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MicroRNA-21/PDCD4 proapoptotic signaling from circulating CD34+ cells to vascular endothelial cells: a potential contributor to adverse cardiovascular outcomes in patients with critical limb ischemia

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Abstract

Objective. In patients with type 2 diabetes (T2D) and critical limb ischemia (CLI), migration of circulating $CD34^+$ cells predicted cardiovascular mortality at 18 months post-revascularization. This study aimed to provide long-term validation and mechanistic understanding of the biomarker.

Research Design and Methods. The association between $CD34^+$ cell migration and cardiovascular mortality was reassessed at 6 years post-revascularization. In a new series of T2D-CLI and control subjects, immuno-sorted bone marrow (BM)-$CD34^+$ cells were profiled for microRNA expression and assessed for apoptosis and angiogenesis activity. The differentially regulated microRNA-21, and its pro-apoptotic target PDCD4, were titrated to verify their contribution in transferring damaging signals from $CD34^+$ cells to endothelial cells.

Results. Multivariable regression analysis confirmed $CD34^+$ cell migration forecasts long-term cardiovascular mortality. $CD34^+$ cells from T2D-CLI patients were more apoptotic and less proangiogenic than controls and featured microRNA-21 downregulation, modulation of several long non-coding RNAs acting as microRNA-21 sponges, and upregulation of the microRNA-21 proapoptotic target PDCD4. Silencing miR-21 in control $CD34^+$ cells phenocopied the T2D-CLI cell behavior. In coculture, T2D-CLI $CD34^+$ cells imprinted naïve endothelial cells, increasing apoptosis, reducing network formation, and modulating the TUG1 sponge/microRNA-21/PDCD4 axis. Silencing PDCD4 or scavenging ROS protected endothelial cells from the negative influence of T2D-CLI $CD34^+$ cells

Conclusions. Migration of $CD34^+$ cells predicts long-term cardiovascular mortality in T2D-CLI patients. An altered paracrine signalling conveys anti-angiogenic and pro-apoptotic features from $CD34^+$ cells to the endothelium. This damaging interaction may increase the risk for life-threatening complications.

Introduction

The chemokine stromal-derived factor-1 (SDF-1) participates in cardiovascular repair through the mobilization of bone marrow (BM)-derived $CD34^+$ progenitor cells that express the CXCR4 receptor. $CD34^+CXCR4^+$ cells positively interact with vascular endothelium by releasing trophic soluble factors and extracellular vesicles (EVs). Risk factors, ageing, and age-related diseases compromise this homeostatic mechanism by perturbing the BM microenvironment[1, 2]. Interestingly, both biased myelopoiesis and deficit/dysfunction of $CD34^+$ cells are associated with an increased risk of cardiovascular morbidity and mortality[3-10].

We showed that $CD34^+$ cell migration predicted cardiovascular mortality in patients with type 2 diabetes (T2D) undergoing revascularization of critical limb ischemia (CLI) [10]. Phenotypic changes in $CD34^+$ cells may cause systemic vascular damage in these high-risk patients through anti-angiogenic and pro-apoptotic microRNAs (miRs)[10-13].

The present study investigated (1) if $CD34^+$ cells predict cardiovascular mortality long-term and (2) how $CD34^+$ cells cause vascular damage instead of repair.

Methods

See Supplementary Data online for supplemental Tables and Figures

Study 1: CD34$^+$ cell migration predicts long-term mortality

We performed a phone survey of T2D-CLI patients recruited in the NCT01269580 study, which demonstrated that migration of peripheral blood (PB) CD45^{dim}CD34$^+$CXCR4$^+$KDR$^+$ cells predicted cardiovascular death after angioplasty[10]. At 6 years,15 patients were lost to follow-up and therefore excluded. Baseline characteristics of the remaining 104 patients are summarized in **Supplemental Table 1**.

Study 2: Molecular mediators of CD34$^+$ cell-induced vascular damage

Clinical characteristics of a new cohort, comprising 47 controls and 41 patients with T2D, of which 30 affected by CLI, are reported in **Supplemental Table 2**. T2D and CLI were defined according to the American Diabetes Association and TASC 2007, respectively. Exclusion criteria: acute disease/infection, immune diseases, current/past hematological disorders or malignancy, unstable angina, recent (within 6 months) myocardial infarction or stroke, liver failure, renal failure, and pregnancy. All participants signed an informed consent to donate the BM leftovers from femoral head otherwise discarded during hip replacement surgery (ctr and subjects with T2D without complications) or BM aspirates of the iliac crest performed ad hoc for the study (T2D-CLI patients).

