk of CVD mortality according to a higher neutrophil count. This analysis took into consideration a competing risk event (i.e. death due to a non-CVD related cause) which may have otherwise impeded the event of interest (CVD mortality). Though after full adjustment we observed a slight attenuation in the risk, neutrophils in the

upper-most quartile remained strongly associated with death due to CVD, HR (95% CI) = 1.54 (1.16, 2.03), *P* = 0.01 (data not shown).

3.5. Discrimination, reclassification and calibration

Based on the conventional risk factors, we employed a backward regression procedure using competing risks methodology to determine the best risk-estimation model (Table 4). We then computed the c-statistic and the area under the receiving operating characteristic (AUC) curve to compare the conventional risk model with a model based on a combination of the retained traditional risk factors and the neutrophil count. The results are shown in Fig. 3. Here, the conventional model had already obtained a high discrimination, with a c-statistic of 0.767. In comparison, adding the neutrophil count only marginally improved discrimination (0.776, *P* = 0.04). However, due to the known discriminatory limitations of the c-statistic and AUC, we also computed the reclassification statistics of integrated discrimination improvement (IDI) and net reclassification improvement (NRI) [22]. When the IDI was considered, inclusion of the neutrophil count achieved a significant improvement in model discrimination (0.012, *P* < 0.001). For the NRI, we chose *a priori* meaningful risk category of <6, 6–20, and

