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ORIGINAL ARTICLE

Effect of normalization of fasting glucose by intensified insulin therapy and influence of eNOS polymorphisms on the incidence of restenosis after peripheral angioplasty in patients with type 2 diabetes: a randomized, open-label clinical trial

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Abstract Primary objective was to evaluate whether an intensified insulin therapy (IIT) incorporating the target of normal fasting glucose and HbA1c levels could halve the incidence of restenosis/amputation/SCA/death at 6 months after peripheral angioplasty compared with standard care (SC) in patients with type 2 diabetes (DMT2) affected by critical limb ischemia (CLI). Forty-six consecutive patients with DMT2 and CLI were randomly assigned to a parallel, open-label study with IIT (basal-bolus glulisine + glargine administrations) or SC (glargine administration + oral antidiabetic drugs). A SNP of eNOS (rs753482-A>C) and circulating $CD34^+$ and $CD34^+KDR^+$ progenitor cells were determined. At the end of the study, although HbA1c levels were lower in IIT than in SC (6.9 ± 1.3 % vs. 7.6 ± 1.2 %, $p < 0.05$), IIT did not reduce the cumulative incidence of restenosis/amputation/SCA/death (52 and 65 %, respectively, odd ratio 0.59; CI 95 %: 0.21–1.62, $p = 0.59$). rs753482AC+CC as compared with rs753482AA increased the cumulative incidence of restenosis/amputation/SCA/death (79 and 42 %; odd ratio 5.3; CI 95 %: 1.41–19.5, $p < 0.02$). Baseline $CD34^+KDR^+$ were higher in rs753482AA (166.2 ± 154.0 × 10^6 events) than in rs753482AC+CC (63.1 ± 26.9 × 10^6 events, $p < 0.01$). At the end of the study, the highest circulating $CD34^+KDR^+$ were found in IIT rs753482AA (246.9 ± 194.0 × 10^6 events) while the lowest levels were found in SC rs753482AC+CC (70.9 ± 45.0 × 10^6 events). IIT did not decrease the cumulative incidence of restenosis/

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amputation/SCA/death in DMT2 and CLI patients. These patients correspond to a class of fragile subjects at high risk of cardiovascular events, and new predictors of restenosis should be contemplated, such as of eNOS polymorphism, (rs753482-A>C SNP) and circulating endothelial progenitor cells.

Keywords Intensified insulin therapy · Type 2 diabetes · Critical limb ischemia · eNOS polymorphism · Endothelial progenitor cells · Restenosis · Amputation

Introduction

Arteriosclerosis is the most common cause for peripheral arterial disease and among other modifiable risk factors; type 2 diabetes mellitus represents a major risk both among men and women [1]. The effectiveness of PTA in achieving limb salvage has been established [2] even if few reports have evaluated the long-term results [3]. Major indications for lower-extremity percutaneous transluminal angioplasty are (1) incapacitating intermittent claudication in persons interfering with work or lifestyle and (2) limb salvage in persons with limb-threatening ischemia as manifested by rest pain, non-healing ulcers, infections, or gangrene [4]. The loss of a limb is a major problem in diabetic foot and is one of the most important causes for disability in patients with the disease and may be life-threatening if the patients are not provided with effective intervention directed at healing. The principles of good wound care include revascularization, control of glucose levels, mainly fasting glucose levels and careful monitoring of the ulcer [5].

It is well known that overt diabetes is a clear risk factor for cardiovascular disease, and in recent meta-regression analyses, a continuous relationship was noted between fasting glucose levels and the risk of cardiovascular events and mortality [6, 7]. Further, it has been demonstrated that diabetes correlates with quality of life and coronary disease prevalence and severity [8, 9] and that glucose dysmetabolism has a prognostic role in myocardial infarction [10, 11]. However, until now, the benefit/risk ratio of intensive glucose-lowering treatment in the prevention of macrovascular and microvascular events remains uncertain since the harm associated with severe hypoglycemia might counterbalance the potential benefit of intensive glucose-lowering treatment. Therefore, whether the above-mentioned potential benefits of diabetes control yield improvements in functional lower limb outcomes such as limb salvage or freedom from repeated revascularization in CLI patients has yet to be determined. As far as we know, the effects of a strict control of fasting glucose levels (associated with a glucose control monitored by phone on weekly basis in order to avoid hypoglycemic episodes) on the degree of restenosis after peripheral arterial angioplasty were never evaluated.