The studies received ethical authorization from the IRCCS MultiMedica, Milan, Italy (PROT 20/2010), Bristol University, Bristol, UK (REC14/SW/1083 and REC14/WA/1005), and Santa Maria 'Ca Foncello Hospital, Treviso, Italy (DDG 2333/2017)[13, 14]. BM-CD34$^+$ cells were assessed *in situ* or following immunomagnetic beads isolation as described in [13]. Some assays were conducted using the CD34$^+$ cells-derived conditioned medium (CCM) and respective EVs collected using Exospin kit (Cell Guidance System) following the manufacturer's instructions.

MicroRNAs and gene expression analyses of CD34⁺ cells

MiR profiling was conducted at Exiqon Services, Denmark. Total RNA (30ng) was reverse-transcribed using the miRCURY LNA™ Universal RT miR PCR, Polyadenylation and cDNA synthesis kit (Exiqon). The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. A total number of 372 miRs were tested. A cut-off (Ct>37) was used to consider a miR as expressed and included in the subsequent analyses.

For biological validation and expressional studies, RNA was extracted from cultured cells, cell-derived CCM/EVs, plasma, and plasma-derived EVs using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Quantitative RT-PCR was performed with the QuantStudio 6 Flex Real-Time PCR using miR TaqMan probes (Thermo Fisher) and lncRNA/gene primers listed in **Supplemental Table 3**. MiR-21 was silenced in CTRL-BM-CD34⁺cells by transfecting them with 50nmol/L anti-miR-21-5p (AM10206Ambion) while controls were transfected with a non-targeting sequence or scramble (SCR) (AM4611, Silencer® Negative Control, Ambion), employing transfection reagents (Genlantis) according to the manufacturer's protocol.

In vitro migration and flow cytometry

PB-MNCs *in vitro* migration assays using SDF-1 as chemoattractant were performed using transwell chambers as described[10]. Migrated cells were stained for surface antigens CD45 (FITC, BD Bioscience), CD34 (PeCy7, BD Bioscience), CXCR4 (APC, BD Bioscience), then fixed in Met-OH, permeabilized in PBS-Tween 0.1%-BSA 0.5% and stained for intracellular antigen PDCD4 (Abcam) with secondary antibody anti-rabbit-PE (BD Bioscience). Cells were analyzed using a FACSCanto flow cytometer with the FACSDiva software (both from BD Biosciences)

Apoptosis assay

BM-CD34⁺ cells or HUVECs were stained with 7-AAD/Annexin-V and analyzed on FACSCanto II flow cytometer (BD Biosciences). The 7-AAD⁻/Annexin V⁺ combination identified apoptotic

cells. Caspase-3/7 Assay (Promega) was performed on HUVECs exposed to $CD34^+$ cells CCMs.

PDCD4 silencing

HUVECs were transfected with 50 nmol/L siPDCD4 (Dharmacon) or non-targeting sequence, (AM4611, Silencer Negative Control, Ambion).

Immunohistochemistry

Paraffin embedded-BM sections were incubated with: rabbit anti-PDCD4 (1:200; abcam), mouse anti CD34 (1:5, Dako), rabbit anti 4-Hydroxy-2-Nonenal (4-HNE) (1:500, Bioss). Nuclei were counterstained with DAPI (1 µg/mL, Sigma Aldrich). Images were acquired with a fluorescent microscope (Leica microsystem DM6 B) at 63X magnification and analyzed using Fiji software. Cultured HUVECs were fixed with 4% PFA (Electron Microscopy Science), permeabilized with 0.3% triton (Sigma Aldrich), and stained with rabbit anti-PDCD4 (1:200). Nuclei were counterstained with DAPI (1µg/mL, Sigma Aldrich). Microphotographs were captured using a Zeiss microscope equipped with digital image processing software (AxioVision Imaging System).

Immunoistochemistry: BM sections were stained with rabbit anti-PDCD4 (1:200) by BenchMark ULTRA system (Roche). Ten images for each sample were acquired with an optical microscope (Nikon Eclipse E800) at 40X magnification and quantified by using ImageJ software.

In vitro angiogenesis

BM-$CD34^+$ cells, their CCM, or isolated EVs were cocultured with HUVECs (1:1 ratio) on Matrigel™ (Corning® Incorporated Life Sciences)[15].

Statistical analyses

Continuous variables were expressed as mean ± standard deviation or standard error as indicated, tested for normality by the Kolmogorov-Smirnov test, and compared using parametric (t test or ANOVA) or non-parametric tests (Wilcoxon or Kruskal Wallis), as

appropriate. Categorical variables were expressed as frequency and percentage, and compared by Chi-Square test or Fisher's Exact test. A p-value < 0.05 was considered statistically significant. SAS (version 9.4), R (version 3.4.4), and GraphPad Prism (version 7) were used for analyses and graphics.