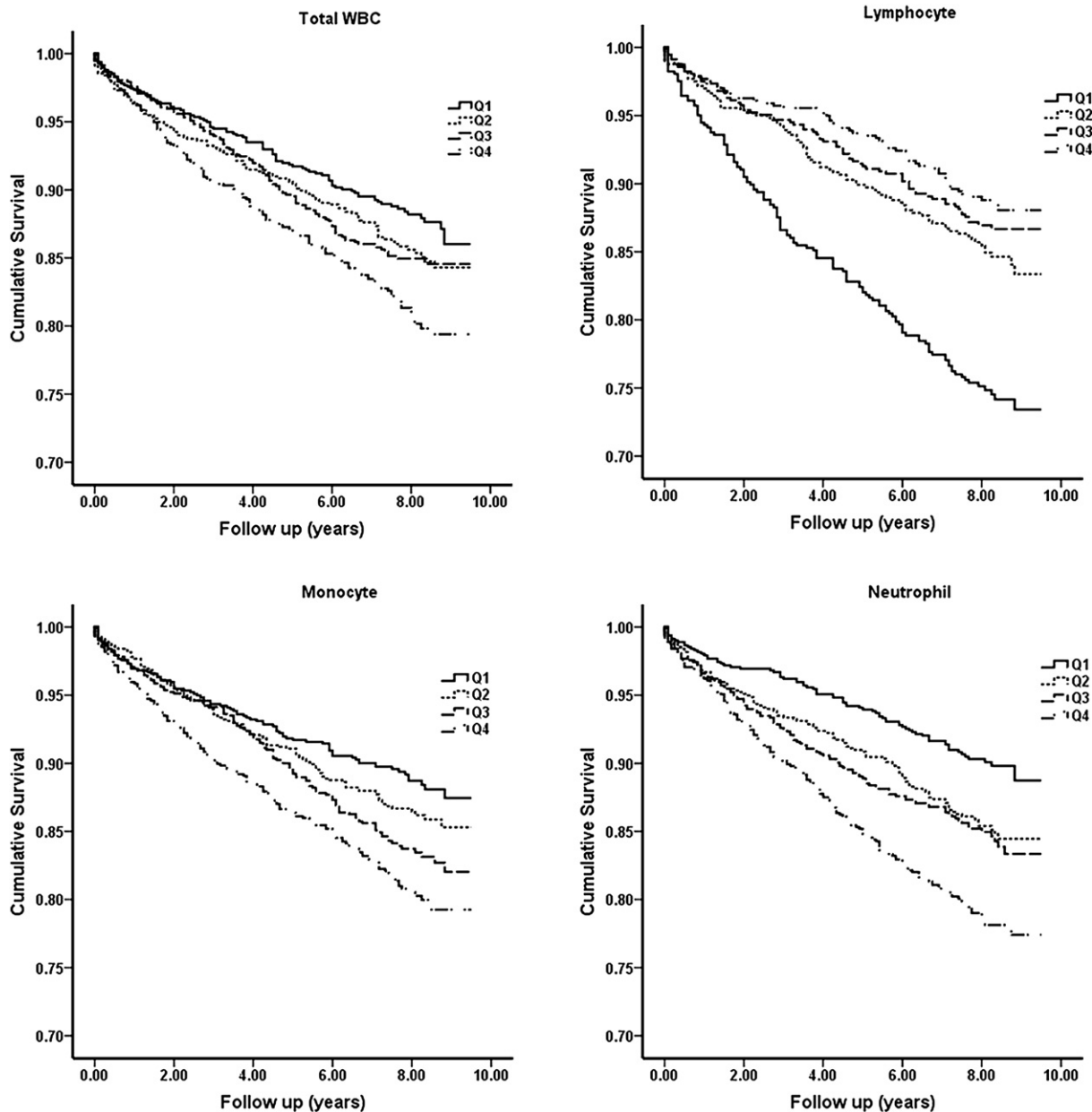


Fig. 1. Kaplan–Meier plots for cardiovascular mortality according to the numbers of total white blood cells (WBC) ($P = 0.001$, Log-rank test), lymphocyte ($P < 0.001$), monocyte ($P < 0.001$), and neutrophil ($P < 0.001$) quartiles. Lymphocyte count excluded CD16/CD56 sub-population.

$>20\%$ 10-year risk of coronary heart disease (CHD) based on the Third Adult Treatment Panel (ATP III) risk classification [23]. Here, adding the neutrophil count to the conventional risk model resulted in correct reclassification of an individual to a different risk category by 5% ($P = 0.003$, Table 5). Lastly, model calibration using the Hosmer–Lemeshow goodness-of-fit test yielded a chi-square of 7.62 ($P = 0.47$), indicating no significant deviation between predicted and observed risk (Fig. 4).

4. Discussion

The present study investigated the predictive utility of leukocyte subsets in an intermediate to high-risk population. One of the main findings was that, of all leukocyte subsets assessed, a neutrophil count in the upper 25% ($>7.3 \times 10^3/\mu\text{l}$) emerged as the strongest

predictor for CVD mortality, yielding an almost two-fold increased adjusted risk of death. This association showed only modest attenuation after correcting for an extensive number of established risk markers.

Comparative analysis between neutrophils and hs-CRP, which is the most commonly measured inflammatory marker in cardiovascular epidemiology, indicated that the neutrophil count is a more robust predictor (both in terms of effect size and stability) in this population. Specifically, the HR for hs-CRP steadily declined with incremental adjustment, and became non-significant when patients who reported a current infection (e.g., a current cold, hepatitis B) were removed from the model. Indeed, the low robustness of CRP has been addressed by other authors [24,25]. Further convincing evidence proposes that CRP may well be a non-causal factor of CVD mortality. A recent meta-analysis of 18

Table 2
Hazard ratios (with 95% CIs) for cardiovascular mortality according to total and differential white blood cell counts.