Among the many environmental signals in ischemic tissue, hypoxia is one of the strongest stimuli inducing angiogenesis, and nitric oxide plays a major role in changes of vascular tone and organ function in the setting of hypoxia. Very soon after the cloning and functional characterization of the endothelial nitric oxide synthase (eNOS), studies on the interaction between O_2 and nitric oxide made the finding that hypoxia modulates eNOS expression and function. We previously reported a genetic association of eNOS gene variants, Glu298Asp rs1799983G>T and rs753482-A>C with type 2 diabetes and metabolic syndrome [12]. In addition, coronary artery disease (CAD) and restenosis after coronary stent implantation were shown to be associated with the same eNOS variants with a specific phenotype characterized by elevated basal NO levels, insensitivity to insulin stimulus, and reduced reactive hyperemia [13]. Even if it is known that eNOS is essential for progenitor cells mobilization and function [14] and increasing eNOS expression by eNOS enhancers improved functional activity of patient-derived cells [15] while eNOS null mice are characterized by impaired ischemia-induced neovascularization [16] and impairment of progenitor cell mobilization from bone marrow [15], little is known about a possible association between variants of eNOS and restenosis after peripheral arterial angioplasty.

Since in patients with diabetes mellitus, the number of circulating endothelial progenitor cells (EPCs) directly correlates with glycemic control [17, 18] and the cells show a reduced capacity to participate in revascularization [19, 20], it could be important to detect in diabetic patients the influence of eNOS variants on circulating EPCs number and furthermore whether eNOS variants in the presence of long-term normoglycemia could influence circulating EPCs number in diabetic patients with CLI.

Therefore, the primary objective of the study was to test whether an IIT incorporating the target of almost normal fasting glucose ($\sim$100 mg/dl) and glycated hemoglobin <6.5 % was able to halve the incidence of angiographic restenosis at 6 months after peripheral angioplasty compared with SC to achieve a glycated hemoglobin <7.0 % in patients with type 2 diabetes and CLI.

Secondary objectives included the identification of genetic markers of eNOS rs753482-A>C associated with, and predictive of, restenosis and the investigation of the underlying pathophysiological background, with specific focus on the role of circulating $CD34^+$ and $CD34^+$ KDR^+ progenitor cells number with flow cytometry analysis.

Subjects and methods

Patient population

This is a randomized, open-label clinical trial comparing two regimens of insulin therapy having as an outcome measure the incidence of angiographic restenosis at 6 months after peripheral angioplasty. Forty-six consecutive patients (33 males and 13 females) with type 2 diabetes and peripheral arterial disease undergoing peripheral angiography and subsequent angioplastic procedure were studied. CLI diagnosis is detailed in the on-line supplementary data. The study was approved by the local Ethical Committee; all patients gave their written informed consent, and the study is registered as Clinical Trial.gov Identifier NCT01150617.

Mean age was 70.6 ± 9.2 years, mean weight 80.5 ± 14.5 kg, mean BMI 28.9 ± 4.6 kg/m^2, systolic blood pressure 135 ± 17, and diastolic blood pressure 82 ± 23 mmHg, mean duration of diabetes 18.1 ± 6.8 years, all patients showed a GFR >60 ml/min 1.73 m^2. All patients had other microvascular (retinopathy, microalbuminuria) and macrovascular complications (coronary or carotid vascular disease). Patient characteristics are depicted in Table 1 in the on-line supplementary data.

Study design

Before the start of the study, hypoglycemic therapy consisted in one or more oral antidiabetic drug (sulfonylureas, metformin, neglitinides) at half-maximum dose or greater in the presence or in the absence of once-daily insulin therapy.

In order to achieve normal glucose levels during the interventional procedure, in the 24 h before and after interventional procedure, a continuous iv insulin infusion was started, and monitoring of blood glucose was performed every 120 min. The steady-state glucose levels during the night before intervention was 108 ± 16 mg/dl in intensive insulin treatment (IIT group) and 105 ± 20 mg/dl in standard care (SC) group.

After the procedure of peripheral angiography and angioplasty and before randomization, all patients underwent a baseline physical examination, and blood samples were collected to evaluate both the metabolic parameters and the degree of glucose control. At the time of this visit, patients were randomly assigned to IIT or SC for glycemic control, added to their usual cardiovascular treatment. A similar diet and physical activity program was planned for both groups.

Table 1 Metabolic characteristics and circulating CD34$^+$ progenitor cells in type 2 diabetic patients (DMT2) affected by critical limb ischemia (CLI) before and 6 months after peripheral arterial revascularization, submitted to Intensive Insulin Treatment (IIT) or Standard Treatment (SC) (mean ± SD)

