Migratory Activity of Circulating Mononuclear Cells Is Associated With Cardiovascular Mortality in Type 2 Diabetic Patients With Critical Limb Ischemia

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OBJECTIVE

Prediction of clinical outcome in diabetic patients with critical limb ischemia (CLI) is unsatisfactory. This prospective study investigates if the abundance and migratory activity of a subpopulation of circulating mononuclear cells, namely, $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells, predict major amputation and cardiovascular death in type 2 diabetic patients undergoing percutaneous transluminal angioplasty for CLI.

RESEARCH DESIGN AND METHODS

A consecutive series of 119 type 2 diabetic patients with CLI was enrolled. $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells were assessed by flow cytometry upon isolation and also after spontaneous or stromal cell-derived factor 1α−directed migration in an in vitro assay. The association between basal cell counts and migratory activity and the risk of an event at 18-month follow-up was evaluated in a multivariable regression analysis.

RESULTS

Time-to-event analysis of amputation (n = 13) showed no association with the candidate predictors. Sixteen cardiovascular deaths occurred during 18 months of follow-up. Abundance of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells was not associated with cardiovascular mortality. Interestingly, in vitro migration of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells was higher in patients with cardiovascular death compared with event-free subjects (percentage of migrated cells median value and interquartile range, 0.03 [0.02–0.07] vs. 0.01 [0.01–0.03]; P = 0.0095). Multivariable regression model analysis showed that cell migration forecasts cardiovascular mortality independently of other validated predictors, such as age, diagnosed coronary artery disease, serum C-reactive protein, and estimated glomerular filtration rate. In this model, doubling of migrated cell counts increases the cardiovascular death hazard by 100% (P < 0.0001).

CONCLUSIONS

The new predictor could aid in the identification of high-risk patients with type 2 diabetes requiring special diagnostic and therapeutic care after revascularization.

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Critical limb ischemia (CLI), the most advanced form of peripheral artery disease, requires foot amputation in 25% of cases within 1 year from the diagnosis. Furthermore, 25% of patients with CLI will die during the same period (1). Unfeasibility of revascularization results in more severe outcomes in terms of major amputation and mortality rates (2). However, our recent survey on 564 consecutive diabetic patients with CLI shows that local and systemic complications are not rare even after successful revascularization, with a reported annual incidence of restenosis and amputation of 6.4 and 2.3%, respectively, and a mortality rate of 11.9% (3). Altogether, acute myocardial infarction, heart failure, and sudden death represented 65% of all-cause deaths in that cohort. This observation is in line with the concept that patients with ischemic diabetic foot have active arterial disease elsewhere in the body (4–11). Therefore, predictors of residual risk of cardiovascular events are urgently needed.

Cellular biomarkers, in contrast to conventional clinical predictors, unify the positive and negative influences on the vascular wall in one biomarker. They are easily accessible and directly involved in pathogenic processes underlining the importance as treatment targets. Recently, attention is focusing on circulating mononuclear cells (MNCs), which comprise a heterogeneous population of cells endowed with regenerative, inflammatory, and scarring activities (12–14). Circulating angiogenic MNCs have been reported to be quantitatively reduced in patients with diabetes. Furthermore, the abundance of $CD34^{pos}$ MNCs that coexpress the vascular endothelial growth factor receptor 2 (or KDR) reportedly predicts the risk of adverse events in subjects with cardiovascular disease (15,16). A more defined population characterized by the coexpression of various surface antigens (i.e., CD34, CD133, KDR, and the stromal cell-derived factor 1α [SDF-1α] receptor CXCR4) has been measured as a potential marker of vascular dysfunction and cardiovascular risk stratification in diabetes (17,18). Functional alterations of circulating MNCs are also gaining attention as predictors of clinical outcome after an acute ischemic event. The SDF-1α/CXCR4 duo plays a pivotal role in this recruitment process, and its dysfunction might be responsible for impaired vascular repair and worse outcomes after an ischemic event. In this respect, we were the first to demonstrate that the failure of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ MNCs to migrate in vitro toward SDF-1α represents a valued predictor of mortality in patients with acute myocardial infarction. Here the failure of regenerative cells to migrate toward a chemoattractant in vitro might mirror the incapacity of the these cells to be recruited at the ischemic myocardium level in vivo, resulting in an imperfect healing and an excess of late adverse events (19). To the best of our knowledge, no study has been conducted to date to verify if circulating MNCs forecast cardiovascular complications in diabetic patients receiving revascularization for CLI. In these subjects, cell migration might reflect an ongoing response to residual ischemia and thus predict local adverse events and cardiovascular death.

