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HIGHLIGHTED ARTICLE

Frontline Science: Low regulatory T cells predict perioperative major adverse cardiovascular and cerebrovascular events after noncardiac surgery

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Abstract

Immune cells drive atherosclerotic lesion progression and plaque destabilization. Coronary heart disease patients undergoing noncardiac surgery are at risk for perioperative major adverse cardiac and cerebrovascular events (MACCE). It is unclear whether differential leukocyte subpopulations contribute to perioperative MACCE and thereby could aid identification of patients prone to perioperative cardiovascular events. First, we performed a hypothesis-generating post hoc analysis of the LeukoCAPE-1 study ($n = 38$). We analyzed preoperative counts of 6 leukocyte subpopulations in coronary heart disease patients for association with MACCE (composite of cardiac death, myocardial infarction, myocardial ischemia, myocardial injury after noncardiac surgery, thromboembolic stroke) within 30 d after surgery. Regulatory T cells (Tregs) were the only leukocyte subgroup associated with MACCE. We found reduced Tregs in patients experiencing MACCE versus no-MACCE (0.02 [0.01; 0.03] vs. 0.04 [0.03; 0.05] Tregs nl^{-1}, $P = 0.002$). Using Youden index, we derived the optimal threshold value for association with MACCE to be 0.027 Tregs nl^{-1}. Subsequently, we recruited 233 coronary heart disease patients for the prospective, observational LeukoCAPE-2 study and independently validated this Treg cutoff for prediction of MACCE within 30 d after noncardiac surgery. After multivariate logistic regression, Tregs < 0.027 cells nl^{-1} remained an independent predictor for MACCE (OR = 2.54 [1.22; 5.23], $P = 0.012$). Tregs improved risk discrimination of the revised cardiac risk index based on ΔAUC (area under the curve; ΔAUC = 0.09, $P = 0.02$), NRI (0.26), and IDI (0.06). Preoperative Treg levels below 0.027 cells nl^{-1} predicted perioperative MACCE and can be measured to increase accuracy of established preoperative cardiac risk stratification in coronary heart disease patients undergoing noncardiac surgery.

KEYWORDS

biomarker, leukocytes, preoperative risk assessment

Abbreviations: AKI, acute kidney injury; ASA, American Society of Anesthesiologists; AUC, area under the curve; CD, cluster of differentiation; CI, confidence interval; ECG, electrocardiogram; FSC, forward scatter; Hs-cTnT, high-sensitive cardiac troponin T; IDI, integrated discrimination improvement; IQR, interquartile range; LeukoCAPE, Leukocytes and cardiovascular perioperative events study; MACCE, major adverse cardiovascular and cerebrovascular events; MI, myocardial infarction; MINS, myocardial injury after noncardiac surgery; NRI, net reclassification improvement; NT-proBNP, N-terminal pro-brain natriuretic peptide; OR, odds ratio; POD, postoperative day; PPV/NPV, positive/negative predictive value; RCRI, revised cardiac risk index; ROC, receiver operating characteristic; sCD14-ST, soluble CD14-subtype; SSC, side scatter; Tregs, regulatory T cells; TRIPOD, transparent reporting of a multivariable prediction for individual prognosis or diagnosis; VISION, vascular events in noncardiac surgery patients cohort evaluation.

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1 | INTRODUCTION

Cardiovascular diseases are substantially driven by innate and adaptive immune effector mechanisms.[1] In long-term prospective clinical trials, leukocyte subpopulations including classical,[2] intermediate,[3] and nonclassical monocytes,[4] as well as natural killer[5] and regulatory T cells (Tregs),[6] were ascribed an association with cardiovascular disease and have been shown to predict cardiovascular events. Acute perioperative stress during major noncardiac surgery implies significant immunomodulatory and inflammatory changes[7,8] associated with patients' susceptibility to major adverse cardiovascular and cerebrovascular events (MACCE).[7,9] MACCE related to noncardiac surgery are among the leading causes of perioperative morbidity and mortality.[7] Mechanisms underlying perioperative MACCE are incompletely understood. As a consequence, tools to accurately identify vulnerable patients prone to develop cardiovascular complications are limited and uncertainty exists regarding the optimal risk stratification model.

Clinical practice guidelines on perioperative risk evaluation[10–12] advocate the use of clinical risk indices such as the revised cardiac risk index (RCRI).[13] However, the RCRI shows only low discriminatory power in high-risk and vascular surgery patients[14,15] as it is based on conditions omnipresent in high-risk cardiovascular patients.

Recent perioperative guidelines encourage additional preoperative biomarker measurements in high-risk patients to identify patients with underestimated severity of cardiovascular disease.[12,16] Still, evidence is scant and their clinical benefit is under debate.[17] Moreover, cardiac biomarkers such as troponins and natriuretic peptides rise as a result of myocardial injury and heart failure, respectively. Thus, they certainly reflect severity of preexisting diseases, but show limited utility for identification of patients with vulnerable, yet silent atherosclerotic lesions prone to cause near-future events. Experimental studies established a causal role for certain leukocyte subpopulations in atherosclerotic lesion progression or regression. Using such cell populations as risk markers may substantially improve preoperative risk prediction by identifying additional patients prone to the development of new cardiovascular events.

Dysregulated activation of inflammatory cells is critical for development and progression of cardiovascular diseases. We recently demonstrated that elevation of atherogenic monocyte subsets during noncardiac surgery and high concentrations of the monocyte activation marker presepsin (soluble CD14-subtype; sCD14-ST) are associated with perioperative MACCE.[18] However, it remains unknown whether a patient's individual immune status renders him prone to perioperative cardiovascular events. In particular, evidence for a potential relation between preoperative levels of leukocyte subsets with perioperative MACCE is scarce.[19]

Therefore, we evaluated preoperative values of 6 predefined leukocyte subsets in a post hoc analysis of the LeukoCAPE-1 study for association with perioperative MACCE. Based on these results, we subsequently conducted the prospective LeukoCAPE-2 study to validate the predictive cutoff value derived for preoperative Treg findings in an independent cohort.

2 | MATERIAL AND METHODS

2.1 | Study design and population

The leukocytes and cardiovascular perioperative events-1 and -2 (LeukoCAPE-1 and -2) studies are 2 single-center, prospective, observational cohort studies performed at the Department of Anesthesiology, University Hospital Heidelberg, Heidelberg, Germany. The studies were registered prior to patient enrolment at clinicaltrials.gov (LeukoCAPE-1: NCT02874508, date of registration: August 22, 2016; LeukoCAPE-2: NCT03105427, date of registration: March 8, 2017). The study protocol conformed to the principles of the Declaration of Helsinki and the World Medical Association and the article adheres to the applicable transparent reporting of a multivariable prediction for individual prognosis or diagnosis (TRIPOD) guidelines for transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (Table S1). After approval by the local ethics committee (medical faculty of the Ruprecht-Karls University Heidelberg, S-351/2016, LeukoCAPE-1: August 4, 2016; LeukoCAPE-2: February 14, 2017) and after informed consent, we enrolled consecutive general, vascular, and urologic surgery patients for the LeukoCAPE-1 study between August and October 2016. A detailed description of blood sample collection and inclusion and exclusion criteria has been published together with the results of the main analysis for the LeukoCAPE-1 study.[18] Between April and November 2017, consecutive patients with documented coronary heart disease undergoing elective, in-patient, noncardiac surgery with overnight hospital admission were considered eligible for the LeukoCAPE-2 study. For both studies, we excluded patients younger than 18 yr, pregnant or breastfeeding, and individuals with leukemia or leukopenia (<4 nl^{-1}, detected in the last clinical routine measurement before surgery). Further exclusion criteria were emergency surgery, history of organ transplantation or splenectomy, immunosuppression, chemotherapy, GM-CSF or cortisone treatment less than 14 d ago, or the occurrence of myocardial ischemia, myocardial infarction (MI), embolic or thrombotic stroke, congestive heart failure, or serious cardiac arrhythmia within the past 28 d before enrolment. Preoperative leukocytosis (>10 nl^{-1}) and intraoperative dexamethasone administration were further exclusion criteria in the LeukoCAPE-1 study. Patients were identified by daily screening of surgical lists and during anesthesia consultation sessions.