	IIT	SC
Basal SMBG (mg/dl)	164.1 ± 48.0	155.3 ± 46.9
2-month SMBG (mg/dl)	124.1 ± 31.5	147.0 ± 37.7
4-month SMBG (mg/dl)	127.5 ± 36.6	132.6 ± 36.8
6-month SMBG (mg/dl)	123.1 ± 28.4	142.7 ± 36.6
Basal HbA1c (%)	7.9 ± 1.7	8.1 ± 1.9
2-month HbA1c (%)	7.3 ± 1.4	7.3 ± 1.1
4-month HbA1c (%)	7.4 ± 1.6	7.4 ± 1.0
6-month HbA1c (%)	6.8 ± 1.2 a,b	7.6 ± 1.1 b
Hypogl.episodes (<70 mg/dl)	57	33
Hypogl. episodes/patients/year	1.24	0.83
Total cholesterol (mg/dl)		
Before revascularization	139.9 ± 34.1	139.9 ± 32.2
After revascularization	144.9 ± 35.3	145.9 ± 32.0
HDL cholesterol (mg/dl)		
Before revascularization	39.9 ± 13.6	34.4 ± 11.7
After revascularization	38.8 ± 13.6	40.1 ± 13.2
Triglycerides (mg/dl)		
Before revascularization	103.0 ± 36.7	113.7 ± 51.2
After revascularization	102.5 ± 43.6	118.3 ± 53.2
CD34$^+$ (× 10^6 events) before revascularization	575.6 ± 491.1	466.8 ± 293.4
CD34$^+$ (× 10^6 events) after revascularization	729.6 ± 658.8	410.9 ± 36.5 b

SMBG self-monitoring blood glucose, *HbA1c* glycated hemoglobin, *Hypogl.* hypoglycemic

(a) $p < 0.05$ versus SC

(b) $p < 0.05$ versus basal

(c) $p < 0.03$ versus before revascularization

The IIT consisted in three pre-prandial administrations of short-acting insulin analogue glulisine combined with the long-acting insulin analogue glargine while SC consisted in a once-daily long-acting insulin analogue glargine and oral antidiabetic agents. Routine visits were scheduled after 1, 2, 4 weeks and then at 2, 4, and 6 months. The algorithm of glargine and glulisine therapy is detailed in the on-line supplementary data. At 2, 4, and 6 months, a physical examination and blood samples to evaluate metabolic parameters and the degree of glucose control were repeated.

End points

The following end points were recorded: above-the-ankle or by tarsal-metatarsal, amputation, ulcer recurrence, clinical restenosis after PTA, CLI recurrence and survival.

Restenosis was suspected when ischemic rest pain reappeared or an ulcer worsened or did not heal. In these situations, ankle pressure and TcPO2 were reassessed, and duplex scanning was performed [21]. If ankle pressure and TcPO2 again showed values diagnostic for CLI and were significantly deteriorated (<15 % of the post-PTA value) and Duplex scanning was positive, a further PTA was performed. Morphological restenosis was not investigated; we did not perform revascularizations in the absence of ischemic resting pain or ulcerations; therefore, we considered morphological restenosis clinically irrelevant [22]. The reappearance of a foot lesion after the primary ulcer had healed was considered an ulcer recurrence.

Choice of the sample size

Corpus et al. [23] demonstrated in diabetic patients a significant decrease in coronary target vessel revascularization from 35 % with glycated hemoglobin >7.0 to 15 % in the presence of good glycemic control (glycated hemoglobin <7.0 %), superimposable to those found in non-diabetic patients.

Similarly, after coronary stent implantation, MACEs steadily increased from 10 % at glycated hemoglobin of 6.1 to 50 % at glycated hemoglobin more that 7.5 % [24, 25].

Exner et al. demonstrated that the occurrence of restenosis (≥50 %) was 35 % at 6-month follow-up after PTA in patients with intermittent claudication or critical limb ischemia [26]. In a similar population, Mlekusch et al. [27] found a 50 % degree of restenosis at 6-month follow-up after angioplasty. A rate of restenosis after angioplasty of lower limb arteries in a range of 30–50 % was also confirmed by Mongiardo et al. [28]. However, these studies did not characterize patients for the presence of diabetes, while it was previously demonstrated that the degree of restenosis was increased by 13 % in diabetic patients compared with non-diabetic patients 6 months after coronary stent implantation [29]. In light of these results, we could expect that the rate of restenosis after infrapopliteal PTA will be between 40 and 55 % and that the intervention study with intensive insulin therapy will be able to determine a 65 % reduction in restenosis compared with the standard care. Therefore, we designed the study to be large enough to be able to detect, with 80 % power ($\beta = 20$ %) at the two-tailed 5 % significance level ($\alpha = 5$ %), a reduction in the degree of restenosis from 50 % in standard care to 10 % in intensive insulin treatment. The sample size required in each of the two groups is 23 patients, for a total number of 46 patients.

Measurements

At the start, at 2, 4 months, and at the end of the study period, fasting samples were taken for the measurement of glucose, cholesterol, HDL cholesterol, triglycerides and HbA1c. All the parameters were assayed by spectrophotometric automated methods, using commercial kits except for HbA1c that was essayed by HPLC method.

At the start of the study, a blood sample was collected from the participants, and genomic DNA was isolated and an intronic SNP of eNOS gene was evaluated (rs753482-A>C). Before and at the end of the study period, samples were drawn to evaluate endothelial progenitor cells (EPCs). DNA extraction and genotyping and methods of endothelial progenitor cells isolation and evaluation are detailed in the on-line supplementary data.