RESEARCH DESIGN AND METHODS

See Supplementary Data online for expanded methods and supplementary data.

Study Design

This registered clinical trial (NCT01269580) has been designed to verify the predictive value of the cellular biomarker with respect to two clinical outcomes (major amputation and cardiovascular death) in a cohort of diabetic patients with CLI at 18-month follow-up after successful revascularization by percutaneous transluminal angioplasty (PTA). The study was approved by the Ethics Committee of IRCCS MultiMedica and adhered to the principles of the Declaration of Helsinki.

Subject Inclusion Criteria

All consecutive type 2 diabetic patients referred to the MultiMedica Diabetic Foot Center for revascularization of CLI were deemed eligible, pending provision of informed consent and meeting inclusion and exclusion criteria. Diabetes was defined according to American Diabetes Association criteria. CLI was diagnosed based on TASC 2007 criteria, i.e., pain at rest, and/or ulcer or gangrene due to peripheral artery disease, transcutaneous oximetry at the dorsum of the foot $<$30 mmHg, and/or ankle pressure $<$70 mmHg.

Subject Exclusion Criteria

Exclusion criteria included type 1 diabetes, dialysis, cancer with adverse prognosis in months or chemotherapy, any type and dose of steroid-associated immunosuppression, organ transplantation, recent trauma or surgery, ongoing or planned pregnancy, and lack of consent to participate to the study.

For all the patients, we recorded the following data: age, gender, history of hypertension, actual smoking, lipid profile, fasting plasma glucose, HbA_{1c}, and creatinine levels. History of diabetic retinopathy was identified based on the patient's personal record of previous fundoscopic examination. Diabetic nephropathy was defined as a urinary albumin/creatinine ratio $>$3.4 mg/mmol or an estimated glomerular filtration rate (eGFR) $<$60 mL/min/1.73 m^2. Coronary artery disease was defined as a history of myocardial infarction or angina, confirmed by imaging tests (radionuclide, coronary angiography, computed tomography, or positron emission tomography), available in the patient's medical history documentation. Cerebrovascular disease was defined as a history of stroke. We also recorded data on medications. All patients received the best available treatment according to international guidelines (20).

Clinical Outcomes

Major Amputation

Limb salvage was considered successful when the plantar stance was maintained, even if achieved by tarsal–metatarsal amputation. Conversely, any above-the-ankle amputation was considered a major amputation. Amputation was performed when therapies failed to relieve patients from resting pain or when infected or ischemic gangrene had extended beyond the Chopart joint.

Vital Status and Cause of Death

Cardiac death was deemed when a fatal event occurred after myocardial infarction, ventricular fibrillation, sudden death, or heart failure. Diagnosis of ventricular fibrillation and heart failure was documented by an expert cardiologist during hospitalization and/or ambulatory visits. Ischemic stroke was considered cerebrovascular death. All the other deaths were classified as noncardiovascular.

Analytical Assessment of the Cellular Biomarker

Assessment of cellular count and migration activity was performed by an investigator blind to clinical outcomes.

Cellular Abundance

Thirty milliliters of peripheral blood were withdrawn by venipuncture from each subject enrolled in the study on the morning of the day after PTA. Peripheral blood MNCs were separated by gradient centrifugation (Ficoll, Sigma-Aldrich, St Louis, MO). Cells were then fixed with 0.5% paraformaldehyde; stained for 15 min in the dark with anti-CD34-PE-Cy7, anti-CD45-APC-H7, anti-CXCR4-APC (all from BD, Franklin Lakes, NJ), and anti-KDR-PE (R&D Systems, Minneapolis, MN) antibodies; and analyzed using FACSCanto flow cytometer equipped with FACSDiva software (both BD) according to International Society of Hematotherapy and Graft Engineering guidelines (21). For each test, 1×10^6 total events were analyzed. The abundance of total $CD45^{pos}CD34^{pos}$ and $CD45^{dim}CD34^{pos}CD133^{pos}CXCR4^{pos}$ MNCs was then calculated.