2.2 | Data collection and conventional risk assessment

After enrolment, we recorded previous cardiovascular and cerebrovascular events, demographic data, the American Society of Anesthesiologists (ASA) physical status classification, smoking status, and current medication. Blood levels of creatinine, C-reactive protein (CRP) and the estimated glomerular filtration rate (eGFR using the Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI]) were documented before surgery. Conventional risk evaluation was based on high-sensitive cardiac troponin T (hs-cTnT) and N-terminal pro-brain natriuretic peptide (NT-proBNP). Patients with hs-cTnT

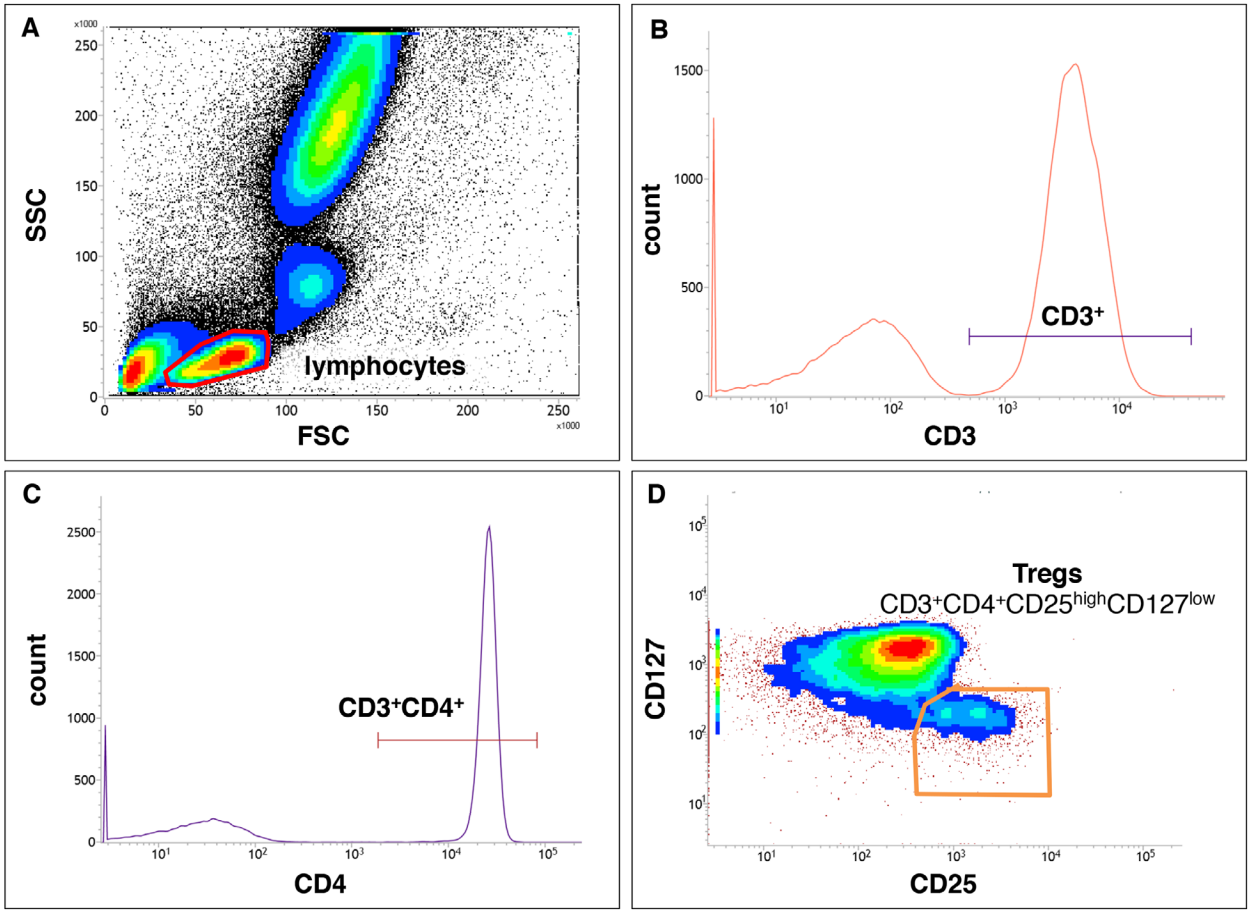

FIGURE 1 **Identification of Tregs by flow cytometry.** (**A**) Lymphocytes (red circle) were identified based on their characteristic forward (FCS) and side scatter (SSC) properties, and were further gated on simultaneous expression of (**B**) CD3 and (**C**) CD4. (**D**) Identification of regulatory T cells (Tregs, orange circle) was based on their high expression of CD25 and low CD127 expression. CD: cluster of differentiation

≥14 ng l^{-1} and NT-proBNP ≥300 ng l^{-1} were considered as at risk for perioperative MACCE.[16,20]

2.3 | Laboratory measurements

Blood samples were collected and processed as described before.[18] NT-proBNP was measured preoperatively (Immulite, Siemens Healthcare Diagnostics, Erlangen, Germany); hs-cTnT was determined preoperatively and daily on postoperative days 1 until 3 (POD1-3; Cobas E4111, Roche Diagnostics, Mannheim, Germany). Automated differential blood counts were performed in the central laboratory. As previously published, leukocyte subpopulations were quantified by flow cytometry (FACSVerse; BD Biosciences, Heidelberg, Germany) in an observer-blinded fashion and using the following monoclonal antibodies: anti-CD4 APC (clone RPA-T4), anti-CD25 PerCP-Cy5.5 (clone M-A251), anti-CD14 FITC (clone M5E2), anti-CD16 PE (clone 3G8), anti-CD56 PE (clone HCD56; all from BioLegend, London, United Kingdom), anti-CD3 PE-Cy7 (clone SK7), and anti-CD127 FITC (clone HIL-7R-M21; both from BD Biosciences). Tregs were identified based on their characteristic $CD3^+CD4^+CD25^{high}CD127^{low}$ surface expression profile (Fig. 1). Data were analyzed using BD FACSuite Software (version 1.0.5.3840, BD Biosciences). Absolute cell counts were calculated as the product of absolute lymphocyte counts from differential blood counts multiplied with the frequency of each subpopulation determined by flow cytometry and are reported as the number of cells per nanoliter blood (nl^{-1}).

2.4 | Outcome analysis

Study participants were followed up until 30 d postsurgery for the occurrence of the composite primary endpoint MACCE including cardiovascular death, MI, myocardial ischemia, myocardial injury after noncardiac surgery (MINS), and embolic or thrombotic stroke. For each patient, time from surgery to first event was documented. Secondary endpoints were individual components of the primary endpoint MACCE, all-cause mortality, new-onset atrial fibrillation in postoperative ECG or documented in patient's chart, peripheral vascular occlusion confirmed by angiography or documented in patient's chart, and acute kidney injury (AKI; increase in creatinine of ≥0.3 mg dl^{-1} [≥26.5 μmol l^{-1}] within 48 h or an increase of ≥1.5× from baseline within 7 d[21]). For outcome analysis, a postoperative 12-lead ECG was recorded at POD3, patient charts were screened and participants or their family doctors participated in a scripted telephone interview at the end of follow-up. Pre- and postoperative ECGs were analyzed by

two independent physicians unaware of the clinical or flow cytometric data. All disagreements in ECG interpretation between the two physicians were discussed with a third physician and were resolved in consensus.