	Quartiles				P for trend
	1st	2nd	3rd	4th	
Total WBC count^a	5.86 (5.81, 5.90)	7.20 (7.18, 7.22)	8.37 (8.34, 8.40)	10.66 (10.57, 10.75)	
No. of study participants at risk	829	824	825	826	
No. of deaths	97	116	119	149	
Median follow-up time (yr)	7.9	7.8	7.8	7.5	
Model 1	1.00 (reference)	1.18 (0.89, 1.55)	1.26 (0.96, 1.66)	1.68 (1.29, 2.17)	0.001
Model 2	1.00	1.07 (0.80, 1.41)	1.18 (0.90, 1.55)	1.62 (1.24, 2.11)	0.001
Model 3	1.00	1.07 (0.76, 1.34)	1.14 (0.86, 1.50)	1.44 (1.09, 1.91)	0.02
Model 4	1.00	1.05 (0.78, 1.43)	1.19 (0.88, 1.60)	1.41 (1.04, 1.06)	0.10
Total lymphocyte count^{a,b}	2.13 (2.11, 2.15)	2.59 (2.58, 2.60)	2.97 (2.96, 2.99)	3.68 (3.65, 3.71)	
No. of study participants at risk	826	826	826	826	
No. of deaths	136	84	73	63	
Median follow-up time (yr)	7.5	7.7	7.8	7.8	
Model 1	1.00 (reference)	0.57 (0.43, 0.75)	0.47 (0.35, 0.63)	0.42 (0.31, 0.56)	<0.001
Model 2	1.00	0.72 (0.55, 0.96)	0.61 (0.46, 0.82)	0.61 (0.44, 0.84)	0.002
Model 3	1.00	0.73 (0.55, 0.97)	0.64 (0.48, 0.87)	0.65 (0.47, 0.89)	0.009
Model 4	1.00	0.77 (0.56, 1.05)	0.71 (0.51, 0.99)	0.70 (0.50, 0.99)	0.10
Total monocyte count^a	0.26 (0.25, 0.27)	0.37 (0.36, 0.37)	0.46 (0.45, 0.46)	0.64 (0.63, 0.65)	
No. of study participants at risk	827	827	827	827	
No. of deaths	90	109	131	153	
Median follow-up time (yr)	7.2	7.1	7.0	6.7	
Model 1	1.00 (reference)	1.23 (0.92, 1.63)	1.49 (1.14, 1.96)	1.85 (1.42, 2.41)	<0.001
Model 2	1.00	1.15 (0.87, 1.53)	1.29 (0.98, 1.69)	1.54 (1.17, 2.01)	0.01
Model 3	1.00	1.09 (0.81, 1.46)	1.31 (0.99, 1.73)	1.43 (1.08, 1.89)	0.05
Model 4	1.00	1.07 (0.78, 1.46)	1.26 (0.94, 1.70)	1.37 (1.01, 1.84)	0.14
Total neutrophil count^a	3.59 (3.56, 3.62)	4.61 (4.60, 4.63)	5.50 (5.48, 5.52)	7.31 (7.23, 7.39)	
No. of study participants at risk	825	825	826	825	
No. of deaths	80	116	120	165	
Median follow-up time (yr)	8.0	7.7	7.6	7.5	
Model 1	1.00 (reference)	1.44 (1.08, 1.93)	1.56 (1.17, 2.07)	2.33 (1.78, 3.05)	<0.001
Model 2	1.00	1.38 (1.03, 1.85)	1.47 (1.10, 1.96)	2.21 (1.67, 2.92)	<0.001
Model 3	1.00	1.27 (0.94, 1.71)	1.32 (0.98, 1.78)	1.96 (1.47, 2.62)	<0.001
Model 4	1.00	1.26 (0.92, 1.73)	1.37 (1.00, 1.88)	1.90 (1.39, 2.60)	<0.001
Monocyte/lymphocyte ratio^{a,b}	0.14 (0.14, 0.15)	0.20 (0.20, 0.21)	0.26 (0.25, 0.26)	0.40 (0.39, 0.41)	
No. of study participants at risk	824	824	824	824	
No. of deaths	86	97	106	189	
Median follow-up time (yr)	7.8	7.7	7.6	7.5	
Model 1	1.00 (reference)	1.09 (0.82, 1.47)	1.27 (0.95, 1.68)	2.35 (1.82, 3.03)	<0.001
Model 2	1.00	0.95 (0.70, 1.27)	1.01 (0.76, 1.35)	1.61 (1.23, 2.11)	<0.001
Model 3	1.00	0.88 (0.65, 1.20)	0.98 (0.73, 1.32)	1.34 (1.02, 1.78)	0.008
Model 4	1.00	0.84 (0.61, 1.16)	0.97 (0.71, 1.32)	1.24 (0.92, 1.67)	0.05
Neutrophil/lymphocyte ratio^a	1.33 (1.31, 1.35)	1.97 (1.96, 1.98)	2.62 (2.60, 2.63)	4.09 (4.01, 4.16)	
No. of study participants at risk	825	826	826	825	
No. of deaths	83	96	116	186	
Median follow-up time (yr)	7.9	7.8	7.7	7.5	
Model 1	1.00 (reference)	1.21 (0.90, 1.62)	1.47 (1.10, 1.95)	2.57 (1.98, 3.33)	<0.001
Model 2	1.00	1.13 (0.84, 1.53)	1.30 (0.98, 1.73)	1.95 (1.49, 2.56)	<0.001
Model 3	1.00	1.11 (0.82, 1.50)	1.16 (0.87, 1.56)	1.74 (1.32, 2.31)	0.008
Model 4	1.00	1.13 (0.82, 1.56)	1.20 (0.88, 1.63)	1.68 (1.24, 2.27)	0.003

Model 1 was unadjusted. Model 2 adjusted for age, sex, body mass index, smoking and high-sensitivity C-reactive protein. Model 3 additionally adjusted for dyslipidaemia, type 2 diabetes mellitus, resting heart rate, systolic and diastolic blood pressure, hypertension, clinical indication of coronary angiography, angina pectoris, atrial fibrillation, family history of cardiovascular disease, symptoms of heart failure, coronary angioplasty, and cardiovascular treatment (i.e., ACE-inhibitors, angiotensin receptor blockers, β -blockers, statins and aspirin). Model 4 also excluded patients reporting a current infection.