Cell Migration

The in vitro assay was performed by seeding MNCs (5×10^6) onto the filter of a Transwell chamber (Corning, New York, NY; pore size, 3 μm), using SDF-1α (100 ng/mL) as chemoattractant to verify directed migration. The SDF-1α vehicle (endothelial basal medium-2 containing 0.1% BSA) was used in parallel to study spontaneous migratory activity (22). Migrated cells were counted after 18 h. Moreover, nonmigrated and migrated cells were analyzed by flow cytometry using the above-mentioned combination of antibodies to compute the relative abundance of antigenically defined cells within each fraction.

Statistical Analysis

Normally distributed continuous variables were expressed as mean $\pm$ SD and compared between groups using the unpaired Student *t* test. Nonnormally distributed continuous variables were expressed as median (interquartile range) and compared with the Wilcoxon–Mann–Whitney test. Categorical variables were compared using the χ^2 or the Fisher's exact test.

Cumulative incidences of events were estimated according to methods described by Kalbfleisch and Prentice (23), taking into account the competing causes of event. When the event of interest was amputation, death was considered as a competitive event. When the event of interest was death from cardiovascular cause, death from other causes was considered competitive. Time-to-event during the 18 months of follow-up was defined as the time from hospital admission for revascularization to event occurrence. Patients who were free of event at the end of the 18 months of follow-up were censored at that time.

To evaluate the association between basal cell counts and migratory activity and the risk of an event, the event-specific hazard ratios (HRs) and 95% CIs were calculated using a regression model for competitive risks based on Fine and Gray method (24). Basal cell counts and migratory activity were $\log_2$ transformed prior to regression analysis since their distribution was positively skewed. A multiple regression analysis was subsequently performed adjusting for prognostic features, which was significantly associated with the considered event in the univariate analysis.

For each significant basal cell count or migratory activity, we fitted two logistic regression models, one including significant prognostic factors for the event and one including prognostic factors plus the basal cell counts or migratory activity as independent predictors of risk of the event. We used the area under the receiver operating characteristic curve (AUC) to determine the discriminatory capability of the two models. AUCs were compared using a nonparametric approach (25). Moreover, the added predictive value of each studied basal cell counts or migratory activity with respect to other significant prognostic factors was evaluated using the integrated discrimination improvement (IDI) index as described by Pencina et al. (26). This index is based on the difference between the individual risk predicted by a model considering as covariates the significant prognostic factors and the basal cell counts or migratory activity (full model) and a model excluding the latter (reference model). A positive difference for an observed event and a negative difference for an observed nonevent imply better prediction ability of the new model, whereas the opposite implies worse prediction ability.

All reported *P* values are two sided. A *P* value <0.05 was considered statistically significant. Statistical analyses were performed with STATA 11 software (StataCorp, 2009, Stata Statistical Software, Release 11, College Station, TX).

Sample Size

For calculation of sample size, we took into account that the predicted incidence of cardiovascular death in a similar population is 20% (3). In addition, we hypothesized that diabetic patients with circulating progenitor cell counts exceeding the median value will have a mortality for cardiovascular causes of 30% while those below the median will experience a mortality of 10%. Hence, a group size of $\sim$120 patients would be necessary to detect a threefold increase in the annual incidence rate in the high-risk versus low-risk group, with a type 1 error rate $\alpha = 0.05$ and a power of 80%.

RESULTS

Study Population

Baseline clinical characteristics of the 119 diabetic patients with CLI enrolled in the study at the time of hospitalization for revascularization are summarized in Table 1.