Further details on the methodology of the intervention technique, antiplatelet therapy during this period, indices of vascular success, and evaluation of the end point of the study are reported in the on-line supplementary data.

Statistical analysis

Data were given as average values and SDs for continuous variables or as percentages for discrete variables. We explored the role of continuous variables across the treatment groups with the Student's t test and ANOVA. The presence of prognostic factors was assessed with the $\chi 2$ test with Yate's correction. The 95 % confidence interval (CI) was adopted, and 5 % was considered significant for the null hypothesis. The proportion of surviving patients was plotted with a Kaplan–Meier curve, and differences in survival among subgroups were tested by the log-rank test.

Results

IIT versus SC

Table 1 reports the metabolic parameters during the 6-month treatment study. At entry into the study, in IIT, fasting blood glucose and HbA1c levels were 164.1 ± 48.0 mg/dl and 7.9 ± 1.7 %, respectively, while in SC, blood glucose and HbA1c levels were 155.3 ± 46.9 mg/dl and 8.1 ± 1.9 %, respectively (N.S. vs. IIT).

At the end of the study, in IIT, fasting glucose and HbA1c levels were 123.1 ± 28.4 mg/dl and 6.8 ± 1.2 %, while 142.7 ± 36.6 mg/dl e 7.60 ± 1.1 % in SC ($p < 0.05$; IIT vs. SC), respectively.

Six months after the procedure, restenosis and amputation was observed in 48 % of the patients in IIT group while in SC group, restenosis and amputation were present in 65 % of patients. In IIT, there were also 2 deaths, one for heart failure and one possibly related to nocturnal hypoglycemia, and a SCA while none were reported in the SC group. Three patients in both groups presented foot ulceration during this period.

The number of participants who had any episode of hypoglycemia was similar between treatment groups (10/23 vs. 10/23, respectively). No episodes of severe hypoglycemia occurred in either group. During the entire study, the frequency of all hypoglycemia averaged 1.24 and 0.83 episodes per patient per year with IIT and SC, respectively (N.S, Table 1).

Figure 1a reports the Kaplan–Meier estimates and the cumulative incidence of restenosis/amputation/ulceration/SCA/death after peripheral arterial revascularization, in the IIT group and in SC group. During the 6-month period after the procedure of peripheral arterial revascularization, the cumulative incidence of restenosis/amputation/ulceration/SCA/death occurred in 52 % of patients in the IIT group and in 65 % of patients in the SC group (hazard ratio, 0.59; 95 % CI: 0.21 to 1.62; $p = 0.59$).

During this period, total cholesterol, HDL, and triglyceride levels were not significantly modified in both groups (Table 1).

Similar numbers of $CD34^+$ were demonstrated before revascularization in both groups of study. Compared with baseline levels, at the end of the study, circulating $CD34^+$ numbers were significantly decreased in SC while no changes were demonstrated in IIT (Table 1).

Circulating $CD34^+KDR^+$ numbers were similar before revascularization in both groups. Compared with baseline levels, at the end of the study, only IIT significantly increased $CD34^+KDR^+$ numbers. Moreover, at the end of study, $CD34^+KDR^+$ numbers were significantly higher in the IIT group than in the SC group (Fig. 2).

Association between eNOS polymorphism and circulating $CD34^+KDR^+$ and their influence on restenosis and/or amputation

Table 2 reports the metabolic parameters during the 6-month treatment study according to eNOS rs753482-A>C polymorphism. At entry into the study, fasting glucose and HbA1c levels were 151.1 ± 50.9 mg/dl and 7.4 ± 2.1 % in patients carrying rs753482AC+CC (variants allele carriers) and 165.8 ± 44.0 mg/dl e 8.3 ± 1.4 %, in patients carrying rs753482AA (wild-type homozygotes), respectively (N.S).

At the end of the study, fasting glucose and HbA1c levels were 131.0 ± 33.4 mg/dl and 7.0 ± 1.3 % in patients carrying rs753482AC+CC and 131.6 ± 33.2 mg/dl and 7.4 ± 1.2 % in patients carrying rs753482AA, respectively (NS).

Figure 1b reports the Kaplan–Meier estimates and the cumulative incidence of restenosis/amputation/ulceration/SCA/death after peripheral arterial revascularization, in patients carrying rs753482AC+CC and in patients carrying rs753482AA. During the 6-month period after the procedure of peripheral arterial revascularization, the cumulative incidence of restenosis/amputation/ulceration/SCA/death occurred in 79 % of patients carrying rs753482AC+CC and in 42 % of patients carrying rs753482AA (hazard ratio, 5.3; 95 % confidence interval: 1.41 to 19.5; $p = 0.02$).