In study 1, cumulative incidences of events were drawn overall and for data stratified by cells (above vs. below the median) that significantly differed between participants with or without events. This analysis considered the competitive causes of the event;[16] specifically, in the case of cardiovascular death, other-causes-of-death were considered as a competitive event, and *vice-versa.* Comparisons between incidence curves were assessed fitting the 'proportional sub-distribution hazards' regression model[17]. Time-to-event was defined as the time from revascularization to death (cardiovascular or for other causes). Patients lost to follow-up were excluded from the analyses. The 15th day of a given month and the month of June were imputed if the day or month of follow-up were missing, respectively. Incidence rate and 95% Confidence Interval (CI) at 3 years and 6 years of follow-up were calculated for cardiovascular death and other-causes-of-death.

To evaluate the association between basal cell counts and migratory activity and risk of death, the event-specific hazard ratio (HR) and 95% CI was calculated. HRs associated with cell migration were evaluated for one-year increase, for presence of history of coronary artery disease, and for 0.01 unit increase in the percentage of $CD45^{dim}CD34^{+}CXCR4^{+}KDR^{+}$ migrated cells toward SDF-1 over total MNCs. All models were performed for presence of investigated variable, if dichotomous, and for 1-unit increase of continuous variables, if not otherwise specified. A multivariable regression model was subsequently implemented, adjusting for prognostic features that were found significantly associated with the event in the univariate analysis.

Results

CD34$^+$ cell migration and cardiovascular mortality

Supplemental Table 1 illustrates clinical/laboratory data of the 104 T2D-CLI patients who completed the 6-year follow-up.

Three outcomes were considered: no-event (N=54), cardiovascular death (N=32), and other-causes-of-death (N=18). Age at recruitment was the only clinical data that differed among the 3 outcomes (p=0.0067) (**Supplemental Table 4**). Regarding CD45^{dim}CD34$^+$CXCR4$^+$KDR$^+$ cells, migration toward SDF-1 (experimental setting illustrated in **Figure 1Ai**) was higher in the cardiovascular death group compared with no-event or other-causes-of-death groups (p=0.0312), whereas there was no difference in PB levels of CD45^{dim}CD34$^+$CXCR4$^+$KDR$^+$ cells or in the migration of total MNCs and CD45^{dim}CD34$^+$CXCR4$^+$KDR$^+$ cells exposed to the SDF-1 vehicle (**Supplemental Table 5** and **Supplemental Figure 1**).

As shown in **Figure 1Aii** and **Supplemental Table 6**, patients with values of SDF-1-migrated cells ≥ the median had higher cumulative incidence of cardiovascular death compared with those with values < median (p=0.0012). Cell migration was associated with an increased cardiovascular risk (HR; 95%CI=1.10; 1.04-1.17. p = 0.0005, data not shown), further confirmed by multivariable Cox analysis simultaneously assessing the effect of age and prevalence of coronary artery disease (**Supplemental Table 7**).

BM-CD34$^+$ cell viability and angiogenic activity

To obtain mechanistic insights into the observed association, we studied the functional characteristics of BM-CD34$^+$ cells from a new series of patients with T2D or T2D-CLI and non-diabetic subjects (controls).

In line with previous studies[13], flow cytometry analyses demonstrated that CD34$^+$ cells immune-magnetically sorted from the BM of T2D-CLI patients have a 2.5-fold higher abundance of 7-AAD$^-$/Annexin V$^+$ events compared with controls (**Figure 1B**).

Moreover, using an *in-vitro* Matrigel assay, we demonstrated that the coculture of human umbilical vascular endothelial cells (HUVECs) with T2D-CLI BM-CD34$^+$cells resulted in a lower number of branches compared with the coculture of HUVECs and control BM-CD34$^+$ cells (**Figure 1C**). A marked decrease in endothelial network formation was also observed when incubating HUVECs with T2D-CLI BM-CD34$^+$ cell-derived CCM or EVs (**Figure 1C**). These data demonstrate that BM-CD34$^+$ cells from T2D-CLI patients have reduced viability and can transfer destabilizing signals to endothelial cells through factors secreted as soluble molecules or packaged in EVs.

MiR signature in BM-CD34$^+$ cells

Diabetes influences the expression of several miRs in hematopoietic cells.[11-13] An unbiased miR profiling of CD34$^+$ cells isolated from the BM of controls (N=6) and T2D patients with (N=6) or without CLI (N=7) identified a suppressive effect of T2D on the quantity of expressed miRs (**Supplemental Figure 2A&B**), which might be attributable to the downregulation of Dicer (**Supplemental Figure 2C&D**), an endoribonuclease involved in miRNA maturation, as also described by others[18].