Clinical Outcomes

The distribution of single and combined events registered during the 18-month follow-up is shown in Supplementary

Table 1—Characteristics of study population

Number of recruited subjects	119
Male gender	80 (67.2)
Age (years)	71.3 ± 9.3
Duration of diabetes (years)	18.1 ± 11.2
Hypertension	79 (66.4)
Actual smoking	3 (2.5)
TCO_2 prerevasc (mmHg)	17.8 ± 9.9
Complications	
History of coronary artery disease	58 (48.7)
History of retinopathy	25 (21.0)
Medications	
Diet	5 (4.2)
Oral antidiabetic drugs	37 (31.1)
Insulin	77 (64.7)
Statins	62 (52.1)
Antithrombotic agents*	119 (100.0)
Laboratory tests	
CRP (mg/dL)	4.7 ± 6.2
Leukocytes ($\times 10^3/\mu L$)	9.3 ± 3.2
Hemoglobin (g/dL)	11.8 ± 1.8
Hematocrit (%)	36.6 ± 5
Platelets ($\times 10^3/\mu L$)	283.2 ± 108.9
HbA_{1c}, % (mmol/mol)	7.8 (62) ± 1.6 (17.5)
eGFR (mL/min/1.73 m^2)	54.9 ± 22.2

Data are presented as *n* (%) or mean ± SD. Distribution of clinical variables, concomitant diseases, and therapies of the enrolled subjects at baseline. TCO_2 prerevasc, transcutaneous oximetry prerevascularization. *Including at least one of the following treatments: acetylsalicylic acid, ticlopidine, clopidogrel, anticoagulants.

Table 1. No patients were lost to follow-up. Cumulative incidence of amputation was 11%, 95% CI 6–17 (13 events) (Supplementary Fig. 1*A*). Cumulative incidence of cardiovascular mortality was 13%, 95% CI 8–20 (16 events) (Supplementary Fig. 1*B*).

Thirteen patients died from cardiovascular causes in the absence of other events, while three cardiovascular deaths occurred after major amputation. Seven patients died from other causes (5.9%), one after major amputation, which were considered as competitive events in the analysis. No death was registered during the time of hospitalization, but six patients (5.0%) died within the first month after the PTA procedure.

Association Between Clinical Variables and Outcomes

No significant association was detected between clinical variables and major amputation. Instead, when examining the distribution of clinical variables, concomitant diseases, and therapies in relation to fatal cardiovascular events (Table 2), we found that the age of patients who died from cardiovascular causes was significantly higher than that of event-free subjects (78.2 ± 4.9 vs. 69.6 ± 9.4 years; $P = 0.0005$). In addition, serum C-reactive protein (CRP) and eGFR at recruitment were significantly higher in patients incurring fatal cardiovascular events than in event-free subjects (9.2 ± 8.8 vs. 4.0 ± 5.4 mg/dL [$P = 0.0019$] and 158.3 ± 22.2 vs. 42.6 ± 15.4 mL/min/1.73 m^2 [$P = 0.0078$], respectively). In addition, cardiovascular mortality was associated with the concomitant presence of coronary artery disease ($P = 0.037$). All the other clinical variables or therapies did not differ significantly between the two groups.

Cellular Parameters and Outcomes

Amputation was not significantly associated with cell abundance or migratory activity. Likewise, there was no difference in circulating levels of $CD45^{pos}CD34^{pos}$ or $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells between patients who died for cardiovascular causes and event-free patients (Supplementary Fig. 2*B*).

When analyzing the association between mortality and indexes of cells migratory activity, we observed that percentages of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells migrated toward vehicle or SDF-1α were significantly higher in patients with cardiovascular death as compared with event-free subjects ($P = 0.0201$ and 0.0095, respectively), whereas no group differences were found with regard to total or $CD45^{pos}CD34^{pos}$ MNCs (Table 3). Furthermore, patients with percentage of migrated $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells above or equal to the median value had higher cumulative incidence of cardiovascular death (vehicle migrated, 19%; SDF-1α migrated, 22%) as compared with patients with migration values below the median (7 and 4%, respectively). Thus cell migration toward vehicle or SDF-1α was associated with an increased risk of cardiovascular death ($P = 0.041$ and 0.005, respectively) (Fig. 1).