2.5 | Detailed definitions of primary outcome variables

Cardiac death was defined as any death presumably of cardiac origin. Diagnosis of MI was based upon the third universal definition of MI.[20] Myocardial ischemia was defined as ST segment elevation (≥2 mm in leads V2 or V3; or ≥1 mm in the other leads), new ST segment depression of ≥1 mm, new symmetric T wave inversions, new left bundle branch block, or development of new pathologic Q waves detected in postoperative ECG. Criteria for MINS differed between LeukoCAPE-1 and -2. When designing LeukoCAPE-1 there was no generally accepted MINS definition available that used troponin values measured with the high-sensitive assay used in our study. Therefore, in the LeukoCAPE-1 study, MINS[22,23] was defined as any raise in postoperative hs-cTnT ≥50 ng l^{-1}[24] judged due to myocardial ischemia. Raising hs-cTnT was defined as an increase of at least 50% from baseline.[25] In LeukoCAPE-2, any postoperative peak hs-cTnT between 20 ng l^{-1} and <65 ng l^{-1} with an absolute increase of ≥5 ng l^{-1} or any new hs-cTnT ≥65 ng l^{-1} with peak hs-cTnT postoperatively was defined as MINS.[26] Stroke was diagnosed as new focal neurologic deficit with radiologic or angiographic evidence of embolic or thrombotic cause.[23]

2.6 | Statistical analysis

Continuous data are presented as median (interquartile ranges [IQR]); categorical data as total and relative counts, unless otherwise stated. Patients were categorized for the occurrence of MACCE. Differences between groups were assessed using nonparametric Mann-Whitney *U* and Fisher Yates test for continuous and categorical data, respectively. Two-sided *P* values <0.05 were considered significant. To account for multiple comparisons in the LeukoCAPE-1 study, statistical analyses of preoperative leukocyte subset counts were adjusted according to Bonferroni ($\alpha < 0.05/6$). If patients were discharged home before the POD3 visit was completed, hs-cTnT data were imputed (last observation carried forward analysis). Receiver operating curve (ROC) analyses were performed to evaluate the discriminatory power of preoperative Treg levels in association with MACCE. The optimal threshold was calculated based on the maximized Youden index.[27] Odds ratios (OR; 95% confidence intervals [CI]) were calculated using Woolf and Baptista-Pike method as appropriate. For the LeukoCAPE-2 study, univariate logistic regression models were calculated for baseline characteristics and corresponding OR are given. Multivariable logistic regression modelling includes factors that revealed a *P* value below 0.1 in univariate analysis. The Hosmer-Lemeshow test was performed to assess goodness-of-fit of the multivariate model. To test for collinearity, variance inflation factor was used. Patients were divided into 2 groups according to the Treg threshold (0.027 cells nl^{-1}) derived from the LeukoCAPE-1 cohort and were stratified into quartiles according to their preoperative Treg level. Kaplan-Meier curves were calculated and cumulative incidence of MACCE (time until first MACCE) were compared by log-rank (Mantel Cox) test. The additive predictive value of Tregs to the basic risk model including RCRI, NT-proBNP, and hs-cTnT was evaluated using ROC curves, net reclassification improvement (NRI), and integrated discrimination improvement (IDI).[28] ROC curves were compared as described by DeLong et al.[29] As appropriate, 95% CI are given. IBM SPSS Statistics 25.0 (SPSS, Chicago, IL, USA), MedCalc 16.8 (MedCalc Software, Ostende, Belgium), and Prism 7.02 (GraphPad Prism Software, Inc., San Diego, CA, USA) were used for statistical analyses.

2.7 | Sample size calculation

The sample size ($n = 40$) of the LeukoCAPE-1 study was initially calculated for testing the association of leukocyte subpopulation counts with noncardiac surgery.[18] For this post hoc analysis we included data from all 38 patients. Sample size for the LeukoCAPE-2 study was calculated to validate the risk predictive value of preoperative Tregs for MACCE. We powered this validation study to reach a 95% CI of the Treg count's area under the curve (AUC) for prediction of MACCE with a maximum width of 9% at an AUC of 0.87 or greater. Based on these assumptions, we calculated a sample size of 221 patients. To compensate for 5% dropouts, we recruited 233 patients into the LeukoCAPE-2 study.

3 | RESULTS

3.1 | Study population

A detailed description of the LeukoCAPE-1 patient flow and baseline characteristics have been published before.[18] In brief, a total of 44 coronary artery disease patients scheduled for elective, noncardiac surgery were screened for eligibility. Of those, 40 patients were successfully enrolled into the study. During follow-up, two patients withdrew consent and were excluded, resulting in a final study population of 38 participants. For the LeukoCAPE-2 study, we screened 295 patients with coronary heart disease scheduled for elective, noncardiac surgery. Of these, 233 patients fulfilling eligibility criteria were successfully enrolled. After study inclusion, seven patients withdrew consent, two patients did not undergo surgery after enrollment, and in two cases exclusion criteria were found after enrolment. Another two subjects were excluded because of technical issues with the FACS analysis for Treg quantification resulting in 220 subjects for the final analysis (Fig. 2).

Baseline characteristics of the LeukoCAPE-1 study population are reported in Table S2 and did not differ between MACCE and no-MACCE patients except for higher creatinine values and lower glomerular filtration rates in individuals suffering MACCE. Seven (18%) patients experienced MACCE. During follow-up three patients died. No patient experienced cardiovascular death.

Baseline characteristics of the subsequent LeukoCAPE-2 study cohort are reported in Table 1. Complications of coronary heart disease

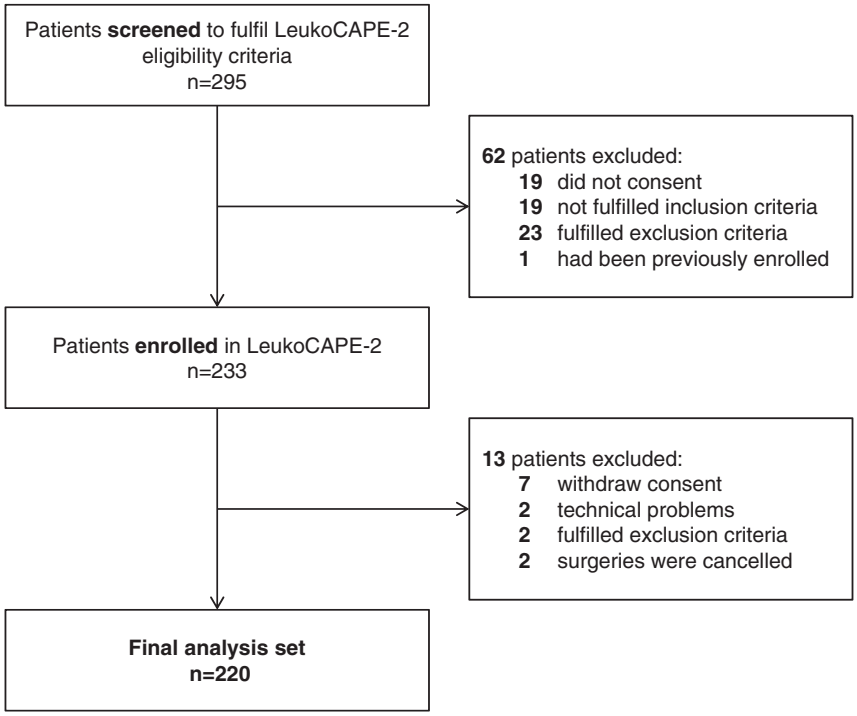

FIGURE 2 Patient flow chart of LeukoCAPE-2 study

such as MI or heart failure were highly prevalent. Most of the participants received cardiovascular medication and had previous interventional or surgical coronary revascularization. Mean age in the study cohort was 69 yr, ranging from 41 to 90 yr. 80% of the participants were male. During 30 d follow-up, the predefined composite primary endpoint MACCE occurred in 84 patients (38%). Of those, 6 patients died (3%), 15 subjects suffered MI (7%), 15 suffered myocardial ischemia (7%), 78 experienced MINS (36%), and 1 patient had a stroke (1%). Several patients experienced more than one event of interest. The majority of MACCE patients (80%) experienced the first event during the first 2 d after noncardiac surgery. Four patients died because of sepsis, one after gastrointestinal bleeding and one because of unknown reasons. No cardiovascular deaths were observed. Study participants who experienced MACCE were older, were more likely to be male, had a higher prevalence of previous percutaneous coronary intervention (PCI) or MI, and presented with more advanced kidney disease. Proportion of ASA 2 status was lower in the MACCE group. The majority of patients underwent general anesthesia alone (59%) or in combination with regional anesthesia (25%). Regional anesthesia (8%) and analgo-sedation alone (8%) were conducted less frequently. Surgical risk according to the European Society of Cardiology/European Society of Anaesthesiology[11] did not differ between the two groups (Table 1). Overall, baseline characteristics in both studies were similar.