Supplemental Table 8 shows that 56 miRs were commonly expressed in the three groups. Moreover, two sets of 11 different miRs were shared by controls and T2D-CLI patients or controls and T2D, respectively. Of those not shared, 49 were unique to controls and 1, namely miR-146a, to T2D-CLI, whereas none was exclusive to T2D. As shown in **Supplemental Table 9**, 18 miRs were differentially expressed in cells from T2D subjects, with or without CLI, compared with controls, with two of them, miR-21 and miR-30e, being shared by the two T2D groups. The heatmap diagram in **Figure 2A** indicates a marked separation of miR expression in T2D-CLI compared with controls, while T2D values were spread between controls and T2D-CLI. Among the miRs that showed a differential expression between T2D-CLI and controls, we studied a set of 6 miRs (miR-125a, miR-222, let-7e, miR-93, miR-21, and miR-30e) known to control cell survival, differentiation, hematopoiesis, and angiogenesis

(**Supplemental Table 10**). To validate the profiling results, we performed single PCR analyses for the 6 miRs of interest on BM-CD34$^+$ cells isolated from a new set of donors (controls, N=5; T2D, N=4; T2D-CLI, N=4) using an Applied Biosystem platform and normalizing miR expression to U6snoRNA, which showed a stable expression among the 3 groups. Three miRs from the set of choice, miR-125a, miR-21, and miR-30e, were significantly modulated in the new cohort (**Figure 2B**).

Downregulation of miR-21 associated with reciprocal changes in its target PDCD4

MiR-21, one of the most highly expressed miRs in mammalian cells, is modulated in cardiovascular disease.[19-23] However, little is known regarding the expression of miR-21 in hematopoietic progenitor cells. Data of RT-PCR confirmed the downregulation of mature miR-21 in sorted T2D-CLI BM-CD34$^+$ cells (**Figure 2Ci**), whereas the levels of the corresponding pri-miR were similar to controls (data not shown). MiR-21 was also found in CD34$^+$ cell-derived CCMs and EVs, but no difference was seen when comparing T2D-CLI patients and controls (**Figure 2Ci**). Interestingly, miR-21 levels were decreased in plasma and EVs isolated from the PB of T2D-CLI patients (**Figure 2 Cii**).

Long ncRNAs can act as miR sponges, thereby interfering with regulation of miR targets. We investigated the expression of several long ncRNAs reportedly implicated miR-21 modulation [24-28]. As shown in **Figure 2Ci**, TALNEC2 was upregulated in T2D-CLI BM-CD34$^+$ cells and respective CCM; TUG1 was decreased in cells and increased in the CCM; MEG3 was not altered; and TCONS was downregulated in CCM. These data suggest that TALNEC2 could inhibit miR-21 at intracellular level, and, together with TUG1, at extracellular level. Moreover, all the studied sponges were upregulated either in plasma, EVs, or both, suggesting they may synergize in inhibiting miR-21 in the circulation (**Figure 2Cii**).

The pro-apoptotic factor PDCD4 is a validated target of miR-21 [19]. In line with the miR-21 downregulation, we found higher *PDCD4* mRNA levels in T2D-CLI BM-CD34$^+$ cells (**Figure 2D**). Likewise, *in-situ* immunohistochemistry confirmed the higher expression of PDCD4 in the

BM (**Figure 2E**). Altogether, these data point at a novel molecular mechanism involving the downregulation of miR-21 and induction of PDCD4 in T2D-CLI $CD34^+$ cells.

Migration toward SDF-1 enriches a population of $CD34^+/CXCR^+/PDCD4^+$ cells

$CD34^+CXCR4^+$ cells represented a small fraction (1.5±0.2%) of the total $CD34^+$ cell population in PB of T2D-CLI patients. PDCD4 was more abundant in the $CD34^+CXCR4^+$ (77.9±0.4%) than in the $CD34^+CXCR4^-$ cell fraction (5.8±0.2%). Moreover, SDF-1-stimulated migration of PB-MNCs resulted in an enrichment of cells expressing both CXCR4 and PDCD4 in the migrated fraction (**Supplemental Figure 3**).

Silencing miR-21 in control BM-$CD34^+$ cells recapitulates the negative features of T2D-CLI $CD34^+$ cells

Next, we sought confirmation of a direct link between T2D-CLI-associated miR-21 downregulation and BM-$CD34^+$ cell dysfunction. To this aim, we silenced miR-21 in control BM-$CD34^+$ cells using an anti-miR strategy. The effective miR-21 knock-down (**Figure 3A**) was associated with *PDCD4* upregulation (**Figure 3B**), and increased apoptotic events compared with SCR-treated cells (**Figure 3C**). Moreover, miR-21 silencing conferred anti-angiogenic properties to $CD34^+$ cells as well as to the $CD34^+$ cell-derived CCM and EVs (**Figure 3D**).

