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# Glycemic Variability Affects Ischemia-Induced Angiogenesis

## In Diabetic Mice

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# Summary.

The aim of this study was to investigate the role of glycemic variability (GV) on diabetic vascular complications and to explore the molecular pathways modulated by glycemic “swings”. We performed a murine model. 30 diabetic mice received once daily basal insulin administration plus 2 oral bolus of glucose solution (GV group, V) and 30 diabetic mice received once daily basal insulin plus 2 oral bolus of saline solution (stable hyperglycemia group, S) for a period of 30 days. Glycemia was measured 8 times daily to detect GV. Finally, post-ischemic vascularization, induced by hindlimb ischemia 30 days after diabetes onset, was evaluated.

We found that GV was significantly different between S and V groups, whereas no significant difference of the mean glycemic values was detected. Laser Doppler perfusion imaging and histological analysis revealed that the ischemia-induced angiogenesis was significantly impaired in V mice compared to S group, after ischemic injury. In addition, immunostaining and western blot analyses revealed that impaired angiogenic response in V mice occurred in association with reduced VEGF production and decreased eNOS and Akt phosphorylation.

In conclusion, we describe a murine model of GV. GV causes an impairment of ischemia-induced angiogenesis in diabetes, likely to be independent of changes in average blood glucose levels, and this impaired collateral vessel formation is associated with an alteration of the VEGF pathway.

**Keywords:** Angiogenesis, Diabetes complications, Vascular endothelial growth factor.

## Introduction.

Cardiovascular complications are mainly responsible for the high morbidity and mortality in people with diabetes mellitus (DM). Indeed, diabetic subjects have 3 to 5-fold higher risk for cardiac ischemic disease death [1, 2] and 2 to 5-fold for stroke and peripheral arterial disease than non-diabetic subjects [3]. Furthermore, long-term prognosis after a coronary event is significantly worse among people with DM than among those without DM [4]. Usually, the risk factors for cardiovascular complications in DM are grouped in glycemic and non-glycemic. Non-glycemic risk factors include hypertension, dyslipidemia, inflammation, microalbuminuria, abdominal obesity and smoking, whereas fasting plasma glucose (FPG), postprandial glucose (PPG), glycemic variability (GV) and glycated hemoglobin (HbA<sub>1c</sub>, index of glycemic control of the last 3/4 months) belong to the first group. Recently, several epidemiological studies performed in both type 2 and type 1 diabetes [5, 6] have suggested that FPG [7], PPG [8] and GV [6] are HbA<sub>1c</sub>-independent risk factors for vascular complications, leading to the concept of “the glucose tetrad” (HbA<sub>1c</sub>, FPG, PPG and GV) [9]. In particular, the role of GV in diabetic complications was at first hypothesized by DCCT study’s authors who surmised that the mean HbA<sub>1c</sub> is not the most complete expression of the degree of glycemia and that the risk of complications may be mostly dependent on other factors; indeed, even when HbA<sub>1c</sub> values were comparable between intensively and conventionally treated subjects, the latter group experienced a markedly higher risk of progression to retinopathy [10]. A possible explanation is that glycemic excursions were of greater frequency and magnitude among conventionally treated patients (who received fewer insulin injections), generating more reactive oxygen species (ROS) in endothelial cells (ECs). Subsequent *in vitro* and *in vivo* studies have confirmed this hypothesis [5, 11-13]; however, to date, the cause-effect relationship between GV and in-vivo diabetic vascular disease is still not clear.

Several of the long-term complications of diabetic vasculopathy are associated with aberrant angiogenesis. In fact, excessive angiogenesis plays a role in diabetic retinopathy, nephropathy and neuropathy whereas inhibited angiogenesis contributes to impaired wound healing, impaired coronary and peripheral artery diseases [14]. After vascular damage, post-ischemic angiogenesis is an important reparative mechanism and can ameliorate the outcome of diabetic vascular pathology. Impaired collateral vessels’ formation in response to ischemic injury has been demonstrated in murine models of DM: hindlimb ischemia created by ligation of the femoral artery has been associated with a reduced formation of capillaries and a reduction in blood flow to the ischemic limb in diabetic compared to non-diabetic mice [15]. Nevertheless, previous studies have focused mostly on chronic hyperglycemia consequences, while there is lack of evidence on the importance of GV-related effects. Therefore, the aim of the present study was to demonstrate a cause-effect relationship between GV and vascular complications, evaluated as impaired response to hindlimb ischemia and to analyzed if the severity of vascular dysfunction was higher with GV than with stable hyperglycemia. Furthermore, we focused our attention on the VEGF/Akt/eNOS pathway, as it has been implicated in vascular alterations due to chronic hyperglycemia [16].