^a Values are geometric means (95% confidence interval), $10^3/\mu\text{l}$.

^b Lymphocyte count did not include CD16/CD56 sub-population. WBC = white blood cell.

genome-wide significant loci associated with CRP levels confirmed there was no evidence to indicate that variations in these genetic loci explained the association between CRP and CHD [26]. In that study, neither the individual single nucleotide polymorphisms nor their combined genetic risk score were significantly related to the risk of MI and CHD [26]. In agreement, a separate report by Elliot and colleagues [27] indicated that genetically elevated CRP was not found to be associated with risk of clinical events due to MI and CHD. Collectively, it seems the lack of concordance in support of CRP genotypes and CRP levels towards the risk of CVD would argue against a causal role for CRP in CVD. On the other hand, the

incremental adjustments did not affect the HR seen for neutrophils. Thus, the present observation and those of others indicate that the neutrophil count, which can be assessed reliably and inexpensively, may well be a more useful and relevant predictor of CVD risk in clinical practise [6,7,28–33].

Though perhaps not yet conclusive, compelling evidence does suggest a causal role for neutrophils in atherosclerosis [34,35], consistent with the current study observation that the HR remained unperturbed by multiple adjustments, including the inflammatory marker CRP. Neutrophils traffic to inflamed arteries where their short lifespan is extended by inflammatory cytokines

Table 3
Hazard ratios (quartile 4 vs quartile 1) for the prediction of cardiovascular mortality according to subset compartments.

	Univariable analysis			Multivariable analysis		
	HR	95% CI	P	HR	95% CI	P
Lymphocyte subsets						
CD3 cells	0.60	0.46–0.80	<0.001	0.85	0.61–1.18	0.32
CD4 cells	0.55	0.41–0.74	<0.001	0.76	0.54–1.07	0.12
CD8 cells	0.83	0.62–1.10	0.19			
CD4:CD8 ratio	0.84	0.63–1.12	0.23			
CD19 cells	0.44	0.32–0.60	<0.001	0.76	0.54–1.09	0.13
Monocyte subsets						
CD11b cells	1.03	0.80–1.33	0.80			
CD18 cells	1.05	0.81–1.38	0.67			
CD31 cells	1.18	0.90–1.54	0.23			
CD40 cells	1.02	0.78–1.33	0.89			
CD58 cells	1.34	1.03–1.73	0.03	0.98	0.73–1.32	0.90
Neutrophil subsets						
CD11b cells	1.28	0.93–1.74	0.13			
CD18 cells	1.26	0.94–1.69	0.13			
CD31 cells	0.73	0.54–0.99	0.04	0.78	0.56–1.10	0.16
CD40 cells	1.11	0.88–1.41	0.38			
CD58 cells	1.19	0.90–1.57	0.22			

Multivariable analysis was adjusted as abbreviated in Table 2.

and there they further promote vascular inflammation and plaque instability. The mechanisms by which neutrophils promote plaque instability are still under investigation but include the release of reactive oxygen species, myeloperoxidase, proteolytic enzymes, and arachidonic acid metabolites [4,5,7]. Recently studies in patients with ACS have suggested that neutrophil elastase promotes CD163 shedding from macrophages, leading to a decreased clearance of haemoglobin by macrophages, which may in turn favour plaque destabilization [36]. Neutrophils also swiftly infiltrate the luminal-plaque regions, residing near the fibrous cap [34,35,37]. In response to inflammation, neutrophils become more rigid, obstructing small nutrient vessels which may promote myocardial ischemia/infarction [38–40]. On the background of these observations, it is somewhat surprising, therefore, that neutrophils have not been more thoroughly investigated in the context of atherogenesis. This may, perhaps, be a consequence of their sparse presence during the development of human