The detected association between cell migration and risk of cardiovascular death was additionally tested in a multivariable model adjusted by features that resulted significant at the univariate analysis (i.e., age, coronary artery disease, serum CRP, and eGFR). Results of the multivariable analysis confirm that percentages of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells migrated toward vehicle or SDF-1α were significantly associated with cardiovascular death (HR = 1.7 [95% CI 1.3–2.4, $P < 0.0001$] for vehicle and HR = 2.0 [95% CI 1.4–2.8, $P < 0.0001$] for SDF-1α). Given that cell values were expressed in $\log_2$ scale, this means that for a doubling of the predictor, the hazard of a cardiovascular death increases by 70 and 100%, respectively. We also examined the interaction of cell migration with the concomitant presence of coronary artery disease by adding product terms to the regression model adjusted for age, CRP, and eGFR. Results of the analysis indicate that the predictive value of cell migration for cardiovascular death does not differ between patients with or without coronary artery disease at recruitment (heterogeneity test, $P = 0.46$ for vehicle and $P = 0.49$ for SDF-1α).

Table 2—Clinical characteristics of patient death for cardiovascular causes

	Event free (n = 96)	Cardiovascular death (n = 16)	P value
Male gender	64 (66.7)	12 (75.0)	0.509
Age (years)	69.6 ± 9.4	78.2 ± 4.9	0.0005
Duration of diabetes (years)	17.9 ± 11	16.5 ± 9.9	0.6505
Hypertension	61 (63.5)	13 (81.3)	0.166
Actual smoking	3 (3.1)	0 (0.0)	1.000*
TCO_2 prerevasc (mmHg)	18.5 ± 10.2	15.5 ± 8.8	0.2735
Complications			
History of CAD	45 (46.9)	12 (75.0)	0.037
History of retinopathy	19 (19.8)	5 (31.3)	0.329*
Medications			
Diet	4 (4.2)	1 (6.3)	0.544*
Oral antidiabetic drugs	32 (33.3)	2 (12.5)	0.141*
Insulin	60 (62.5)	13 (81.3)	0.145
Statins	52 (54.2)	8 (50.0)	0.757
Antithrombotic agents†	96 (100.0)	16 (100.0)	1*
Laboratory tests			
CRP (mg/dL)	4.0 ± 5.4	9.2 ± 8.8	0.0019
Leukocytes ($\times 10^3/\mu L$)	9.4 ± 3.3	9.0 ± 3.3	0.6583
Hemoglobin (g/dL)	11.7 ± 1.8	11.8 ± 1.7	0.8617
Hematocrit (%)	36.5 ± 4.9	37.7 ± 4.5	0.3536
Platelets ($\times 10^3/\mu L$)	290.6 ± 109.2	256.1 ± 99.8	0.2394
HbA_{1c} (%)	7.8 ± 1.6	7.9 ± 1.2	0.8479
eGFR (mL/min/1.73 m^2)	58.3 ± 22.2	42.6 ± 15.4	0.0078

Data are presented as n (%) or mean ± SD. Distribution of clinical variables, concomitant diseases, and therapies between patients dead for cardiovascular causes (n = 16) and event-free patients (n = 96) during the 18 months of follow-up. TCO_2 prerevasc, transcutaneous oximetry prerevascularization; CAD, coronary artery disease. *Fisher's exact test. †Including at least one of the following treatments: acetylsalicylic acid, ticlopidine, clopidogrel.

Finally, we verified the additive value of the cell predictor by comparing individual risks of cardiovascular death assessed by a reference model considering only age, coronary artery disease, CRP and eGFR and a full model including also $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cell migration. The predictive value measured by AUC of a reference model was 0.8961. The inclusion in the above-referenced model of the $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cell migration toward vehicle yielded a gain in AUC of 0.0281 (P = 0.4183) while cell migration toward SDF-1α yielded a gain in AUC of 0.0238 (P = 0.3989), with the difference being not statistically significant in both cases.

As shown in Supplementary Fig. 3, significant improvement in predictive ability of the full model with respect to the model considering only clinical variables was observed (IDI = 0.179, SE = 0.061, and P = 0.0034 for $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cell migration toward vehicle and IDI = 0.195, SE = 0.060, and P = 0.00108 for $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cell migration toward SDF-1α).