## Materials and Methods.

### Mouse model of glycemic variability.

The investigation was approved by A. Gemelli University Hospital Institutional Animal Care and Use Committee. Male 8-12-week-old C57BL/6J mice were used for experiments. All animals were allowed free access to the same standardized diet and water during the entire study. Diabetes was induced by administering 50 mg/kg body wt streptozotocin (STZ; Sigma) in citrate buffer (pH 4.5), intraperitoneally (i.p.) during the fasting state and consecutively for 5 days, as previously described [17]. Hyperglycemia was verified on blood obtained from the tail vein, by an Accu-Check Active glucometer (Roche) 2 days after STZ injections. We considered mice to be diabetic when blood glucose was at least 16 mmol/l (normal 5–8 mmol/l). Overall, 60 mice showed a blood glucose level of at least 16 mmol/l, both 1 and 2 weeks after the STZ injections and, therefore, were included in the experimental group. Then, the diabetic animals were divided in two groups (30 mice each): the first group (glycemic variability group, named “V”) was treated with a subcutaneous injection of 0.5 units of long-acting insulin glargine at 8 am, plus 2 boluses of 0.7 ml 33% glucose solution (at 10 am and 2 pm) by gavage daily for 30 days; the second group (stable hyperglycemia group, named “S”) received 0.5 units of long-acting insulin glargine at 8 am, plus 2 boluses of 0.7 ml saline solution (at 10 am and 2 pm) by gavage daily for 30 days (**Fig. 1**). During the night, both groups received no intervention and lived in the same environmental conditions. For 30 days, glycemic values of each mouse were measured in tail blood by glucometer at 8 am, 10 am, 12 am, 2 pm, 4 pm, 6 pm, 8 pm and 10 pm (**Fig. 1**). After the treatment and until the end of the study (subsequent 28 days; see below), both V and S received a subcutaneous injection of 0.5 units of glargine at 8 am plus 2 boluses of 0.7 ml saline solution (at 10 am and 2 pm); the glycemic profiles were measured as previously described. GV was measured by three well-known indices, namely standard deviation (SD), mean amplitude of glycemic excursions (MAGE, the mean of glycemic excursions > 1 SD) [18], continuous overlapping net glycemic action (CONGA<sub>n</sub>, SD of differences between any glucose value and another one exactly n hours later) [19]. Finally, 30 untreated normoglycemic C57BL/6J mice (control group, named “C”) were also included in the model and glycemic profiles were measured for 30 days as in V and S.

### Mouse hindlimb ischemia model.

After 30 days, unilateral hindlimb ischemia was induced in control group mice (30 mice), in V and S mice (30 mice each), as previously described [20]. Briefly, all animals were anesthetized with an i.p. injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). The proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. The arteries and all side branches were dissected free and excised. The skin was closed with 5-0 surgical sutures. A laser Doppler perfusion imager system (PeriScan PIM II, Perimed) was used to measure hindlimb blood perfusion before and immediately after surgery and then weekly, until the end of the study, for a total follow-up of 28 days after surgery. Before imaging, excess hairs were removed from the limbs using depilatory cream and mice were placed on a heating plate at 40 °C. To avoid the influence of ambient light and temperature, results were expressed as the ratio between perfusion in the right (ischemic) versus left (non-ischemic) limb.

### Histological Assays.

At 1 and 4 weeks after surgery, mice were sacrificed by i.p. injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight. The femora were carefully removed, and the ischemic thigh muscles were embedded in paraffin. All the specimens were routinely fixed overnight in 4% buffered formalin and embedded in paraffin. Four-micrometer sections of tissue samples were subjected to immunoperoxidase biotin–avidin reaction in the Labeled Streptavidin Biotin method (LSAB) to determine the CD31 and VEGF expression. CD45 was used as a marker for inflammatory infiltrate. Ki67 was used as a marker for proliferation. The cytoplasmic expression of Ki67, a nuclear protein expressed in cell cycle, may be considered a functional phenomenon that is shared by normal tissues undergoing postnatal remodeling [21]. The sections for immunohistochemistry were cut and mounted on 3-aminopropyltriethoxy-silane-coated (Sigma) slides, allowed to dry overnight at 37° C to ensure optimal adhesion, dewaxed, rehydrated, and treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min to block endogenous peroxidase. For antigen retrieval (not necessary for VEGF) the section were microwave treated in 1 mM EDTA at pH 8 (for CD31) and pH 6 (for CD45 and Ki67) for 10 min and allowed to cool for 20 min. Endogenous biotin was saturated using a biotin blocking kit (Vector Laboratories). The sections were incubated at room temperature for 30 min with the following antibodies: purified rat anti-mouse CD31 [dilution 1:30; monoclonal (IgG2a); BD Bioscience], rabbit anti-mouse VEGF (dilution 1:100, polyclonal, Santa Cruz Biotechnology) and rabbit anti-mouse CD45 (dilution 1:50, polyclonal, AbCam), and incubated overnight with rabbit anti-mouse Ki67 [dilution 1:200; monoclonal (SP6); Novus Biological]. Binding was visualized using biotinylated secondary antibody (1 h of incubation) and the Streptavidin-biotin peroxidase complex developed with diaminobenzidine. Finally, slides were counterstained with hematoxylin. Capillary density, proliferating cells and leukocyte infiltration were measured by counting six random high power (magnification ×200) fields for a minimum of 200 fibers from each ischemic and non-ischemic limb on an inverted light microscope, and were expressed by the number of CD31+, Ki67+ or CD45+ cells per square millimeter. VEGF-positive cells were counted from four fields of view using a ×20 objective lens and the mean cell number per mm<sup>2</sup> was established. Apoptosis was demonstrated in situ using the Mebstain Apoptosis kit II (Immunotech, France), and the apoptotic index was determined by dividing the total number of myocytes showing nuclear positivity by the total number of cells in the fields examined [22]. Area was measured with a NIH Image analysis system (ImageJ 1.41). Two operators extracted independently the results.