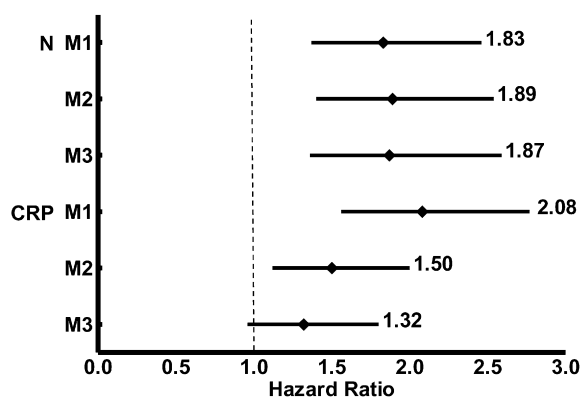


Fig. 2. Mutually adjusted hazard ratios (quartile 4 vs quartile 1) for the risk of cardiovascular mortality according to baseline neutrophil (N) count and high sensitivity C-reactive protein (CRP). Model 1 was unadjusted. Model 2 corrected for age, sex, body mass index, smoking, lipids, type 2 diabetes mellitus, resting heart rate, systolic and diastolic blood pressure, hypertension, clinical indication of coronary angiography, angina pectoris, atrial fibrillation, family history of cardiovascular disease, symptoms of heart failure, coronary angioplasty, and cardiovascular medication. In Model 3, patients with a current infection were removed.

Table 4
Hazard ratios derived from a multivariable backward regression model using competing risks for the association of various conventional risk factors with cardiovascular mortality.

Variable	HR	95% CI	P
Age	1.03	1.02–1.05	<0.001
Female sex	0.78	0.62–0.98	0.04
Type 2 diabetes mellitus	1.50	1.19–1.89	0.001
Diastolic blood pressure	0.99	0.97–1.00	0.01
Atrial fibrillation	1.52	1.17–1.97	0.002
Angina pectoris			
Stable	0.63	0.50–0.80	<0.001
Unstable	0.46	0.35–0.60	<0.001
New York Heart Association functional class			
1	1.00 reference		
2	1.18	0.94–1.47	0.15
3	1.44	1.11–1.86	0.006
4	1.77	1.06–2.96	0.03
Diseased vessels			
0	1.00 reference		
1	1.26	0.92–1.73	0.14
2	1.39	1.02–1.90	0.04
3	1.55	1.16–2.06	0.003
Aspirin	1.22	1.17–2.40	<0.001
ARBs	1.10	1.01–1.98	0.03
β-blockers	0.80	0.65–0.99	0.04
Neutrophils	1.21	1.10–1.34	<0.001

The model initially included age, sex, body mass index, smoking, high-sensitivity C-reactive protein, dyslipidaemia, type 2 diabetes mellitus, resting heart rate, systolic and diastolic blood pressure, hypertension, clinical indication of coronary angiography, atrial fibrillation, angina pectoris, family history of cardiovascular disease, symptoms of heart failure, coronary angioplasty, ACE-inhibitors, angiotensin receptor blockers, β-blockers, statins and aspirin, and the neutrophil count. Retention criteria was $P < 0.05$. ARB = angiotensin receptor blocker.

atherosclerotic lesions, suggesting a less important role [4,35]. Nonetheless, their low presence may be misleading, and can, at least in part, be explained by the short lifespan of neutrophils, recently estimated at 5.4 days [41], which following recruitment to an area of inflammation will eventually undergo apoptosis. Further, local macrophages rapidly phagocytose both apoptosing and intact neutrophils [42], the latter phenomenon occurring in activated neutrophils and mediated via externalisation of phosphatidylserine during neutrophil activation [43] and possibly also involving galectins in promoting subsequent uptake by macrophages [44]. The local destruction of neutrophils appears functional, however, and enhances the antimicrobial capacity of macrophages through the acquisition and use of neutrophil microbicidal molecules. Thus, while there is good evidence to support a causal role for a raised peripheral neutrophil count in the development of cardiovascular disease, paradoxically their potent role evolves, in part, from cellular processes whereby the evidence for their involvement is being destroyed.

Several limitations of our study bear mentioning. Though prospective in patient enrollment, total and differential WBC parameters were determined at a single time point, and therefore, are cross-sectional in nature. Subjects in the present study were Caucasian of German ancestry referred for coronary angiography. Thus, caution is warranted when transferring our findings towards the understanding of atherosclerosis in other populations. Although we performed multivariable adjustments, we cannot rule out that our results may be influenced by unmeasured or unknown confounders. Due to the large sample size and extensive range of measurements taken, we were however, able to adjust for a range of important confounders which may, in part, address this issue. The associations of neutrophil, monocyte, and lymphocyte sub-compartments with CVD mortality no longer retained their significance after correcting for a range of potential confounders.