Transfer of pro-apoptotic miR-21/PDCD4 signaling to endothelial cells

We next assessed if the negative crosstalk between $CD34^+$ cells and endothelial cells involves the direct transfer of miR-21 or PDCD4 or is mediated by associated factors. To this purpose, using a protocol illustrated in **Figure 3E**, we measured the relative expression of miR-21 and PDCD4 in HUVECs exposed to the CCM from control $CD34^+$ cells (either naïve or transfected with anti-miR-21 or SCR) or to the CCM from T2D-CLI $CD34^+$ cells. Interestingly, plotting the expressional values of miR-21 and PDCD4 from the 4 groups demonstrated an inverse relationship between the miR and its target (**Figure 3E**). This indicates that modulation of the miR-21/PDCD4 duo in BM-$CD34^+$ cells can induce similar expressional changes in the exposed endothelial cells. Next, employing a protocol illustrated in **Figure 3F**, we

demonstrated that *PDCD4* silencing in HUVECs remarkably reduced the apoptosis caused by the exposure to the CCM from T2D-CLI CD34$^+$ cells (**Figure 3F**). A dedicated ELISA could not detect PDCD4 in the CD34$^+$ cells-derived CCM. This data suggests that CD34$^+$ cells induce apoptosis in HUVECs through different paracrine mechanisms.

Implication of TUG1 and oxidative stress

The presence of miR-21 sponges in the CCM from T2D-CLI CD34$^+$ cells suggested they could act paracrinally to inhibit miR-21 in endothelial cells. Therefore, we assessed TALNEC2, TUG1, MEG3, and TCONS in HUVECs either naïve or exposed to the CCM from control or T2D-CLI CD34$^+$ cells. Results confirmed the downregulation of miR-21 and the induction of PDCD4 by the T2D-CLI CCM and demonstrated TUG1 was the only upregulated long ncRNA in conditioned HUVECs (**Figure 4A**).

Reciprocal interactions exist between long ncRNAs, and ROS production and scavenging[29]. Relevant to our study, H_2O_2 and hypoxia reportedly induced TUG1 in cardiomyocytes, thereby increasing ROS production and apoptosis[30]. Hence, we hypothesized that oxidative stress could be involved in the transfer of pro-apoptotic signaling to endothelial cells. This was confirmed by multiple evidences: (1) T2-CLI BM-CD34$^+$ cells showed elevated *in situ* 4-HNE staining, a marker of oxidative damage and lipid peroxidation (**Figure 4B**), (2) treatment of HUVECs with increasing H_2O_2 concentrations induced miR-21 inhibition and PDCD4 upregulation (**Figure 4C**), and (3) ROS scavenging with N-acetylcysteine (NAC) blocked the miR-21 downregulation in HUVECs exposed to the CCM from T2D-CLI CD34$^+$ cells (**Figure 4Di**) or to the CCM from anti-miR-21-transfected control CD34$^+$ cells (**Figure 4Dii**). In parallel, NAC abrogated the induction of PDCD4 by the CCMs (**Figure 4Diii** and **Figure 4Div**). It also counteracted the apoptosis of HUVECs exposed to the CCM from T2D-CLI CD34$^+$ cells (**Figure 4Ei**) but was unable to prevent the apoptosis of HUVECs exposed to the CCM from miR-21-silenced CD34$^+$ cells (**Figure 4Eii**). These findings indicate that ROS in association with TUG1 is the likely mediator for transmission of an altered miR-21/PDCD4

balance from T2D-CLI $CD34^+$ cells to endothelial cells (**Figure 4F**). They also point to the possibility that the total disruption of miR-21 impacts on additional pro-apoptotic inducers that are independent of ROS.

Conclusions

In an extended follow-up of T2D-CLI, $CD34^+$ cell migration maintained a predictive value at 6 years post-angioplasty. In these critical patients, CD34 cells responsive to SDF-1 chemoattraction exert negative effects on the vascular endothelium through a mechanism involving miR-21 inhibition and PDCD4 upregulation.

In the high-migratory group, cardiovascular mortality accrued during the first years of follow-up, with the difference *vs.* the low-migratory group remaining unchanged later. Most patients with the highest cardiovascular risk had already died at that stage, resulting in a reduction of the target for prediction. Moreover, mortality for other causes can act as a strong opposer in an elderly population. Nonetheless, the biomarker maintained its validity in a multivariable analysis accounting for the age of participants.

Reduction and dysfunction of stem/progenitor cells is associated with and predicts adverse outcomes of diabetic complications.[8] Thus, at first glance, our data appear counterintuitive. We hypothesized that sub-fractions of BM-$CD34^+$ cells could become anti-angiogenic and pro-apoptotic due to the adverse metabolic milieu they are exposed to in the BM and circulation. Once entered in the circulation, these cells may convey pathogenic signals to the vascular endothelium, thereby accelerating ischemic complications.