CONCLUSIONS

Diabetic patients with CLI bear high risk of amputation and death. Limb revascularization reduces the rate of amputation and mortality but cannot stop the progression of vascular disease in other districts. Therefore, apart from recurrence of local ischemic complications due to restenosis, the major burden is represented by cardiac disease and stroke, which together account for ~77% of postrevascularization deaths (2,27,28). In the cohort studied here, major amputation was required in 13 patients (incidence per year, 9.1%). In addition,

Table 3—Migration of circulating MNCs in patients with or without events

	Event free (n = 96)	Cardiovascular death (n = 16)	P value
Total MNCs (number of MNCs)			
Migrated cells vs. vehicle	314,583.5 (200,000–512,500)	306,250 (210,416.5–462,500)	0.8549
Migrated cells vs. SDF-1α	446,875 (265,625–712,500)	491,666.5 (269,791.5–711,458.5)	0.8581
Antigenically defined MNCs (%)			
$CD45^{pos}CD34^{pos}$ nonmigrated cells vs. vehicle	0.09 (0.05–0.17)	0.07 (0.05–0.16)	0.5554
$CD45^{pos}CD34^{pos}$ migrated cells vs. vehicle	0.08 (0.05–0.2)	0.13 (0.09–0.43)	0.0837
$CD45^{pos}CD34^{pos}$ nonmigrated cells vs. SDF-1α	0.11 (0.06–0.18)	0.06 (0.04–0.16)	0.2197
$CD45^{pos}CD34^{pos}$ migrated cells vs. SDF-1α	0.09 (0.05–0.14)	0.1 (0.06–0.26)	0.4241
$CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ nonmigrated cells vs. vehicle	0.01 (0.01–0.03)	0.03 (0.01–0.07)	0.0709
$CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ migrated cells vs. vehicle	0.01 (0.01–0.04)	0.04 (0.02–0.11)	0.0201
$CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ nonmigrated cells vs. SDF-1α	0.01 (0.01–0.03)	0.02 (0.01–0.03)	0.3419
$CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ migrated cells vs. SDF-1α	0.01 (0.01–0.03)	0.03 (0.02–0.07)	0.0095

Data are presented as median (interquartile range). Distribution of cell migration-associated variables between patients dead from cardiovascular causes during the 18 months of follow-up (n = 16) and event-free patients (n = 96). Nonmigrated cells are nonmigrated cells found in the upper chamber of the migration chamber.

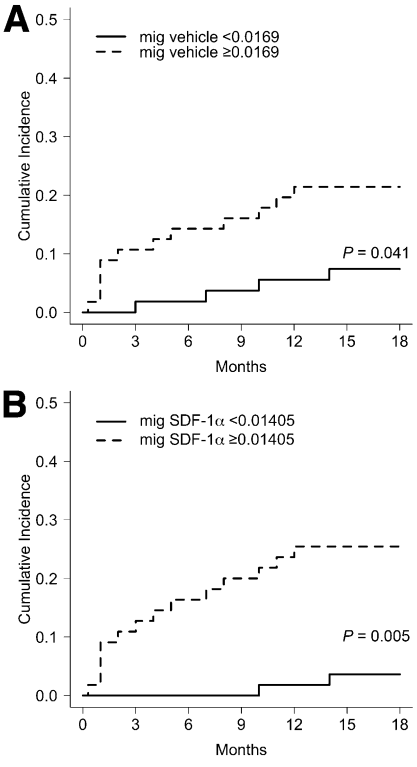

Figure 1—Association between cell migration and cardiovascular mortality. Cumulative cardiovascular mortality during the 18-month follow-up in groups categorized according to median values of migration of $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ cells toward (*A*) vehicle and (*B*) SDF-1α. Dotted lines indicate the cumulative incidence in the groups with migration values equal or above the median value, while continuous lines represent the incidence in the groups with migration values below the median. mig, migrated cells vs.

23 patients died during the 18 months of follow-up (annual death rate, 14.8%): 70% of cardiac or cerebrovascular accidents. These data are in line with our previous report showing an annual mortality rate of 11.9% after revascularization (3). However, the annual incidence of amputation was higher in the present series as compared with our previous study, probably due to the longer follow-up of the latter. In fact, the two studies showed similar cumulative incidences of major amputation during the first 18 months of follow-up when the majority of local adverse events occur.