Evaluation of circulating $CD34^+$ (Table 2) and $CD34^+KDR^+$ (Fig. 3) demonstrated that before revascularization, both $CD34^+$ and $CD34^+KDR^+$ numbers were significantly lower in patients carrying rs753482AC+CC than in patients carrying rs753482AA. In particular, circulating $CD34^+KDR^+$ numbers were significantly higher at baseline in patients carrying rs753482AA ($166.2 \pm 154.0 \times 10^6$ events) than in patients carrying rs753482AC+CC ($63.1 \pm 26.9 \times 10^6$ events; Fig. 3a). Compared with baseline levels, at the end of the study, there was a significant increase in $CD34^+$ and $CD34^+KDR^+$ numbers only in patients carrying rs753482AA, while both $CD34^+$ and $CD34^+KDR^+$ numbers remained unchanged in patients carrying rs753482AC+CC (Table 2; Fig. 3b). Moreover, the highest $CD34^+KDR^+$ numbers were found in patients carrying rs753482AA treated with IIT ($246.9 \pm 194.0 \times 10^6$ events), and the lowest levels were found in patients carrying rs753482AC+CC treated with SC ($70.9 \pm 45.0 \times 10^6$ events; Fig. 3b).

Discussion

The study showed that IIT was unable to reduce the cumulative incidence of restenosis and/or amputation after peripheral revascularization as compared to a SC, and this

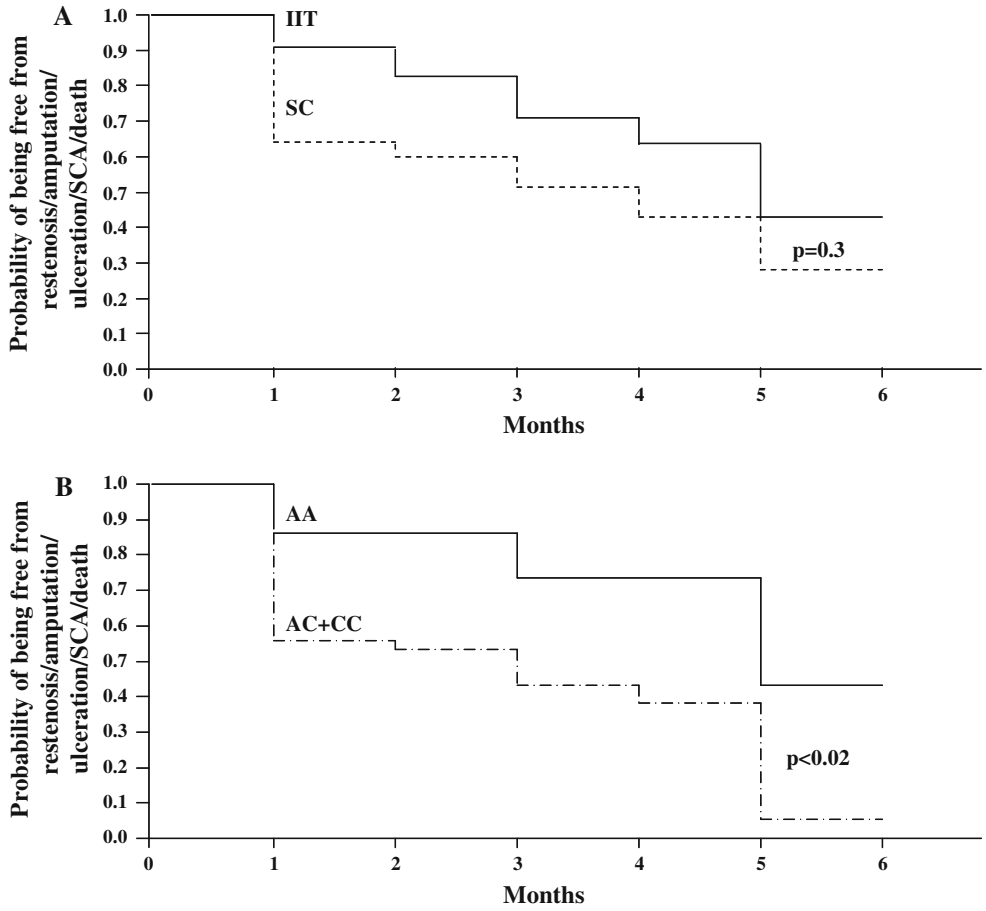

Fig. 1 Kaplan–Meier estimates of restenosis/amputation/ulceration/SCA/death in DMT2 affected by CLI after peripheral arterial revascularization. **a** During the 6 month period after the peripheral arterial revascularization procedure, the cumulative incidence of restenosis/amputation/ulceration/SCA/death occurred in 52 % of patients in the IIT group and in 65 % of patients in the SC group (hazard ratio, 0.59; 95 % confidence interval: 0.21–1.62; $p = 0.59$). **b** During the 6 month period after the peripheral arterial revascularization procedure, the cumulative incidence of restenosis/amputation/ulceration/SCA/death occurred in 79 % of patients carrying AC+CC (variant allele carriers) and in 42 % of patients carrying AA (wild-type homozygotes) (hazard ratio, 5.3; 95 % confidence interval: 1.41–19.5; $p = 0.02$)