3.2 | Derivation of preoperative Treg threshold value derived from LeukoCAPE-1 post hoc analysis

For the main analysis of the LeukCAPE-1 study, WBCs and selected leukocyte subpopulations known to be associated with an increased cardiovascular risk were quantified in elevated-risk patients before and at different time points after noncardiac surgery.[18] Post hoc, we stratified patients for the occurrence of MACCE and analyzed preoperative leukocyte subset counts for their association with MACCE. Whereas no differences were observed in median WBC (Fig. 3A), classical (Fig. 3B), intermediate (Fig. 3C), and nonclassical monocyte (Fig. 3D) counts or natural killer cell levels (Fig. 3E), MACCE patients presented with approximately 50% lower preoperative Treg counts compared to patients without MACCE (0.02 [0.010 to 0.027] vs. 0.04 [0.033 to 0.053] cells nl^{-1}, $P = 0.002$; Fig. 3F). For the different leukocyte subpopulations descriptive data from all time points stratified for MACCE versus no MACCE are reported in Figure S1.

Moreover, ROC curve analysis demonstrated that preoperative Treg levels had a high discriminatory ability for MACCE in elevated-risk patients undergoing noncardiac surgery (AUC = 0.87 [0.74; 0.99], $P = 0.003$; Fig. S2A). Based on the maximized Youden index, the optimal cutoff was calculated to be < 0.027 cells nl^{-1} yielding a sensitivity of 86% and a specificity of 87% (Table 2). Preoperative Treg levels < 0.027 cells nl^{-1} were associated with an increased risk of 30 d MACCE (OR = 41 [3.8 to 430], $P = 0.0005$). OR and descriptive P values for other secondary endpoints in association with preoperative Treg levels are reported in Figure S2B.

3.3 | Preoperative Treg levels predict MACCE

After demonstrating an association between preoperative Treg counts and MACCE in a post hoc analysis, we next aimed at validating the calculated threshold value in a larger, independent cohort. In the prospective LeukoCAPE-2 study, we could confirm preoperative differences for Treg levels. Median preoperative Treg levels were significantly lower in patients who suffered MACCE compared to patients without MACCE (0.038 [0.022; 0.051] vs. 0.050 [0.031; 0.068] Tregs nl^{-1}, $P = 0.0003$). ROC curve analysis of the total Treg

TABLE 1 Clinical Baseline Characteristics of the LeukoCAPE-2 cohort

Variable	Analysis set	MACCE	No MACCE	*P* value
n (%)	220 (100)	84 (38)	136 (62)	
Age (years)	69 [63; 75]	74 [67; 77]	68 [61; 74]	<0.001
Male sex, *n* (%)	175 (80)	78 (93)	97 (71)	<0.001
BMI (kg m^{-2})	27 [24; 29]	26 [24; 28]	27 [24; 30]	0.122
ASA, *n* (%)				
ASA ≤ 2	24 (11)	3 (4)	21 (15)	0.007
ASA 3	188 (85)	76 (90)	112 (82)	0.117
ASA ≥ 4	8 (4)	5 (6)	3 (2)	0.265
RCRI, *n* (%)				
RCRI 1	47 (21)	12 (14)	35 (26)	0.062
RCRI 2	118 (54)	48 (57)	70 (51)	0.487
RCRI 3	43 (20)	19 (23)	24 (18)	0.386
RCRI 4	10 (5)	4 (5)	6 (4)	1
ESC/ESA surgical risk				
Low-risk surgery	20 (9)	6 (7)	14 (10)	0.48
Intermediate-risk surgery	108 (49)	39 (46)	69 (51)	0.58
High-risk surgery	92 (42)	39 (46)	53 (39)	0.325
Medical history, *n* (%)				
Hypertension	207 (94)	81 (96)	126 (93)	0.379
Diabetes mellitus	74 (34)	26 (31)	48 (35)	0.558
Insulin-dependent	33 (15)	12 (14)	21 (15)	0.849
Chronic kidney disease (KDIGO stage ≥3)	49 (22)	26 (31)	23 (17)	0.019
Peripheral artery disease (Fontaine > 1)	69 (31)	22 (26)	47 (35)	0.232
Atrial fibrillation	37 (17)	19 (23)	18 (13)	0.094
Stroke	21 (10)	7 (8)	14 (10)	0.814
Congestive heart failure	4 (2)	2 (2)	2 (1)	0.637
Coronary heart disease	220 (100)			
History of PCI	113 (51)	52 (62)	61 (45)	0.018
History of CABG	50 (23)	19 (23)	31 (23)	1
History of myocardial infarction	83 (38)	40 (48)	43 (32)	0.022
Smokers, *n* (%)				
Active	51 (23)	16 (19)	35 (26)	0.324
Previous	70 (32)	28 (33)	42 (31)	0.766
Medication, *n* (%)				
Betablockers	165 (75)	67 (80)	98 (72)	0.262
ACE inhibitors	112 (51)	43 (51)	69 (51)	1
Angiotensin II receptor blockers	72 (33)	24 (29)	48 (35)	0.375
Calcium antagonists	67 (30)	25 (30)	42 (31)	0.881
Diuretics	102 (46)	45 (54)	57 (42)	0.097
Statins	175 (80)	67 (80)	108 (79)	1
Aspirin	161 (73)	60 (71)	101 (74)	0.642
ADP receptor antagonists	37 (17)	18 (21)	19 (14)	0.194
Vitamin K antagonists	21 (10)	7 (8)	14 (10)	0.814
New oral anticoagulants	36 (16)	15 (18)	21 (15)	0.709
Preoperative hemodynamics[a]				
Systolic blood pressure (mmHg)	150 [140; 165]	150 [140; 170]	150 [136; 165]	0.259

(Continues)

TABLE 1 (Continued)

Variable	Analysis set	MACCE	No MACCE	*P* value
Diastolic blood pressure (mmHg)	80 [70; 90]	80 [70; 88]	80 [70; 90]	0.918
Heart rate (/min)	70 [60; 80]	70 [60; 80]	75 [60; 80]	0.183
Preoperative laboratory values				
Creatinine[b] (mg dl^{-1})	0.9 [0.8; 1,1]	1.0 [0.9; 1.3]	0.9 [0.7; 1.1]	<0.001
eGFR[b] (ml min^{-1} 1.73 m^{-2})	81 [63; 92]	71 [53; 89]	84 [66; 96]	<0.001
C-reactive protein[b,c] (mg dl^{-1})	3.1 [1.9; 11.2]	3.5 [1.9; 9.2]	2.6 [1.9; 12.05]	0.649
Hemoglobin (g/dl)	12.4 [11; 13.4]	12 [10.8; 13.3]	12.5 [11.1; 13.5]	0.264
Leukocytes (cells nl^{-1})	6.1 [5; 7.4]	6 [4.8; 7.1]	6.2 [5.0; 7.7]	0.200
Type of anesthesia, *n* (%)				
General anesthesia only	130 (59)	42 (50)	88 (65)	0.035
Regional anesthesia (neuroaxial)	17 (8)	8 (10)	9 (6)	0.039
Combination anesthesia (general and regional)	56 (25)	28 (33)	28 (21)	0.039
Analgo-sedation only	17 (8)	6 (7)	11 (8)	1

Data are presented as median [interquartile range], or as absolute numbers (percentage). *P* values refer to comparison between MACCE vs. no MACCE patients. Continuous data were compared using Mann Whitney *U* test. Categorical variables were compared using Fisher exact test.