Investigation of predictors of residual risk indicates that age, CRP, creatinine, and history of cardiac disease are independently associated with death in diabetic patients after revascularization of CLI (3,7,10). Our study confirms the utility of the above markers in prediction of mortality. Furthermore, it newly shows that cellular biomarkers can provide additional indication of clinical outcomes after revascularization.

Cellular biomarkers are attracting the attention of scientists and care providers. In patients carrying multiple cardiovascular risk factors, a reduced count of $CD34^{pos}$ MNCs or $CD34^{pos}KDR^{pos}$ MNCs reportedly help with refining the prediction of major adverse cardiovascular events as compared with a model based on classical clinical and laboratory predictors (15,16,29). This is true also for diabetic patients, for whom $CD34^{pos}$ cell count is known to be decreased, with this trend being associated with the progression of vascular complications (18). $CD34^{pos}$ MNCs represent a heterogeneous class of hematopoietic cells at different differentiation stages and with different implications for vascular remodeling (12). Therefore, analysis of more specific populations might increase the accuracy of the predictor. Of note, our study could not detect any association between $CD34^{pos}$ MNCs, either in terms of abundance or migratory activity, and post-PTA adverse events. In contrast, we newly show that the migratory activity of a more defined population of circulating progenitor cells, namely, $CD45^{dim}CD34^{pos}CXCR4^{pos}KDR^{pos}$ MNCs, independently predicts cardiovascular death. Interestingly, the cell biomarker failed to forecast the risk of amputation. Therefore, the new predictor might reflect the activity and severity of vascular disease in organs like the heart and brain. Moreover, cells were collected after revascularization. Hence we cannot exclude that improvement of local perfusion might attenuate the association of the cellular biomarker with outcomes directly related to peripheral vascular disease.

Silent myocardial ischemia is frequent in diabetic patients. The different patterns of symptoms in patients with diabetes could be explained by higher thresholds of pain sensitivity, psychological denial, or the presence of autonomic neuropathy leading to sensory denervation. Importantly, the predictive value of cell migration for cardiovascular death does not differ between patients with or without symptomatic coronary artery disease. Therefore, the biomarker can help identify patients in whom cardiac disease risk is underestimated. Furthermore, model analysis indicates that measurement of cell migration may add incremental predictive value to standard risk assessment based on biochemical predictors.

In conclusion, results of this prospective study show that progenitor cell migration can aid current assessment of cardiovascular risk in patients with type 2 diabetes undergoing revascularization of CLI.

Study Implications and Limitations

In our previous study on patients with acute myocardial infarction, the lack of migration activity in vitro was associated with a higher mortality risk (19). This association could reflect an impaired recruitment of regenerative cells to the ischemic heart, resulting in imperfect myocardial healing. Differently, in the present cohort of CLI patients, the assessment of cell migration was performed after removal of the critical stenosis at the lower limb level. A migration index above the median might implicate the presence of an active ischemic disease elsewhere in the body, which could manifest later on with cardiovascular death. Therefore, data from the two studies should be compared with caution, as they reflect profoundly different clinical situations. Analysis of the cumulative incidence of death based on consideration of the new cell biomarker indicates the presence of two classes of patients with low and high cardiovascular risk. Hence, an important implication of this study is that additional diagnostic and therapeutic care is necessary in the high-risk class. Whether cell-based risk categorization can be extended to the patients who do not receive revascularization remains unknown. Likewise, our study was not designed to dissect pathophysiological mechanisms; therefore additional investigation is required to unravel determinants of the observed association between cell migration and death.

A limitation of the current study is that the multivariable model was based on 16 events, and it is therefore possible that that model was overfit. Moreover, with regard to clinical exploitation, the new biomarker would be accessible only to clinical centers with the capacity to perform cell biology studies and multicolor flow cytometry. Therefore, an independent validation in a larger set of patients, coupled with calculation of the cost-to-benefit ratio, should be performed before proposing the test for routine use in the clinical practice.

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