Accumulating evidence indicates that CLI aggravates the remodeling effect of T2D on the BM niche and induces a senescent phenotype in $CD34^+$ cells, which may be, at least in part, attributable to alteration in miR biogenesis, expression, and degradation[13, 18]. The observed reduction of expressed miRs in T2D BM-$CD34^+$ cells may be attributable to a block in the miR processing, as suggested by Dicer downregulation. Among differentially expressed miRs, we focused on miR-21, because of its involvement in cardiovascular disease [31-34] MiR-21 downregulation in T2D-CLI $CD34^+$ cells was associated with upregulation of the programmed cell death protein PDCD4, a validated inhibitory target of miR-21, and with alterations in viability and proangiogenic activity of $CD34^+$ cells. Silencing miR-21 reproduced

the same phenotype in $CD34^+$ cells of subjects without diabetes. Moreover, data of an SDF-1 migration assay on T2D-CLI PB-MNCs demonstrated the high co-expression of PDCD4 and CXCR4 within the migrated $CD34^+$ cell fraction. This finding provides a key of interpretation for the link between $CD34^+CXCR4^+$ cell migration and cardiovascular death in the follow-up study on T2D-CLI patients.

MiR-21 is the most abundant miRNA in macrophages and its downregulation has been associated with induction of atherosclerosis, plaque necrosis, and vascular inflammation [35]. Silencing of miR-21 in macrophages increases the expression of mitogen-activated protein kinase kinase 3, thereby leading to the activation of the p38-CHOP and cJNK signaling pathways and triggering macrophage apoptosis [35]. Additionally, miR-21-silenced macrophages are unable to remove apoptotic cells, which contributes in delaying the resolution of inflammation[35]. The miR-21 inhibitory target PDCD4 acts as a tumor suppressor protein involved in programmed cell death. Recent cardiovascular research has shown that PDCD4 is upregulated in coronary arteries of atherosclerotic rats, where it participates in the formation of coronary plaques, through destabilization of vascular smooth muscle cells and promotion of inflammatory chemokines [36]. Nonetheless, the regulation of miR-21/PDCD4 interaction is only partially known. Prostaglandins reportedly act as inducers of miR-21 expression and suppressors of PDCD4 protein; whereas, cyclooxygenase 2 inhibitors produce opposite effects[37]. Moreover, several long ncRNAs can act as sponges for miR-21.[24-28] For instance, miR-21 is a direct target of MEG3, and, in hypoxic vascular cells, MEG3 interferes with miR-21 modulation of PTEN resulting in cell proliferation and migration.[27] Likewise, studies on H9c2 cells showed that TALNEC2 modulates miR-21/PDCD4expression under hypoxia, aggravating its consequences.[25] TCONS is an endothelium-associated long ncRNA involved in plaque progression. Binding of miR-21 to TCONS reduces its expression and, for this reason, was proposed as potential treatment to improve endothelial dysfunction and plaque stabilization [28]. Finally, TUG1 was proposed to interact miR-21 and to modulate endothelial cell apoptosis [38].

Both oxidative stress and ischemia induce the sponging activity of TUG1, thereby stimulating intracellular ROS accumulation, and aggravating the ischemic injury[30]. Interestingly, we found that the above long ncRNAs were modulated in $CD34^+$ cells and PB of T2D-CLI patients. Moreover, exposure of HUVECs to the CCM of T2D-CLI $CD34^+$ cells induced the expression of TUG1 and PDCD4, while suppressing miR-21. This mechanism may be ROS dependent when considering that the inhibition exerted by NAC resulted in improved cell survival. We propose that $CD34^+$ cells recruited from PB may exert a sponge-dependent inhibition of the interaction between miR-21 and PDCD4, thereby sustaining vascular damage. Elevated PB levels of miR-21 sponges could strengthen this cellular crosstalk.

These findings have clinical and therapeutic implications, but also raise new questions. Currently, long-term prognosis of CLI is based on clinical parameters[39]. Our study suggests that the assessment of $CD34^+$ cells profile may help identify high-risk patients for whom more aggressive treatments are necessary. Although more sophisticated than traditional biomarkers, cellular biomarkers can be very useful in helping us understand the complex interplay among cellular systems in inducing diabetic vascular disease. In this respect, circulating cells offer a more feasible means for molecular profiling than BM cells, also considering the variability in cell composition of different BM sites. This last aspect represents a limitation of our study, as BM samples were obtained from the femoral head or iliac crest aspirates with the intention of not interfering with clinical practice and patients' care.