[a]measured during induction of anesthesia;

[b]detected in the last clinical routine measurement before surgery; and

[c]clinical routine measurement of CRP was missing in 31 patients and determined post hoc in frozen Lithium-Heparin plasma.

MACCE: major adverse cardiac or cerebrovascular event; BMI: body mass index; ASA: risk classification according to the American Society of Anesthesiologists; RCRI: revised cardiac risk index; ESC/ESA: European Society of Cardiology/European Society of Anaesthesiology[11]; KDIGO: Kidney Disease: Improving Global Outcomes; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; ACE: angiotensin converting enzyme; ADP: adenosine diphosphate; and eGFR: estimated glomerular filtration rate calculated by Chronic Kidney Disease Epidemiology Collaboration.

population revealed a significant, albeit moderate discrimination for prediction of MACCE (AUC = 0.64 [0.57; 0.72], $P = 0.0004$). Whereas MACCE was observed in 60% of patients with preoperative Treg levels < 0.027 cells nl^{-1}, only 32% of patients with higher Tregs suffered MACCE resulting in a significant difference in time-to-event analysis (log-rank [Mantel Cox] $P = 0.0003$; Fig. 4A). The threshold value reached a sensitivity of 36% and specificity of 85% (Table 2). When dividing the cohort by their preoperative Treg levels into quartiles (each quartile $n = 55$), Treg levels of the lowest quartile were associated with the highest cumulative incidence of MACCE (log-rank [Mantel Cox] $P = 0.0034$; Fig. 4B). Severity of coronary heart disease, and incidence of as well as time since last MI, PCI, and bypass surgery were not significantly different between patients with low versus high Tregs (Table 3), suggesting similar advanced lesions in both groups.

Univariable predictors of perioperative MACCE are listed in Table 4. Univariable analysis revealed that patients with low Treg levels were associated with a more than 3-fold increased risk for perioperative MACCE (OR = 3.22 [1.68; 6.18], $P = 0.0005$). A descriptive analysis of preoperative Treg levels and secondary endpoints is reported in Figure 5. Tregs were associated with a 2-fold increased risk for MINS (OR = 2.21 [1.16; 4.2], $P = 0.0185$). No association between preoperative Treg levels and any other secondary endpoint was detected (Fig. 5). All-cause mortality is reported, but was not a prespecified endpoint (OR = 3.55 [0.69; 18.18], $P = 0.132$).

In multivariable logistic regression analysis, Treg levels were entered as dichotomized variable and remained an independent risk factor for perioperative MACCE after adjustment for age, gender, ASA physical status, history of PCI, and creatinine levels (Table 4). The final multivariable model for perioperative MACCE demonstrated a high discriminative ability (AUC = 0.8 [0.74; 0.85], $P < 0.001$]). The overall goodness-of-fit Hosmer-Lemeshow test calculated a good fit ($\chi^2 = 2.66$, $P = 0.954$). As the variables included in the multivariable model showed a variance inflation factor of far less than 10, collinearity was not suggested.

3.4 | Additive risk predictive value of Tregs

We next aimed to elucidate whether the addition of Treg levels improved risk stratification of guideline-recommended perioperative risk predictors. Therefore, we performed three measures (AUC, NRI, and IDI) with all markers being analyzed as continuous variables.

Measures of diagnostic accuracy for preoperative Treg levels and conventional risk predictors are reported in Table 2. Even though the RCRI is recommended by current guidelines and is therefore commonly used, its predictive accuracy is limited especially in cardiovascular risk patients.[30] As recent North American and European guidelines recommend preoperative cardiac risk assessment using both, the RCRI and additional measurements of biomarkers,[11,12,16] we first quantified the additive predictive value of preoperative Treg levels to RCRI. RCRI alone revealed an AUC of 0.57 (0.49; 0.64), which was significantly increased when combined with Tregs (AUC = 0.66 [0.59; 0.73], ΔAUC = 0.09, $P = 0.02$). Addition of Treg cell counts to RCRI significantly improved MACCE risk classification (total NRI = 0.27, $P = 0.005$). Improvement in risk classification