### Western Blotting.

Immunoblotting was performed on homogenates of muscle tissues. Protein concentration of samples was carefully determined by the protein assay (Bio-Rad Laboratories, Inc.). Equal amounts of protein were subjected to SDS-PAGE electrophoresis using 4–12% gradient gels under reducing condition (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (GE Healthcare). To ensure equivalent protein loading and quantitative transfer efficiency of proteins, membranes were stained with Ponceau S before incubating with primary antibodies. Membranes were incubated with antibodies against VEGF (1:500) (Santa Cruz Biotechnology), eNOS (1:1000) (Santa Cruz Biotechnology), phospho-eNOS (Ser1177, 1:1000) (Cell Signaling Technology Company), Akt (1:1000) (Santa Cruz Biotechnology) and phospho-Akt (Ser473, 1:500) (Cell Signaling Technology Company). Immunoreactive bands were detected by an ECL



kit (GE Healthcare). VEGF, eNOS, p-eNOS, Akt and p-Akt expression was normalized using a mouse monoclonal anti- $\alpha$ -actin antibody. Densitometric analysis was performed and the results expressed as a ratio vs  $\alpha$ -actin.

### ELISA for VEGF.

Ischemic and control hindlimbs were harvested 7 days after surgery. VEGF levels were measured by ELISA (R&D Systems, Minneapolis, M, USA). Results were expressed as protein fold increase, calculated as the ratio between VEGF protein levels in ischemic and control hindlimb.

### Statistics.

Statistical analysis was done using STATA software (version 10.0; STATA). Data are expressed as the means $\pm$ SEM. Comparison among groups was done using ANOVA followed by Fisher's post hoc test. Repeated-measures ANOVA was done to assess improvement in perfusion over time within groups. Statistical significance was set at a probability value ( $p$ ) of  $<0.05$ .

### Results.

#### A new model of glycemic variability.

All the three considered indices of GV (MAGE, CONGA2, SD) were significantly different between V, S and C (**Online-Fig. 2a and Online-Fig. 2b and Online-Table**); the difference in mean glucose value (mgv) between V and S did not reach statistical significance ( $p = 0.066$ ), and was not different between S and C ( $p = 0.396$ ), while it was between V and C ( $p = 0.001$ ). Furthermore, there was no difference in glycemic values at 8pm, 10pm, 8am and 10am among the three groups, while it was found after the glucose gavage at 12am, 2pm, 4pm and 6pm (**data not shown**); these data indicate that the difference in GV is a consequence of 12am, 2pm, 4pm and 6pm-glycemic values, with a similar (not statistically different) glycemic control between 8pm and 10am (night profile). Overall, the results of our models clearly indicate that we can consider the V group as having different glycemic variability from S and C, but similar mgv. This is of relevant as, to date, clinical studies regarding diabetes focused on the positive association between diabetic vascular complications and HbA1c, mathematically correlated to mean glucose value [ $HbA1c = f(mgv)$ ] [23]. Therefore, the glucose-related effects demonstrated in our model, in particular the angiogenic response to ischemic injury between V and S, as well as S and C, are more likely to be a result of the effect of glucose oscillations. Furthermore, as insulin was administered in V and S at the same units (0.5 units), its effect on the angiogenic response to ischemic injury is the same in the two groups. Of note, the 28-day analysis of the glycemic data after surgery showed that GV indices and mgv were not different among V, S and C (**data not shown**).

#### Ischemia-induced angiogenesis is significantly impaired in V mice compared with S mice.

Laser Doppler perfusion imaging was performed before, immediately after, and on days 7, 14, 21 and 28 after hindlimb ischemia (**Fig. 3**). Mean blood flow in untreated (C) mice reached 94% of the pre-ischemic flow 28 days after hindlimb surgery. Perfusion recovery was significantly attenuated in S mice compared to C mice on postoperative days 7 ( $p=0.021$ ), 14 ( $p=0.023$ ), 21

( $p=0.018$ ) and 28 ( $p=0.032$ ). Interestingly, the recovery was further impaired in V mice compared to S mice 7 ( $p=0.023$ ), 14 ( $p=0.026$ ), 21 ( $p=0.008$ ) and 28 ( $p=0.002$ ) days after surgery. In addition, histological analysis revealed that the capillary density in ischemic limb was significantly decreased in all diabetic mice compared to control mice and, importantly, there was a statistically significant difference between S and V mice ( $p=0.029$ ) (**Fig. 5**).

To test whether observed angiogenic responses were dependent either on different proliferation, apoptosis or inflammation stimuli between the two diabetic groups or an altered regulation of pro-angiogenic mechanisms, we analyzed, proliferation, apoptosis and leukocyte infiltration and noted there were no differences between S and V mice according to all three aspects (**Fig. 4a and 4b**). Therefore, it is possible to state that the observed difference in post-ischemic vessel formation does not depend on a different proliferation, apoptotic or inflammatory response to hyperglycemia between the two groups.

### **Impaired angiogenic response in V mice occurs in association with reduced VEGF production and decreased eNOS and Akt phosphorylation.**

Immunostaining revealed that VEGF expression in the ischemic tissue of S mice was reduced 7 days after surgery compared to control mice (**Fig. 6a and Fig. 6b**) and that it was significantly impaired in V mice compared to S mice on postoperative day 7 (**Fig. 6b and Fig. 6c**). Western blot analysis and VEGF positive cells evaluation demonstrated that VEGF concentration in ischemic tissue was also significantly higher in S mice than in V mice (**Fig. 6d and Fig. 6e**), underlying the crucial role of VEGF in impaired angiogenic response observed in V mice. To further investigate the mechanism by which the GV inhibits angiogenesis in diabetic mice, we evaluated eNOS and Akt phosphorylation in the ischemic leg 7 days after surgery by Western blotting analysis (**Fig. 7**). GV reduced phosphorylation/activation of eNOS at Ser<sup>1177</sup> and of Akt at Ser<sup>473</sup>. VEGF exerts many of its effects via the Akt pathway [24]. The Akt pathway leads to downstream activation of eNOS and release of nitric oxide, which results in cGMP production. The ratios of p-Akt/total Akt and p-eNOS/total eNOS (**data not shown**) were significantly lower in V versus S group, indicating that there is reduced downstream VEGF signaling.