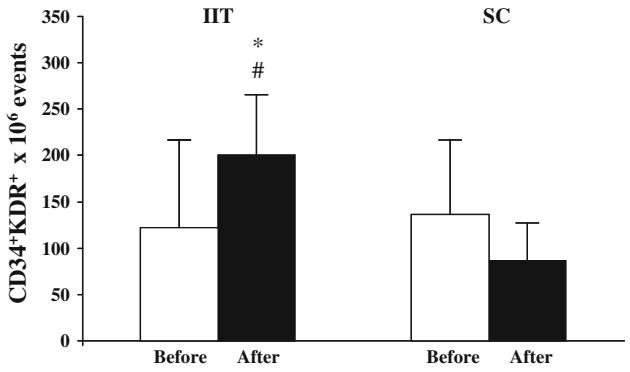

Fig. 2 Circulating $CD34^+KDR^+$ progenitor cells number before (*white histograms*) and after (*black histograms*) peripheral arterial revascularization during IIT and SC in DMT2 affected by CLI. *$p < 0.05$ versus before revascularization; #$p < 0.05$ versus SC

trend was maintained when comparing the two treatments for the cumulative incidence of restenosis/amputation/ulceration/SCA/death. On the other hand, the cumulative incidence of restenosis/amputation found in the SC was similar to that found in a recent meta-analysis evaluating the effect of percutaneous transluminal angioplasty of lower limb revascularization in 2557 both non-diabetic and diabetic patients [30]. Conversely, we found that the patient survival rate in the present study was 95.7 % (44/46 patients), higher than the average of the patient survival rate after 6 month from percutaneous transluminal angioplasty of lower limb revascularization found in a recent meta-analysis (92.3 %), although in this meta-analysis both non-diabetic and diabetic patients were evaluated together [30]. It is important to underline that in the present study,

Table 2 Metabolic characteristics and circulating $CD34^+$ progenitor cells in type 2 diabetic patients (DMT2) affected by critical limb ischemia (CLI) before and 6 months after peripheral arterial revascularization, according to eNOS rs753482-A>C polymorphism (mean ± SD)

	AA	AC+CC
Basal SMBG (mg/dl)	165.8 ± 44.0	151.1 ± 50.9
2-month SMBG (mg/dl)	130.2 ± 33.6	138.6 ± 38.8
4-month SMBG (mg/dl)	128.5 ± 34.1	131.7 ± 39.7
6-month SMBG (mg/dl)	131.6 ± 33.2	131.0 ± 33.4
Basal HbA1c (%)	8.3 ± 1.4	7.4 ± 2.1
2-month HbA1c (%)	7.5 ± 1.1	7.1 ± 1.3
4-month HbA1c (%)	7.6 ± 1.3	7.2 ± 1.4
6-month HbA1c (%)	7.4 ± 1.2 a	7.0 ± 1.3
Hypogl.episodes (<70 mg/dl)	47	43
Hypogl. episodes/patients/year	0.98	1.13
Total cholesterol (mg/dl)		
Before revascularization	136.0 ± 33.1	146.7 ± 31.5
After revascularization	142.4 ± 28.2	150.8 ± 38.9
HDL cholesterol (mg/dl)		
Before revascularization	34.2 ± 13.6	34.3 ± 10.3
After revascularization	40.2 ± 14.7	38.9 ± 10.9
Triglycerides (mg/dl)		
Before revascularization	102.4 ± 41.6	119.6 ± 45.8
After revascularization	106.0 ± 43.6	117.9 ± 52.1
$CD34^+$ ($\times 10^6$ events) before revascularization	606.1 ± 270.9	345.8 ± 258.0 b
$CD34^+$ ($\times 10^6$ events) after revascularization	768.7 ± 71.2 c	307.7 ± 124.8 d

SMBG self-monitoring blood glucose, *HbA1c* glycated hemoglobin, *Hypogl.* hypoglycemic, *AA* wild-type homozygotes, *AC+CC* variant allele carriers