PDCD4 might represent a valuable biomarker and therapeutic target in ischemic disease. The latter assumption is indirectly supported by the established benefit of prostaglandin E1, an inhibitor of PDCD4, in the treatment of limb ischemia. Novel treatments targeting upstream modulators of PDCD4, including miR-21 and related sponges, might be also considered for treatment of CLI. Finally, autologous BM-$CD34^+$ cells are currently used in clinical trials of CLI patients. Our study calls for caution in using $CD34^+$ cells that carry a proapoptotic and antiangiogenic molecular signature, e.g. low miR-21/high PDCD4. New investigation is needed

to determine if this signature can be exploited to increase the safety and efficacy of the cell therapy approach.

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Author Contributions. ES researched data relative to both study 1 and 2. ET performed Study 1 statistical analyses. DM isolated cells and performed in vitro molecular analyses of study 2. OC conducted tissue and cells immune characterization. DFMcC isolated cells and provided biological samples. FC and PO provided human BM samples and contributed to discussion. AP acquired funding and contributed to discussion. AF, PMF, and LS was involved in human BM samples collection and clinical data recording. MS acquired funding, provided human BM samples and corrected the manuscript. GS and PM acquired funding, analyzed data and wrote the manuscript.

GS and PM are the guarantor of this work and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis (person[s] taking responsibility for the contents of the article).

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Figure legends

Figure 1. Migration of $CD34^+$ cells toward SDF-1 predicts cardiovascular mortality and is associated with reduced cell viability and angiogenic capacity. A) Left, schematic view of the *in vitro* migration assay; right, cumulative incidence of mortality for cardiovascular causes during the 6-year follow-up in groups categorized according to the median value of $CD45^{dim}/CD34^+/CXCR4^+/KDR^+$ migrated toward SDF-1. Red line represents the incidence in the group with migration value below the median value, while the blue line indicates the cumulative incidence in the group with migration value equal or above the median. P-value for the difference between the two curves =0.0012. **B**) Typical flow cytometry displacement curves and bar graph showing the percent of bone marrow (BM)-derived $CD34^+$ cells expressing AnnexinV in controls (ctr) and patients with type 2 diabetes with critical limb ischemia (T2D-CLI) (N=3 in each group). **D**) Representative microscopy images of the Matrigel assay (bar = 50μm) and bar graphs showing fold changes in the average number of network branches made by human umbilical vein endothelial cells (HUVECs) in the presence or the absence of $CD34^+$ cells (coculture 1:1), $CD34^+$ cell-derived conditioned medium (CCM), or $CD34^+$ cell-derived extracellular vesiscles (EVs) from controls (empty bars, N=5) or patients with T2D-CLI (black bars, N=4). Values are means ± SE; *p<0.05 *vs.* ctr.

Figure 2. MicroRNA profiling of bone marrow-derived $CD34^+$ cells unveils the modulation of microRNA-21 and its target PDCD4 in patients with type 2 diabetes and critical limb ischemia. A) Heat map showing that bone marrow (BM)-$CD34^+$ cells isolated from subjects with type 2 diabetes either uncomplicated (T2D) or complicated by critical limb ischemia (T2D-CLI) bear a specific microRNA (miR) profile. Quantitative RT-PCR data, N=6 donor/group. **B**) Bar graph showing the average relative expression of a group of 6 miRs assessed in a validation study on BM-$CD34^+$ cells obtained from donors different from those used in profiling

(ctr: N=5, with T2D: N=4, with T2D-CLI N=4). Quantitative RT-PCR. *p<0.05 *vs*. ctr, #p<0.05 *vs.* with T2D. **C**) (i) Relative expression of mature miR-21 and miR-21 sponges in BM-derived $CD34^+$ cells (N=4), $CD34^+$ cell-derived conditioned medium (CCM) (ctr: N=8, T2D-CLI: N=7) and isolated extracellular vesicles (EVs) (ctr: N=8, T2D-CLI: N=7) from new donors. (ii) Relative expression of mature miR-21 and miR-21 sponges in plasma (N=9-10) and EVs (N=4-5). Quantitative RT-PCR. *p<0.05, *p<0.01, and ***p<0.001 *vs.* ctr. **D**) PDCD4 mRNA modulation in T2D-CLI BM-$CD34^+$ cells. Quantitative RT-PCR (N=8) *p<0.05 *vs.* ctr. **E**) (i) Bar graph show the frequency of PDCD4 positive cells over total number of hematoxylin positive nuclei in the BM of ctr and T2D-CLI subjects (N=3) *p<0.05 vs. ctr. (ii and iii) Representative microphotographs of BM using immunohistochemistry (upper images, scale bar 20μm), and immunofluorescent microscopy (lower images, scale bar 10μm). White arrowheads point at $CD34^+$ cells.