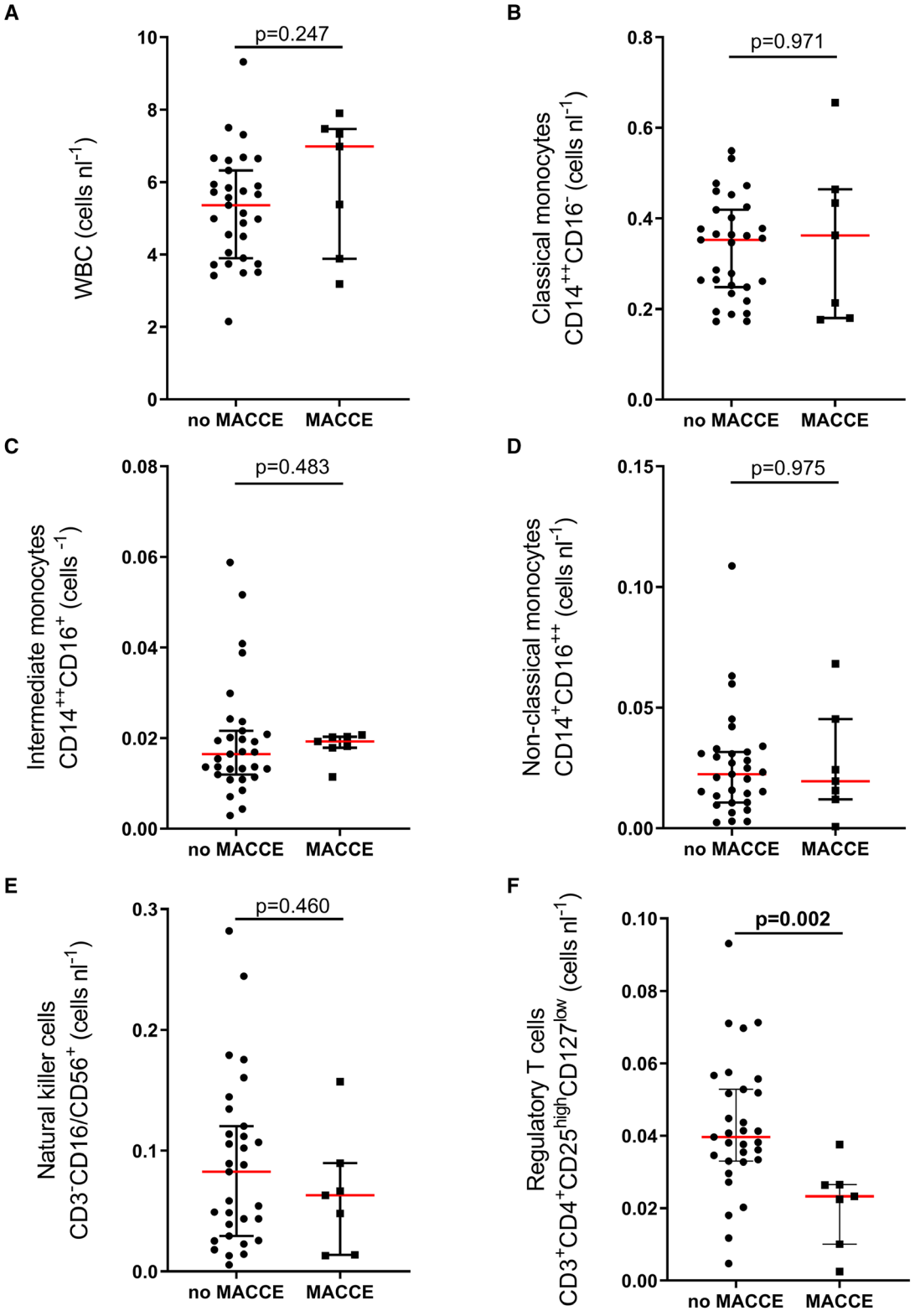

FIGURE 3 **Preoperative leukocyte subset counts in patients with and without 30 d MACCE after noncardiac surgery**. WBCs and 5 different leukocyte subpopulations known to be associated with cardiovascular risk were quantified in 38 coronary artery disease patients before noncardiac surgery. Patients were stratified for no MACCE ($n = 31$) versus MACCE ($n = 7$). Each dot represents an individual patient; horizontal lines indicate the median. Data are presented as median (interquartile range). Two-tailed nonparametric Mann-Whitney *U* test was used to assess statistical significance. To adjust for multiple comparisons, Bonferroni correction was applied. Alpha values <0.008 ($P < 0.05/6$) were considered statistically significant (boldface). (**A**) WBCs, (**B**) classical, (**C**) intermediate and (**D**) nonclassical monocytes as well as (**E**) natural killer cell counts were similar between MACCE and non-MACCE patients. (**F**) Patients who suffered MACCE during 30 d follow-up presented with approximately 2-fold lower preoperative Treg levels compared to patients without MACCE (0.02 [0.01 to 0.03] cells nl-1 vs. 0.04 [0.03 to 0.05] cells nl-1, $P = 0.002$). (MACCE: major adverse cardiovascular and cerebrovascular events; CD: cluster of differentiation; WBC: white blood cell)

TABLE 2 Comparison of test characteristics in the LeukoCAPE-1 and -2 study

Variable	Cutoff	*n* (%)	MACCE (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	OR [95% CI]
LeukoCAPE-1 study									
Tregs (cells nl^{-1})	<0.027	10 (26)	6	86	87	60	96	87	41 [3.8; 430]
LeukoCAPE-2 study									
RCRI	≥2	173 (79)	72	86	26	46	74	49	2.08 [1.01; 4.28]
hs-cTnT (ng l^{-1})	≥14	106 (48)	66	79	71	62	84	74	8.8 [4.65; 16.66]
NT-proBNP (ng l^{-1})	≥300	109 (50)	58	69	63	53	77	65	3.72 [2.09; 6.63]
Tregs (cells nl^{-1})	<0.027	50 (23)	30	36	85	60	68	66	3.22 [1.68; 6.18]

Data are expressed as odds ratios (OR) and 95% CI.MACCE: Major adverse cardiovascular and cerebrovascular events; PPV/NPV: positive/negative predictive value; RCRI: revised cardiac risk index; hs-cTnT: high-sensitive cardiac Troponin T; and NT-proBNP: N-terminal pro-hormone brain natriuretic peptide.

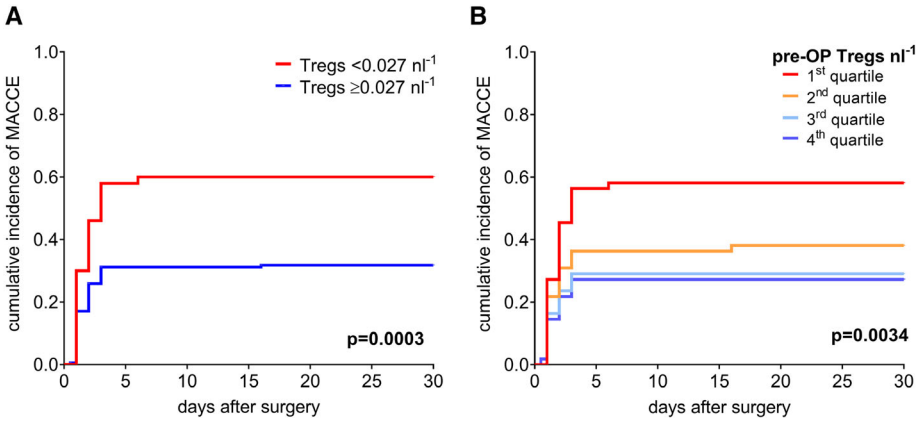

FIGURE 4 **Kaplan-Meier curves.** (**A**) Patients were stratified according to the threshold value of 0.027 Tregs nl^{-1} into two groups: <0.027 Tregs nl^{-1} (red) and ≥0.027 Tregs nl^{-1} (blue). (**B**) Patients were stratified into quartiles (each quartile $n = 55$) according to their preoperative Treg level. Statistical significance was evaluated using log-rank test

TABLE 3 Association between Treg levels and severity of coronary heart disease in the LeukoCAPE-2 study

	Tregs < 0.027 nl^{-1} *n* = 50	Tregs ≥0.027 nl^{-1} *n* = 170	*P* value
Severity of coronary heart disease[a], *n* (%)	46 (100)	149 (100)	
Nonsignificant coronary heart disease	7 (15)	16 (11)	0.436
Single-vessel disease	10 (22)	35 (23)	>0.999
Two-vessel disease	10 (22)	25 (17)	0.510
Three-vessel disease	19 (41)	73 (49)	0.401
History of MI, *n* (%)	19 (38)	64 (38)	>0.999
Time since last MI[b], months	97 [31.5; 212]	53 [18; 163]	0.241
History of PCI, *n* (%)	22 (44)	91 (54)	0.262
Time since last PCI, months	36 [11; 99]	42 [14; 108]	0.825
History of CABG, *n* (%)	12 (24)	38 (54)	0.848
Time since last CABG, months	67 [28; 122]	32 [14; 120]	0.563

Data are presented as absolute numbers (percentages) or median [IQR]. *P* values are calculated using Fisher exact test and Mann Whitney *U* test.
[a]Data were available for 195 patients (46 patients with Tregs < 0.027 nl^{-1} and 149 with Tregs ≥0.027 nl^{-1});
[b]Data were not available in eight patients.
MI: myocardial infarction; PCI: percutaneous coronary intervention; and CABG: coronary artery bypass grafting

mainly resulted from an increased correct reclassification rate within the event group (NRI = 0.25, $P = 0.001$). Category-free IDI was 0.06 ($P = 0.0001$), demonstrating that the difference in the mean predicted probability between subjects with and without MACCE significantly increased when Tregs were added to the RCRI (Figure 6A).

We next considered a combination of RCRI, NT-proBNP, and hs-cTnT as the basic risk model. AUC for the basic risk model was

TABLE 4 Univariable and multivariable logistic regression analysis

	Univariable		Multivariable	
Variable	OR [95% CI]	*P* value	OR [95% CI]	*P* value
Tregs crude (binary, 0.027 cells nl^{-1})	**3.22 [1.68; 6.18]**	**<0.001**	**2.54 [1.22; 5.23]**	**0.012**
Age (years)	1.07 [1.03; 1.11]	<0.001	1.08 [1.03; 1.13]	0.001
Male sex	5.23 [2.1; 12.98]	<0.001	5.25 [1.87; 14.74]	0.002
BMI (kg m^{-2})	0.97 [0.92; 1.02]	0.242		
ASA classification	3.67 [1.49; 9.04]	0.005	3.79 [1.41; 10;18]	0.008
Diabetes mellitus	0.82 [0.46; 1.47]	0.508		
Chronic kidney disease, KDIGO ≥3	2.2 [1.16; 4.19]	0.016		
Atrial fibrillation	1.92 [0.94; 3.91]	0.073		
Stroke	0.79 [0.31; 2.05]	0.631		
History of PCI	2.00 [1.15; 3.48]	0.015	1.78 [0.93; 3.4]	0.08
History of CABG	0.99 [0.52; 1.90]	0.976		
History of myocardial infarction	1.97 [1.12; 3.44]	0.018		
Active Smokers	0.68 [0.34; 1.32]	0.255		
Betablockers	1.53 [0.80; 2.93]	0.202		
ACE inhibitors	1.02 [0.59; 1.76]	0.948		
Diuretics	1.60 [0.93; 2.77]	0.093		
Statins	1.02 [0.52; 2.01]	0.950		
Creatinine (mg dl^{-1})	1.77 [1.12; 2.79]	0.014	1.52 [1.03; 2.24]	0.035
eGFR (ml · min^{-1} · 1.73 m^{-2})	0.98 [0.97; 0.99]	<0.001		
CRP (mg dl^{-1})	1.00 [0.99; 1.01]	0.984		

Data are expressed as odds ratios (OR) with 95% CI. Multivariable logistic regression modeling included factors that revealed a *P* value below 0.1 in univariate analysis and was conducted by means of a forward stepwise (Wald) technique. To avoid redundancy only one criterion for renal impairment was included. We chose creatinine over KDIGO stage, as creatinine is a continuous variable. We did not choose eGFR because it depends on age and gender, two variables already included in the multivariable analysis. Tregs: regulatory T cells; BMI: body mass index; ASA: risk classification according to the American Society of Anesthesiologists; KDIGO: Kidney Disease: Improving Global Outcomes; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; ACE: angiotensin converting enzyme; and eGFR: estimated glomerular filtration rate calculated by Chronic Kidney Disease Epidemiology Collaboration.