### **Discussion.**

DM is a pathological condition of eminent epidemiological importance and several of the long-term diabetic complications are characterized by vasculopathy associated with aberrant angiogenesis. Experimental animal models of peripheral arterial disease in DM have shown attenuated perfusion recovery in response to ischemia [25]. A large number of hypothesis have been postulated to explain the impaired angiogenic post-ischemic response in diabetes, such as the presence of vascular dysfunction characterized by both endothelial and vascular smooth muscle cells impairments [26], the decreased release or defective function of endothelial progenitor cells from the bone marrow [27], the exposure to chronic hyperglycemia that leads to the non enzymatic glycation of proteins and defective formation of new blood vessels [28], or the presence of abnormalities in growth factor signaling and/or expression, with maladaptive dysregulation of vascular growth factors pathways [29]. But it is important to underline, once again, that previous studies on diabetic effects have focused principally on steady state



conditions of constant hyperglycemia, and not on glycemic control, glycemic profiles and degree of GV. For this reason we developed a mouse model of increased GV, to analyze how GV could contribute to ischemia-induced vascularization in diabetic mice, and whose most relevant feature was the presence of a significant difference of glycemia variability comparing V vs S groups. In fact, in our model, an accurate (8 glycemic values per day; for comparison, in the cornerstone study DCCT, GV was evaluated from 7 per day measurements [10]) and prolonged (58 days) evaluation of glycemic profiles evidenced as V and S mice were different primarily between GV, and S and C. Therefore, the found differences can be likely attributed primarily to GV. To our knowledge, this is the first animal model where GV is the primary difference and this represents the first innovative finding of this work. Furthermore, we used this model to analyze if GV has more deleterious effects on vascular function than stable hyperglycemia. In our experimental model, after hindlimb ischemia, the presence of GV caused a significant impairment of post-ischemic angiogenesis, if compared to collateral vessels formation and blood flow recovery observed in untreated control mice and in stable diabetic mice. Moreover, V mice showed a reduced VEGF up-regulation and protein expression in ischemic muscle after the ischemic injury. The role of angiogenesis in diabetic cardiovascular diseases represents a major unresolved issue. Angiogenesis has attracted interest from opposite perspectives. Angiogenic cytokine therapy has been widely regarded as a fascinating approach both for treating ischemic diseases and for enhancing atheroprotective functions of the endothelium [30]; conversely, several studies suggest that vascularization contributes to the development of atherosclerotic lesions and is a key factor for plaque destabilization, leading to plaque vulnerability and rupture [31]. But it is evident that angiogenesis is an important defense mechanism from diabetic micro- and macro-vascular disease that leads to formation of new collateral vessels and consequent blood supply to peripheral ischemic tissue. Previous studies have shown that large glycemic swings exert deleterious effects in endothelial function and cardiovascular complication of DM [26] but, up to now, there are no data about the relationship between GV and ischemia-induced vascularization. To further investigate whether an alteration of VEGF-Akt-eNOS pathway was involved in the post-ischemic angiogenesis in our model, we evaluated eNOS and Akt activity, because Akt-dependent phosphorylation of eNOS at Ser<sup>1177</sup> plays a key role in VEGF mediating vascular formation after ischemic injury [32]. Previously, few studies investigated the modifications of Akt and eNOS pathway in DM and, actually, alterations of Akt or eNOS activity in diabetes have been found in various cells and tissues, depending on experimental and clinical contexts. Defects in VEGF-Akt-eNOS activity have been reported in endothelium of Type 2 DM models, possibly contributing to the development of endothelial dysfunction and loss of angiogenesis under these conditions [33]. In contrast, Akt activity is increased in some tissues and vascular beds affected by complications of Type 1 DM [34]. In the present study we demonstrated that GV is associated with a phosphorylation/activation of eNOS and Akt in diabetic mice, indicating there is impaired downstream VEGF signaling. Of note, as C mice did not receive insulin, the histological and functional differences between C and S could be due to the administration of insulin; however, this hypothesis is not supported by the evidence of the same differences between S and V, both receiving the same type and dose of insulin (glargine 0.5u).

These results are not in conflict with the existing evidence, as the studies that have analyzed the angiogenic response to ischemia in diabetic mice have been performed without insulin treatment and in conditions of constant hyperglycemia [29]. These studies have documented a reduced ischemia-induced vascularization but an increased up-regulation of VEGF, with a secondary

functional alteration of this angiogenic factor. Our results underline that, when the DM is controlled with the administration of insulin, the post-ischemic collateral vessels formation is reduced, with an impaired VEGF up-regulation and not significantly when compared to non-diabetic mice. Particularly, our findings show that when a significant blood glucose variability is induced in the same conditions of diabetes controlled by insulin treatment, the angiogenic response to ischemic injury is further and significantly reduced respect to non-diabetic and to diabetic mice with chronic hyperglycemia. Moreover, our data indicate that both VEGF up-regulation and eNOS and Akt phosphorylation/activation are also significantly reduced in these animals, suggesting a reduced downstream VEGF signaling in GV group mice.