Figure 3. Inhibition of miR-21 in BM-$CD34^+$ cells from control subjects mimics diabetes-associated dysfunction. Silencing miR-21 in $CD34^+$ cells resulted in **A**) miR-21 reduction (RT-PCR, N=3) and **B**) PDCD4 upregulation (RT-PCR, N=6). *p<0.05 *vs.* scramble (SCR). **C**) miR-21 inhibition is associated with increased apoptosis of BM-$CD34^+$ cells, bar graph of Annexin V+/7AAD- cells assessed using flow cytometry. (N=7). **D**) Anti-angiogenic action of miR-21-silenced BM-$CD34^+$ cells (N=4), cell-derived conditioned medium (CCM, N=3) and extracellular vesicles (EVs, N=3). Human umbilcal vein endothelial cells (HUVECs) networking analysis on Matrigel. Scale bar 50μm. **E**) Left, schematic view of the experimental setting; right, negative correlation between PDCD4 and miR-21 in HUVECs treated with the CCM from $CD34^+$ cells. The graph reports data of average PDCD4 protein levels (as percentage of positive HUVECs identified using immunofluorescence microscopy) and average miR-21 expressional levels (by PCR analysis). Colored circles indicate the source of CCMs. **F**) Upper panel, scheme of the experiment; lower panels, bar graph shows the percentage of Annexin $V^+/7AAD^-$ HUVECs

either SCR- or siPDCD4 transfected after treatment with $CD34^+$-CCM. Flow cytometry analysis, N=3 donors/condition.

Figure 4. Exposure of endothelial cells to the conditioned medium of $CD34^+$ cells from patients with type 2 diabetes and critical limb ischemia induces a modulation of the miR-21/PDCD4 axis through a mechanism involving reactive oxygen species and the miR-21 sponge TUG1. A) Bar graph showing the average relative expression of miR-21, PDCD4, and indicated long ncRNA in human umbilical vein endothelial cells (HUVECs) either not treated (NT) or treated with the conditioned media (CCM) from bone marrow (BM)-derived $CD34^+$ cells of controls (ctr) or type 2 diabetes+critical limb ischemia patients (T2D-CLI) (N=4 donors, *p<0.05 *vs.* ctr CCM. **B**) *In situ* detection of reactive oxygen species (ROS) levels assessed by measuring 4-Hydroxy-2-Nonenal (4-HNE) in BM-$CD34^+$ cells. Representative images of immunofluorescence staining (IF) and bar graph of average data (ctr: N=5 and T2D-CLI: N=6) *p<0.05 . White arrowheads in IF microphotographs point at $CD34^+$ cells. Scale bar: 10μm. **C**) Bar graph showing the relative expression of miR-21 (i) and PDCD4 mRNA (ii) (RT-PCR, N=4) and PDCD4 protein (iii) (immunofluorescence staining, NT: N=3 and treated: N=5) in HUVECs exposed to H_2O_2 at the indicated concentrations. *p<0.05 *vs.* NT. **D**) Antioxidant action of N-acetyl-L-cysteine (NAC) results in prevention of miR-21 inhibition in HUVECs that were exposed to the CCM of $CD34^+$ cells from patients with T2D-CLI (i) or to the CCM of miR-21-silenced $CD34^+$ cells from ctr (ii). Bar graphs with average RT-PCR data show there was no difference with respective CCM from ctr $CD34^+$ cells or CCM from ctr $CD34^+$ cells transfected with scramble siRNA (SCR). (iii) Percentage of PDCD4 positive HUVECs exposed to the CCM of $CD34^+$ cells from ctr or T2D-CLI patients with (+) or without (-) NAC; *p<0.05 as indicated by the lines. (iv) Percentage of PDCD4 positive HUVECs exposed to the CCM of ctr $CD34^+$ cells, transfected with either SCR or anti-miR-21, with (+) or without (-) NAC; *p<0.05 and **p<0.01 as indicated by the lines. N=4 in each group for all the experiments. **E**) NAC

scavenging of ROS protects HUVECs from CD34-CCM-induced apotosis. Conditions in (i) and (ii) are the same of panel E (iii) and (iv), respectively. Bar graphs of average caspase 3/7 activity that was measured using ELISA; *p<0.05 and **p<0.01 as indicated by the lines (N=3). **E**) Diagram of propesed molecular interaction between $CD34^+$ cell and endothelial cells, involving the participation of ROS and miR-21 sponge TUG1. Section of $CD34^+$ cell on the left of dotted line shows the inhibition of proapoptotic PDCD4 by miR-21 leading to prosurvival signalling to the vascular endothelium. Section of $CD34^+$ cell on the right illustrating the interference of miR-21 sponge on the inhibitory target, which result in activation of pro-apoptotic signalling in vascular endothelium.