(a) $p < 0.05$ versus basal

(b) $p < 0.05$ versus AA

(c) $p < 0.05$ versus before revascularization SC

(d) $p < 0.03$ versus AA

these results were achieved in diabetic patients affected by multiple cardiovascular and neurological complications thanks to a strict daily capillary glucose monitoring associated with a weekly phone contact with the investigators in order to avoid hypoglycemic episodes. In addition, most studies have shown that insulin therapy plays a beneficial role in the outcome of critically ill patients. It has been demonstrated that, in post-surgical patients treated to achieve fasting glucose of 80–110 mg/dl, there was a significant reduction in overall mortality [31] even if the results of major randomized clinical trials on the benefits of such treatment are controversial. In particular, The ACCORD study (Action to Control Cardiovascular Risk in Diabetes) failed to demonstrate that an intensive treatment to achieve an almost normal HbA1c could reduce mortality in patients with type 2 diabetes [32]. This study randomized participants with type 2 diabetes and cardiovascular disease or additional cardiovascular risk factors to receive intensive therapy (targeting HbA1c < 6.0 %) or standard therapy (targeting a level of 7–7.9 %). The ACCORD study was prematurely stopped after 3.4 years due to an increased mortality in the intensively treated group, although the rates of non-fatal myocardial infarction and stroke were lower in the intensively treated group by that time. At 5 years, the use of intensive therapy for 3.7 years reduced the 5-year non-fatal myocardial infarction but increased the 5-year mortality [33]. In the present study, a IIT treatment based on three pre-prandial administrations of short-acting insulin analogues combined with the long-acting insulin analogue was able to achieve an optimal glycemic control with a very low numbers of hypoglycemic episodes, similar to SC.

The potential importance to achieve an almost normal glucose level in these patients may derive from a recent study showing that elevation of blood glucose for 6 weeks is sufficient to stimulate gene expression of matrix metalloproteinase (MMP)-2, MMP-9, as well as membrane type 1 (MT1)-MMP in both aortic and mesenteric samples to a similar degree [34]. Increased MMP gene expression is associated with vascular remodeling, which is characterized by increased medial thickness and a decreased lumen-to-media ratio [35] with a net effect of reduction in luminal diameter. This effect may contribute to primary arterial stenosis predisposing to peripheral arterial disease [36], as well as the restenosis following an intervention for the treatment of peripheral arterial disease.

The present study confirms that circulating EPCs may be increased by normalizing glucose levels after IIT [37]. Previous studies have also shown that hyperglycemia can reduce both the EPCs and impair EPCs migration and proliferation [38, 39].

Reduced availability and down-regulation of EPCs are some of several important mechanisms that are responsible for the occurrence of endothelial dysfunction and vascular diseases in these patients. [40]. Decreased number and impaired function of EPCs can be involved early in

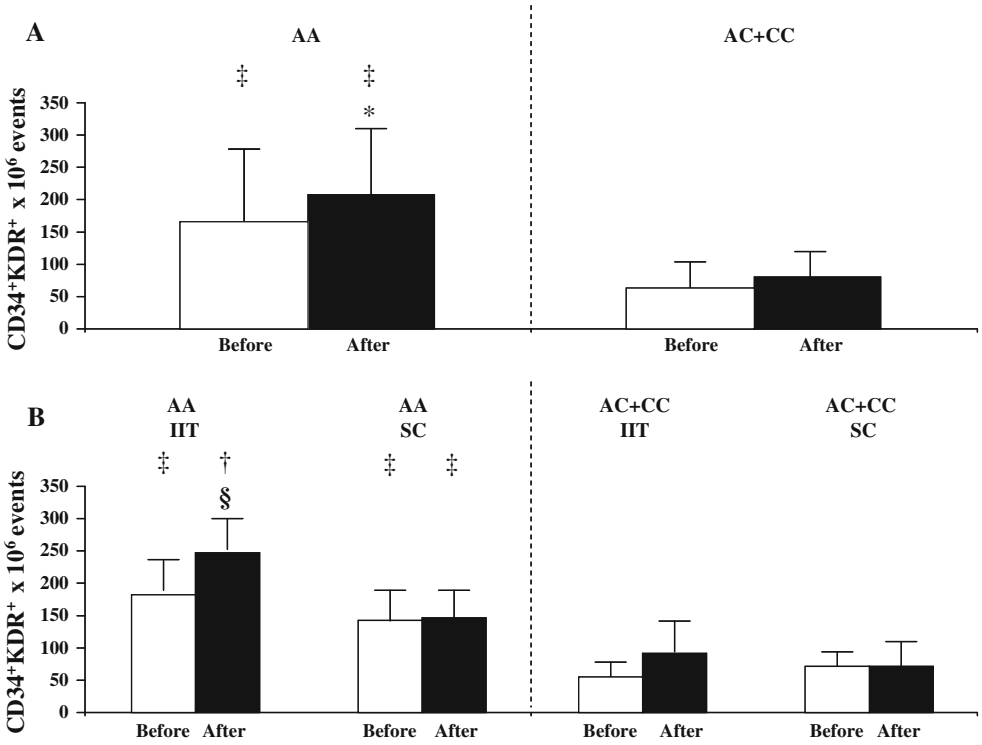

Fig. 3 Circulating $CD34^+KDR^+$ progenitor cells number according to the rs753482-A>C SNP polymorphism before and after peripheral arterial revascularization in patients affected by DMT2 and CLI. **a** Circulating $CD34^+KDR^+$ number before (*white histograms*) and after (*black histograms*) peripheral arterial revascularization, according to genotype. AA, wild-type homozygotes, AC+CC, variant allele carriers. *$p < 0.05$ versus before revascularization; ‡$p < 0.01$ versus AC+CC. **b** Circulating $CD34^+KDR^+$ progenitor cells number before (*white histograms*) and after (*black histograms*) peripheral arterial revascularization, according to genotype and treatment arm. *AA*, wild-type homozygotes, *AC+CC*, variant allele carriers. §$p < 0.01$ versus before revascularization; ‡$p < 0.01$ versus AC+CC; †$p < 0.001$ versus AC+CC