Our experimental findings support the clinical evidence of the role of glycemic control as a new potential risk factor for increasing cardiovascular disease's mortality and morbidity in DM [35]. Indeed, as non-glycemic risk factors are well-recognized and treated in diabetic as well as in non-diabetic subjects (often to lower targets in diabetics), something else should justify a such persistent excess of mortality due to diabetes. Obviously, the difference between diabetics and non-diabetics is the degree of glycemia but, even after an intensive treatment of blood glucose – reduction of HbA<sub>1c</sub>/mean blood glucose – the mortality remains high; so, other glycemic risk factors than HbA<sub>1c</sub> should be considered in the treatment of blood glucose disturbances in diabetes. Indeed, the glycemic control (mgv) of S group was not statistically significantly different from C group. The mechanism through which GV induces loss of angiogenic properties after acute ischemia could be related to an excess of oxidative stress in ECs [36]. Oxidative stress is known to be the key mechanism in the pathogenesis of diabetes-related endothelial dysfunction. Oxidative stress is attributable to excessive production of ROS and the inactivation of nitric oxide (NO) by ROS is recognized to be a crucial factor in reducing NO bioavailability and the development of endothelial dysfunction [37]. Interestingly, both chronic sustained hyperglycemia and acute glycemic fluctuations from peaks to nadirs, the two main determinants of GV, conduce to the activation of oxidative stress with an overproduction of superoxide by the mitochondrial electron-transfer chain [16]. Even if there are not data regarding the role of GV in the pathogenesis of diabetic ischemic damage, our results suggest a possible correlation between the glucose fluctuations and angiogenic defects. This hypothesis deserves further evaluation and will represent the base of our future studies.

In conclusion, this study designed and developed a murine model of GV. This will allow further studies to analyze the effect of GV on diabetic complications. Moreover, our experiments also showed that GV significantly reduces angiogenic reaction to peripheral ischemic damage in diabetic mice, in the presence of similar average blood glucose levels. Finally, our data show that the reduced angiogenesis occurs in association with lowered VEGF up-regulation and with an impaired eNOS and Akt phosphorylation. These data provide support the hypothesis that GV contributes to vascular complications of DM and control of GV plays a potentially important role for the management of diabetic patients.

## **Addendum.**

### **Authors' contributions:**

FB, DP, GS and FZ participated in the design of the study, performed the hindlimb ischemia model, performed data analysis and reviewed the manuscript. PR, RDC, SL and TM carried out the immunoassays. VA and ES performed the immunohistochemical analysis. GG and AF conceived the study, participated in its design and coordination and helped draft the manuscript.

### **Founding.**

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### **Disclosures.**

The authors declare that they have no conflicts to disclose.

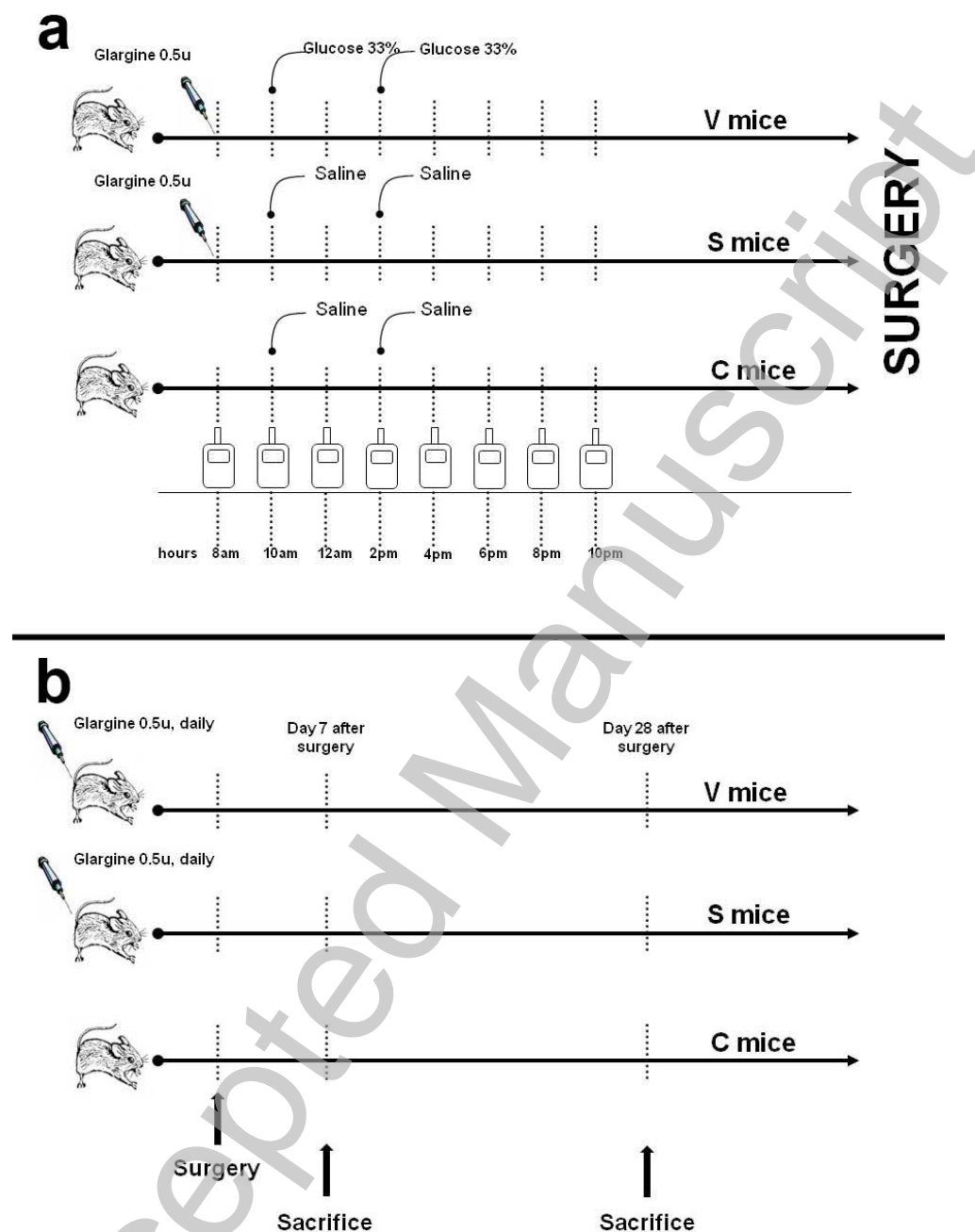
## Table and Figures Legends.