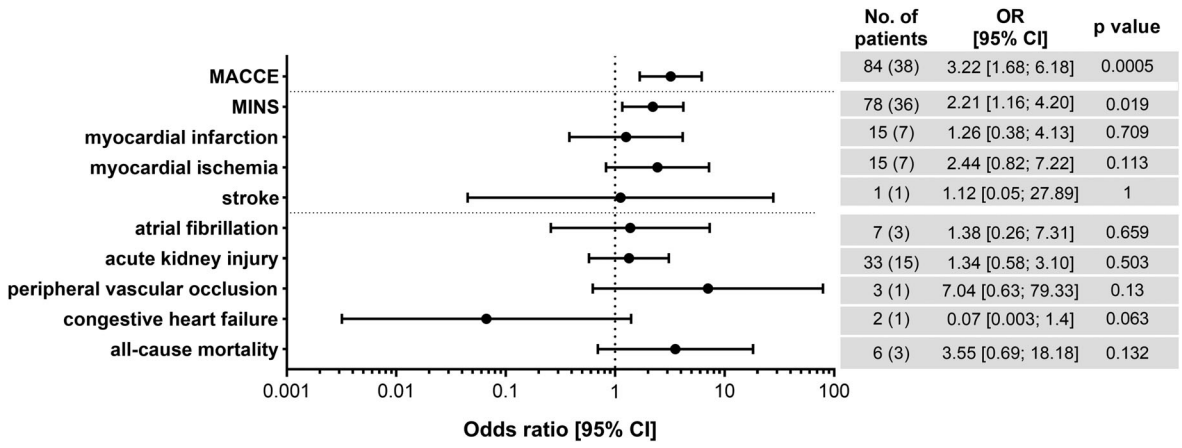

FIGURE 5 **Preoperative Treg levels <0.027 cells nl^{-1} and their association with primary and secondary endpoints in the LeukoCAPE-2 study.** Forest plot illustrating preoperative Treg levels as predictor for the composite primary endpoint MACCE and descriptive analysis of its individual components and other secondary endpoints. Values are absolute numbers of patients experiencing the indicated endpoint ($n = 220$). Relative numbers are reported in parenthesis

0.67 (0.60; 0.74). Combination of the basic risk model with Tregs did not increase the AUC (ΔAUC = 0.001). However, both NRI and IDI revealed a significant improvement in risk classification, when Treg cell counts were added to the basic risk model (total NRI = 0.26, $P = 0.005$; IDI = 0.052, $P < 0.001$; Figure 6B).

4 | DISCUSSION

Here we report the derivation and independent validation of a preoperative cutoff value for circulating Tregs to predict MACCE in coronary artery disease patients undergoing noncardiac surgery. In a

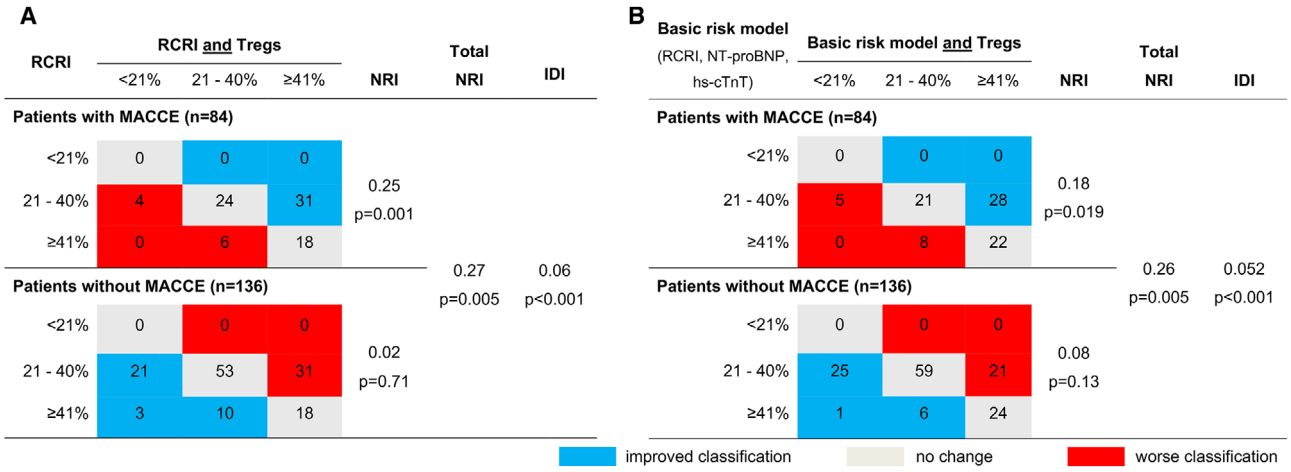

FIGURE 6 **Net reclassification improvement (NRI) and integrated discrimination improvement (IDI) for probability of MACCE.** (A) RCRI compared to RCRI and Tregs: In the event group, adding Tregs to RCRI reclassified 31 patients into a higher and 10 patients into a lower risk category, resulting in a NRI of 0.25 (p=0.001). NRI in the non-event group was 0.02 (p=0.71). The total NRI was 0.26 (p=0.005). Category-free integrated discrimination improvement (IDI) was 0.06 (p<0.001). (B) Basic risk model including RCRI, NT-proBNP, and hs-cTnT compared with the basic risk model and Tregs. In the event group, adding Tregs to the basic risk model reclassified 28 patients into a higher and 13 patients into a lower risk category, resulting in a NRI of 0.18 (p=0.019). NRI in the non-event group was 0.08 (p=0.13). The total NRI was 0.26 (p=0.005). Category-free integrated discrimination improvement (IDI) was 0.052 (p<0.001)

post hoc analysis of the LeukoCAPE-1 study,[18] we identified an association between preoperative Treg counts and MACCE and derived a predictive cutoff value, which we validated in the consecutive LeukoCAPE-2 study including 220 patients. In these two independent studies, we found (i) that patients who experienced MACCE within 30 d postsurgery exhibited reduced preoperative Treg levels compared to patients without MACCE; (ii) that preoperative Tregs < 0.027 cells nl^{-1} were independently associated with an increased risk for perioperative MACCE; and (iii) that addition of Tregs to conventional cardiac biomarker measurements significantly improved preoperative cardiovascular risk category assessment.