endothelial dysfunction and atherogenesis and later in impaired collateralization after artery occlusive diseases, leading to clinical manifestations of vascular diseases [41]. Tepper et al. [42] showed that EPC proliferation from DMT2 patients was inversely correlated with their HbA1c levels, suggesting a relationship between the patients' glycemic control and EPC number and proliferation. Fadini et al. [20] reported that the EPC count in type 2 diabetic patients with PAD is substantially lower than that of healthy subjects, non-diabetic patients with vascular disease, and type 2 diabetic patients without vascular disease.

According with the secondary end point, we observed that patients carrying rs753482AA+AC were associated with increased risk of restenosis/amputation/ulceration/SCA/death with a hazar ratio of 5.3 (95 % CI: 1.41–19.5, $p < 0.02$). Moreover, these patients showed significantly lower circulating EPCs than patients carrying AA. To the best of our knowledge, this is the first report to describe an association with an intronic eNOS variant and circulating EPCs. Knowledge of this AC+CC for eNOS rs753482-A>C SNP could facilitate the identification of those individuals who are at risk of developing in-stent restenosis in response to a peripheral angioplasty. Maybe even more importantly, after further testing of this SNP in a prospective study, this risk stratification could support the intervention of IIT in these high-risk patients. The pharmacogenetics analysis of the present study showed that the best effect in stimulating EPCs and in turn reducing restenosis was found in patients carrying AA haplotype and treated by IIT.

The limitation of the present study is the relatively small sample size determined by the severe selection criteria of the study and before drawing final conclusions on the effects of rs753482-A>C SNP on the cumulative incidence of restenosis/amputation/SCA/death in DMT2 and CLI patients, it is mandatory to perform further studies with a larger number of subjects. Another limitation of the present study is the relatively short period of observation in which patients reached normal glucose levels, not sufficient to overcome all the metabolic abnormalities produced by previous long periods of hyperglycemia. Recent studies have shown, in fact, that excess reactive species are central in the development of hyperglycemia-related diabetic complications. There is a growing mass of evidence supporting the role of oxidative stress in the metabolic memory phenomenon as recently reviewed by Ceriello et al.

[43]. The excess superoxide anion produced by the mitochondria in response to hyperglycemia leads to accumulation of potentially harmful substances such as advanced glycated end products, protein kinase C, and nuclear factor κB, which are directly implicated in the development of vascular complications in diabetes. These adverse effects are not reversed when the high blood glucose is corrected, and some may be permanent because of epigenetic changes, explaining, at least in part, the persistence of the risk for complications even when hyperglycemia is reduced or normalized.

In conclusion, IIT is not able to reduce the cumulative incidence of restenosis/amputation/SCA/death in DMT2 and CLI patients in spite of normalized HbA1c and increased EPCs. These patients corresponded to a class of fragile subjects at very high risk of cardiovascular events and new predictors of restenosis should be contemplate, such as knowledge of eNOS polymorphism (i.e. rs753482-A>C SNP) and EPCs.

The results of the present study are in line with a recent meta-analysis that analyzed the effects of intensive glucose-lowering treatment on all cause mortality or cardiovascular death in 34533 patients included in 13 studies [44]. Overall results of this meta-analysis showed that there are no benefits of intensive glucose-lowering treatment on all cause mortality and deaths from cardiovascular causes in adults with type 2 diabetes and the harm associated with severe hypoglycemia might counterbalance the potential benefit of intensive glucose-lowering treatment. However, in these results, new hypoglycemic agents such as DDP IV inhibitors and GLP-1 analogues were not taken into consideration. In a recent meta-analysis, Phung et al. showed that DPP-4 inhibitors (RR, 0.63; 95 % CI, 0.26–1.71) and GLP-1 analogues (RR, 0.89; 95 % CI, 0.22–3.96) were not associated with increased risk of hypoglycemia compared with placebo [45]. These data are very promising, and the results of ongoing clinical outcome trials with DDP IV inhibitors and GLP-1 analogues for cardiovascular effects and mortality will be needed to substantiate these findings. In the meantime, patients treated with DDP IV inhibitors and GLP-1 analogues should be closely monitored for signs of cardiovascular disease.

The present study adds the evidence that a structured and intensive glucose monitoring in these patients makes it possible to reach a good metabolic control by an IIT in the absence of increased rate of hypoglycemic episodes. However, if it is not possible to perform such a structured program, intensive glucose-lowering treatment of type 2 diabetes should be considered with caution and therapeutic escalation should be limited.

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