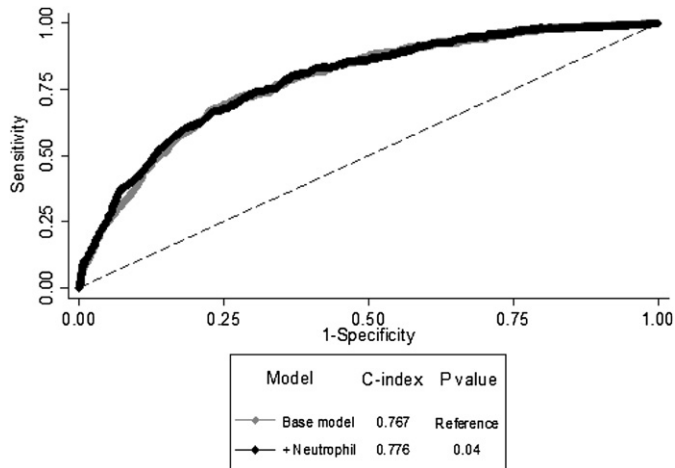


Fig. 3. C-index derived from receiver operating characteristic (ROC) analysis for the prediction of cardiovascular mortality. Base model (gray) includes age, sex, type 2 diabetes mellitus, diastolic blood pressure, atrial fibrillation, angina pectoris, New York Heart Association functional class, number of diseased vessels, aspirin, ARBs and β -blockers; Model 2 (black) additionally includes neutrophils.

Despite these findings, we cannot exclude the possibility that other functionally distinct subsets may be implicated in the process of atherosclerosis, that weren't examined here. For example, within the monocyte compartment, a minor subset (<15% of monocytes) has been identified that may account for 90% of monocyte TNF- α production and may contribute to athero-progression as observed in preclinical studies [45,46]. Clearly, further investigations to dissect the involvement of other functionally distinct cell phenotypes in the initiation, progression and/or development of atherosclerotic-related complications are necessary [11].

In summary, a simple, inexpensive, and readily available marker of inflammation, the neutrophil count should be considered as the mainstay among leukocytes for CVD prediction in a high-risk population. Further understanding of a high neutrophil count in combination with other markers of inflammation or simultaneously within a multiple-marker setting may provide clinically useful incremental information.

Table 5

Reclassification of intermediate (defined as 6–20%) risk patients with and without the cardiovascular endpoint.

Classification according to conventional risk factors	Reclassification accounting for neutrophils			
	Low	Intermediate	High	Total no.
Patients with CVD endpoint				
Low <6% in 10 yrs	28	6	0	34
Intermediate 6–20% in 10 yrs	6	137	24	167
High >20% in 10 yrs	0	13	208	221
Total no. with event	34	156	232	422
Patients without CVD endpoint				
Low <6% in 10 yrs	937	91	0	1028
Intermediate 6–20% in 10 yrs	163	1076	65	1,304
High >20% in 10 yrs	0	70	369	439
Total no. without event	1100	1237	434	2771
Net reclassification improvement	5.0% (P = 0.003)			

Conventional risk factor model included age, sex, type 2 diabetes mellitus, diastolic blood pressure, atrial fibrillation, angina pectoris, New York Heart Association functional class, number of diseased vessels, aspirin, ARBs and β -blockers.

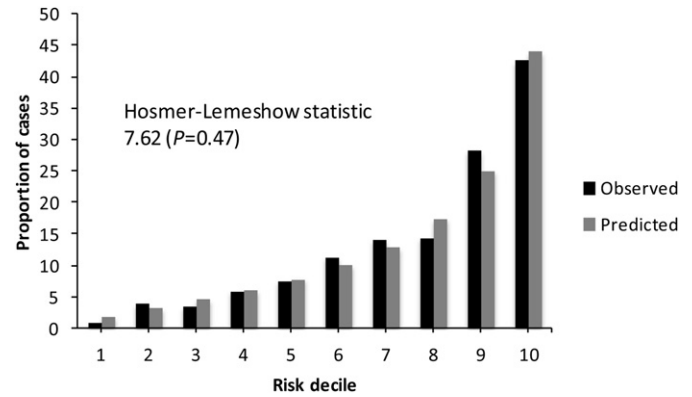


Fig. 4. Proportion of observed and predicted cases according to the risk model that included the neutrophil count. Hosmer–Lemeshow test for goodness-of-fit indicated no significant deviation between observed and predicted risk ($P = 0.47$).

Conflict of interest

The authors declare no conflict of interest.

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