### Online-Table

	V vs S	S vs C	V vs C
Mean Glycaemia	$p = 0.066$	$p = 0.396$	$p = 0.001$
Standard Deviation	$p < 0.001$	$p < 0.001$	$p < 0.001$
MAGE	$p < 0.001$	$p < 0.001$	$p < 0.001$
CONGA <sub>nh</sub>	$p < 0.001$	$p = 0.035$	$p < 0.001$

Indices of glycemic variability and mean glycemia derived from 7,200 per-group glucose measurements (30 mice each group). SD, standard deviation; MAGE, mean amplitude of glycemic excursions; CONGA, continuous overlapping net glycemic action.

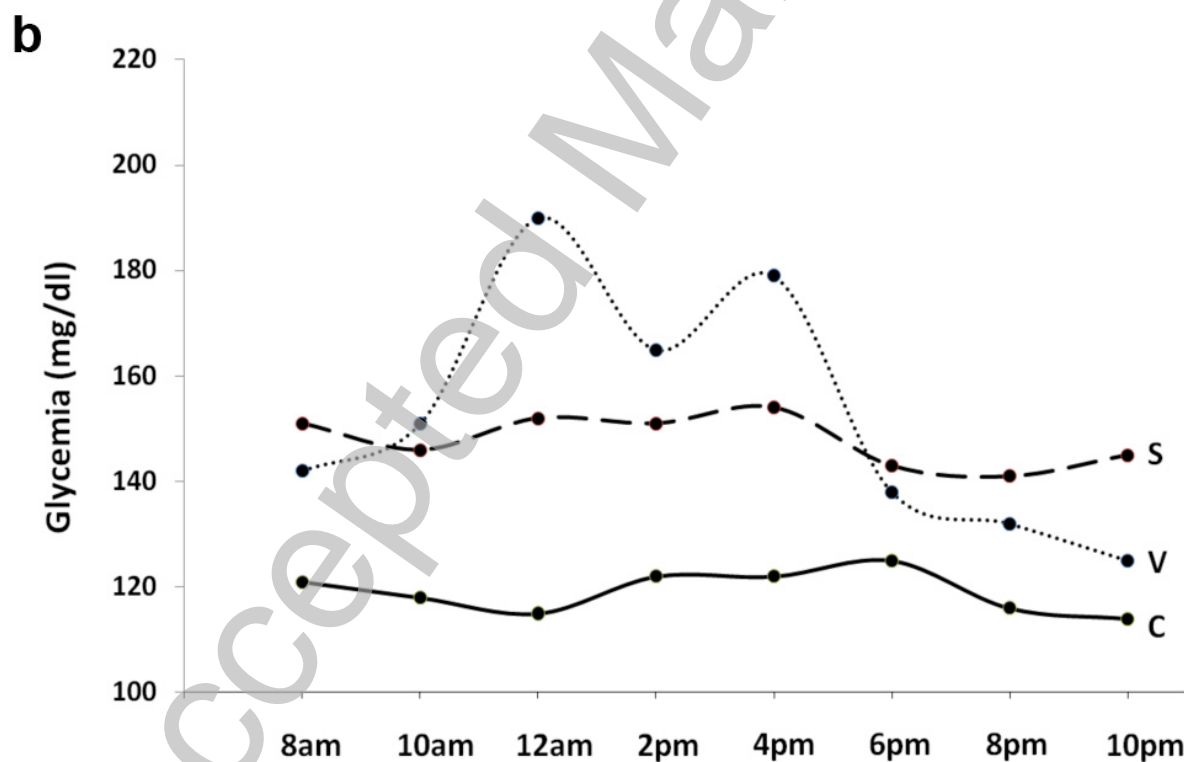
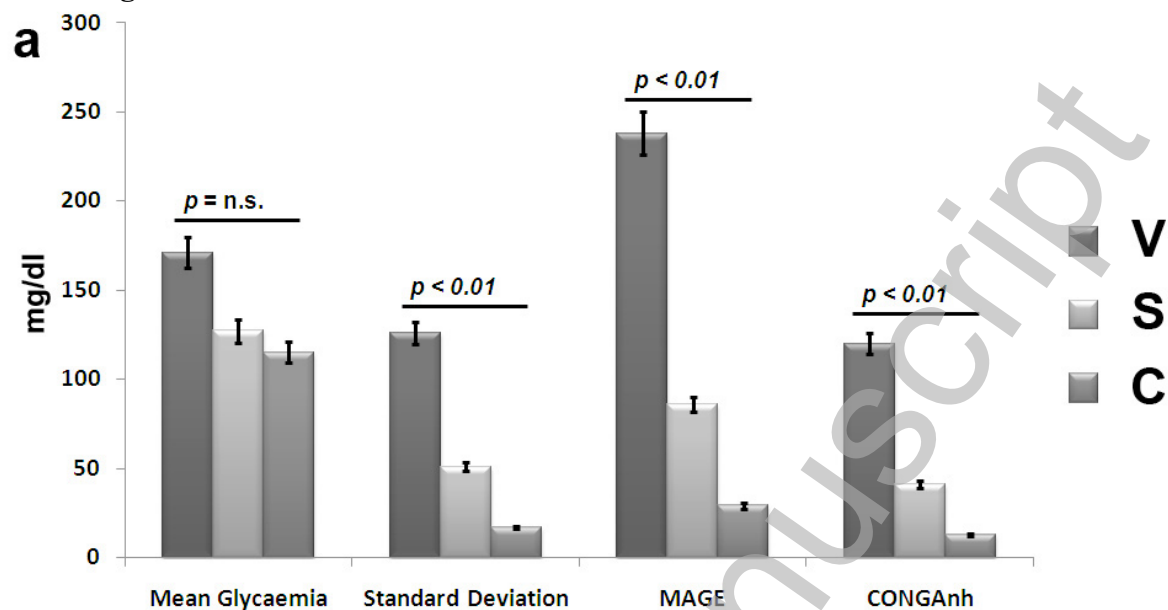
Figure 1