We assessed perioperative levels of 6 different leukocyte populations, which are associated with long-term cardiovascular risk. In our cohort, Tregs were the only cell population that differed between patients with and without perioperative MACCE. Numerous experimental and clinical studies have proven a protective role of Tregs in cardiovascular disease. In one previous study, the authors report small preoperative differences for some lymphocyte populations including Tregs in a highly selected patient population, but did not assess the potential for risk prediction.[19]

Tregs constitute 5–10% of all peripheral $CD4^+$ T lymphocytes and are considered negative regulators of cellular immunity.[31] Tregs help maintaining self-tolerance, T cell homeostasis and they are specialized for the suppression of pathogenic immune responses against self- and foreign antigens.[32] It was shown that cardiovascular diseases such as acute MI, unstable and stable angina are associated with significantly reduced numbers of circulating Tregs and compromised immunosuppressive function.[33–38] However, it remained unclear whether decreased Treg levels follow the clinical incident or if a constellation of low Tregs precedes an acute cardiovascular event. Patients recruited in our study were suffering ischemic heart disease but were free of symptoms characteristic for acute cardiovascular events. Additionally, atherosclerotic lesion severity was similar in patients with low versus high Tregs, which is in line with published evidence. As reported in the study by Ammirati et al., intima-media thickness of the common carotid artery did not correlate with Treg levels.[39] In our LeukoCAPE patients, low Treg levels preceded acute perioperative cardiovascular complications indicating that reduced Treg levels may not only be a consequence of ischemia but can also predict cardiovascular complications.

Atherosclerotic lesions are the underlying substrate for cardiovascular events such as the individual components of the primary endpoint MACCE. Rupture of destabilized atherosclerotic plaques is seen in approximately 25% of all perioperative MIs.[40] Nonspecific inflammation induced by conditions such as surgical trauma, pain, or blood loss constitutes a second hit, which might put surgical patients at risk for atherosclerotic lesion destabilization. Recent murine models demonstrate that atherosclerotic plaque volume and vulnerability can rapidly increase in response to acute perioperative stress[41,42] and strategies to quickly stabilize atherosclerotic plaques are conceivable.[43] Accordingly, the general inflammation marker CRP has been proposed as risk factor for cardiovascular events. Interestingly, in our cohort preoperative CRP values did not significantly differ between MACCE and no-MACCE patients.

Tregs were shown to protect from atherosclerotic plaque development and progression in mouse models.[44] In humans, vulnerable atherosclerotic lesions contain significantly less Tregs compared to stable plaques.[45] Therefore, one may speculate that low preoperative Treg levels might even be actively involved in rapid atherosclerotic lesion progression by promoting a state of reduced immunosuppressive function and thereby reflecting the patient's individual susceptibility to the development of perioperative events. However, owed to

their observational design, results from the LeukoCAPE studies can only demonstrate an association.

MACCE rates differed between the two studies (18% vs. 38% for LeukoCAPE-1 vs. LeukoCAPE-2). This is likely because we did use different definitions for the diagnosis of MINS. When we designed the LeukoCAPE-1 study, there was no generally accepted MINS definition available for the hs-cTnT assay used in our study. Therefore, we chose a reasonable definition based on the evidence available at the time. When designing the LeukoCAPE-2 study, the vascular events in noncardiac surgery patients cohort evaluation (VISION) investigators had meanwhile published a definition, based on a cohort from an international large-scale study using the hs-cTnT assay.[26] We chose this definition for the subsequent LeukoCAPE-2 study accepting the likely increase in the event rate compared to LeukoCAPE-1. However, in Figure S3 we provide an additional post hoc analysis of the LeukoCAPE-1 data set using the newer VISION definition of MINS.[26] When using this analysis, MINS as well as MACCE rates were similar for both studies. Also, the threshold for preoperative Treg counts was unaltered with the alternative definition and remained associated with 30 d MACCE (Fig. S3).

Accurate identification of patients at risk of perioperative cardiovascular events is a prerequisite for individual treatment choices. Awareness of potential perioperative risks allows the physician to analyze and communicate the benefit-to-risk ratio of the proposed procedure. Depending on the patient's predicted risk, preoperative cardiovascular optimization, resource allocation and intensified postoperative monitoring may be initialized, thereby improving individual patient outcome. Recent guidelines recommend the RCRI for preoperative cardiac risk evaluation.[12] As the discriminatory power of RCRI is low in high-risk patients,[30] guidelines suggest considering further preoperative biomarker measurements in this population.[12,16] Data from our LeukoCAPE-2 study suggest that conjunctive use of cardiac biomarkers and preoperative Treg levels significantly improves preoperative risk assessment. Established cardiac biomarkers detect patients with preexisting myocardial damage and heart failure. Based on patients' individual immune status, concurrent quantification of preoperative Treg levels identified additional patients prone to the development of new cardiovascular complications; this allowed to correctly reclassify MACCE patients into the high-risk group that were assigned intermediate risk by the basic risk model (RCRI, NT-proBNP, and hs-cTnT). Those individuals would have gone unrecognized with conventional risk prediction. Sensitivity of Tregs for predicting MACCE was relatively low whereas specificity was excellent. Our findings suggest combining the highly specific preoperative Treg values with a sensitive pretest based on RCRI and conventional cardiac biomarkers such as NT-proBNP and hs-cTnT.

A major strength of our analysis is the evaluation of preoperative Treg levels in two independent study populations. The results of our LeukoCAPE-1 study come with some important limitations. First, the analysis was planned post hoc, rendering the results explorative rather than confirmatory. Observational studies are susceptible to the effect of confounding. Whereas the relatively small number of 38 recruited patients was sufficient for the primary analysis of the effect of surgery on circulating leukocyte subsets,[18] it is certainly underpowered to draw scientifically sound conclusions regarding outcomes. However, preoperative Treg levels and corresponding IQRs did not overlap between MACCE and no-MACCE patients, pointing toward an association between low preoperative Treg levels and an increased risk of 30 d MACCE. Next, we aimed to confirm the predictive value of preoperative Treg levels in the subsequent and sufficiently powered LeukoCAPE-2 study. Additionally, we performed multivariable regression analysis to adjust for confounding factors. However, due to the observational design of our study we cannot prove a causative role of Tregs for perioperative cardiovascular events. Studying Tregs in human cardiovascular disease is hampered because different combinations of phenotypic markers are established. We chose the $CD4^+$ $CD25^{high}$ $CD127^{low}$ marker combination to quantify Tregs, as this combination is commonly used in patients suffering coronary heart disease[38,46] and is proposed by the Human Immunophenotyping Consortium.[47] In addition, CD127 inversely correlates with FoxP3.[48–50] Therefore, selecting $CD127^{low}$ leukocytes yields a population highly enriched with $FoxP3^+$ cells. However, we cannot fully exclude that the $CD4^+$ $CD25^{high}$ $CD127^{low}$ marker combination identified $FoxP3^+$ non-Treg cells or that we missed some $FoxP3^+$ Tregs.

In summary, results from our two independent studies suggest that reduced preoperative Treg levels independently predicted 30 d MACCE in elevated cardiovascular risk patients undergoing elective noncardiac surgery and improved the predictive value of current preoperative cardiac risk stratification. Preoperative Treg quantification holds promise to complement preoperative risk evaluation based on cardiac biomarkers. Also, this work will stimulate future research to investigate whether Tregs exert protective functions in perioperative plaque destabilization. If so, active modulation of preoperative Treg levels might constitute a promising new therapeutic target for the prevention of perioperative MACCE.

AUTHORSHIP

The authors contributed in the following manner: study conception and design: A.S.S., J.H., J.M., J.L.; institutional review board submission: J.M., J.L.; blood sample analysis: A.S.S., J.H., Q.Z.; patient recruitment: H.J., S.D., C.A., F.E., J.L.; acquisition of data: A.S.S., J.H., Q.Z., H.J., S.D., C.A., F.E., F.U., M.A.W., J.L.; statistical analysis: A.S.S., J.H., H.-J.G., J.L.; interpretation of data: A.S.S., J.H., H.-J.G., F.U., M.A.W., J.M., J.L.; manuscript preparation: A.S.S., J.H., J.L.; and critical manuscript revision: H-J.G., H.J., Q.Z., S.D., C.A., F.E., A.S., E.G., F.U., M.A.W., J.M. All authors approved the final version of the manuscript. A.S.S. and J.H. contributed equally to this work.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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