A schematic representation of the GV experimental protocol (30 mice each group). After the diagnosis of diabetes, the V mice received 0.5u s.c. long-acting insulin glargine at 8 am plus 2 oral boluses of 0.7 ml 33% glucose solution (at 10 am and 2 pm) to induce glycemic swings, whereas the S mice received 0.5 units of long-acting insulin glargine at 8 am, plus 2 boluses of 0.7 ml saline solution (at 10 am and 2 pm). Control C mice received an equal amount of 0.7 ml saline solution on the same time schedule. Tail blood samples were measured at 8 am, 10 am, 12 am, 2 pm, 4 pm, 6 pm, 8 pm and 10 pm (a). A schematic representation of the mouse hindlimb ischemia model protocol (b).

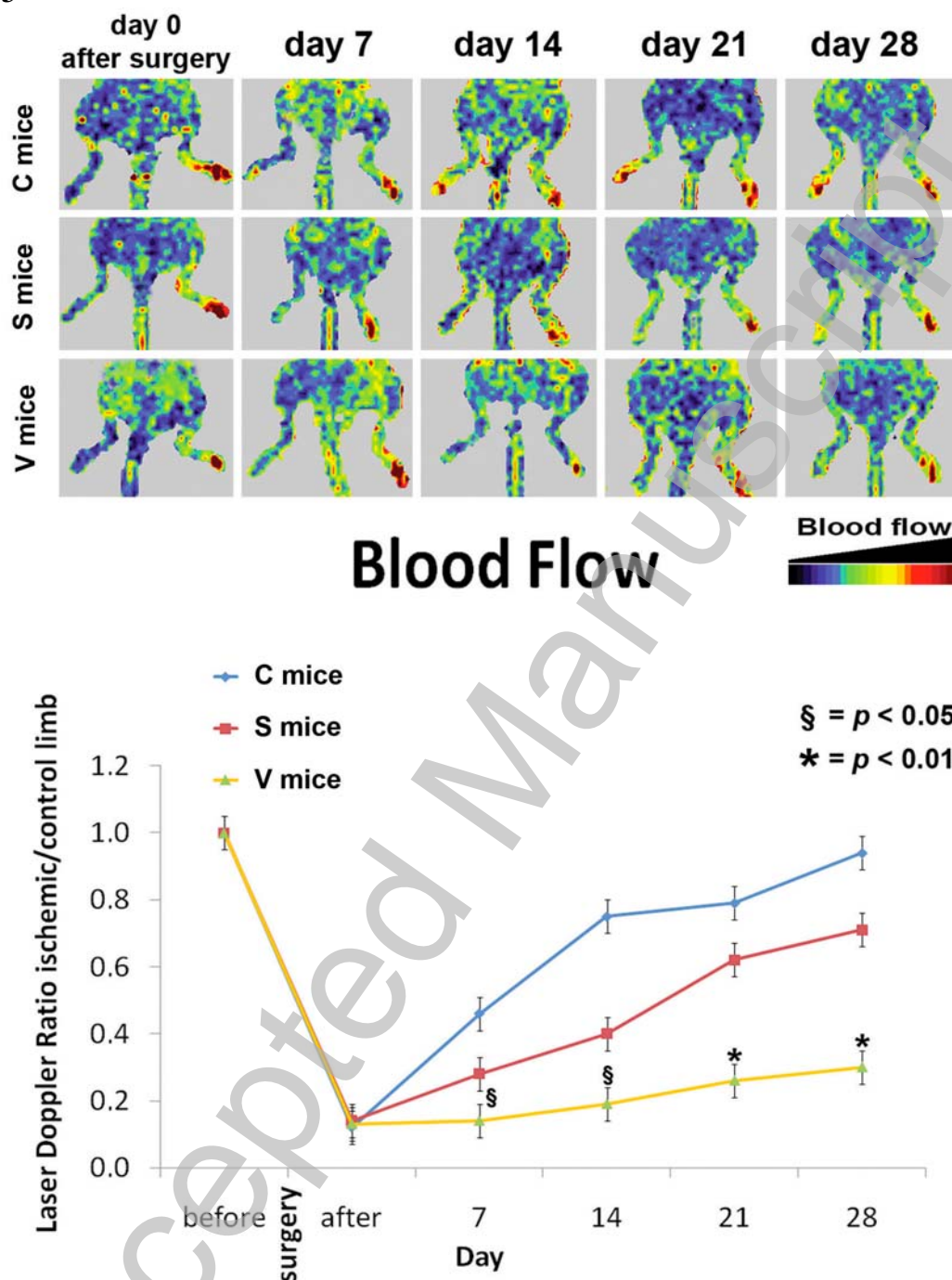


Online-Figure 2



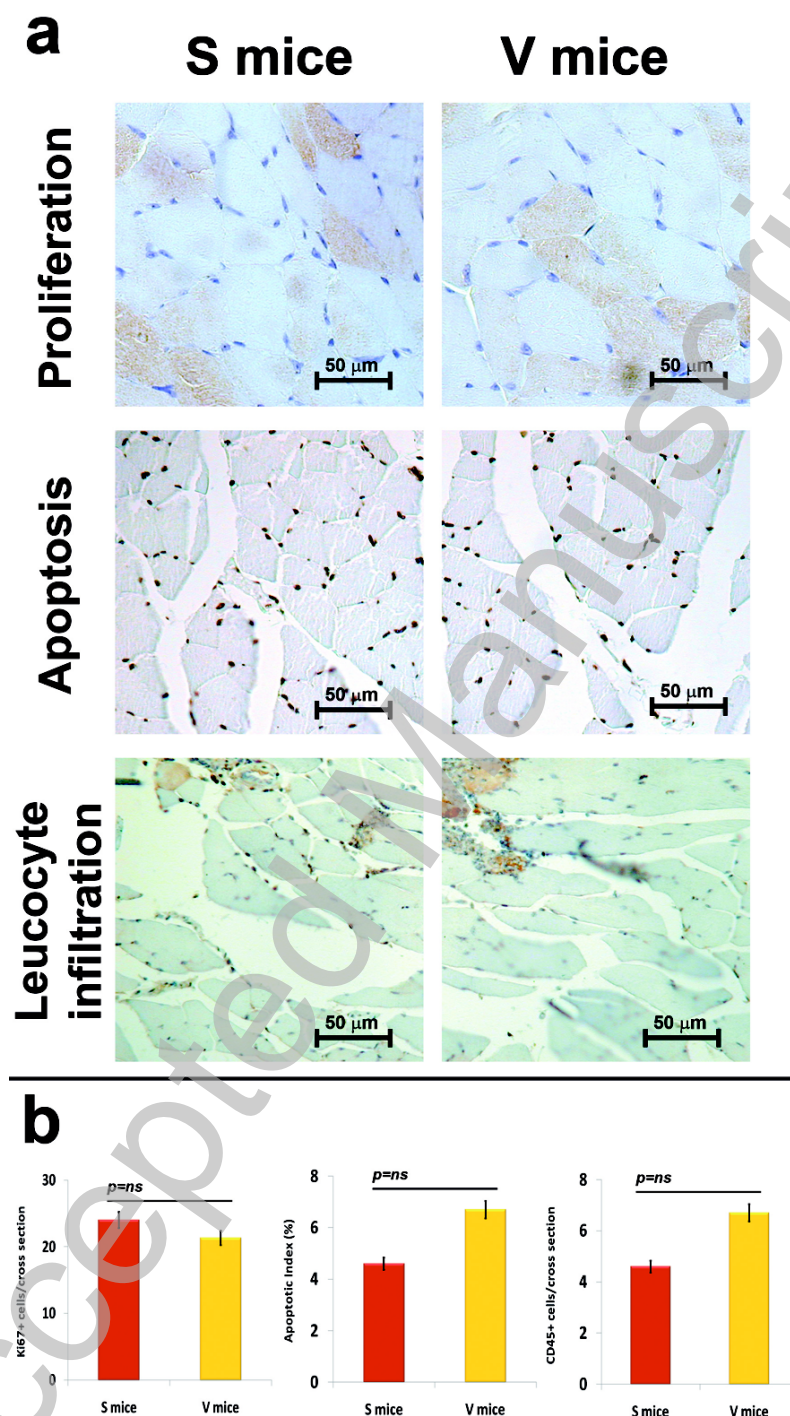
Indices of glycemic variability and mean glycemia derived from 7,200 per-group glucose measurements (30 mice each group). SD, standard deviation; MAGE, mean amplitude of glycemic excursions; CONGA, continuous overlapping net glycemic action (a). Mean glycemic profiles of Stable (S), Variable (V) and Control (C) mice (b).

Figure 3



Foot blood flow monitored *in vivo* by laser Doppler perfusion imaging in C, S and V mice (10 mice each group). Representative evaluation of the ischemic (right) and non-ischemic (left) hindlimbs, immediately after surgery and on postoperative days 7, 14, 21 and 28. In color-coded images, red indicates normal perfusion while blue a marked reduction in blood flow of ischemic hindlimb. Perfusion recovery was significantly attenuated in V mice compared with S mice on postoperative days 7, 14, 21 and 28 (respectively  $p=0.021$ ,  $p=0.023$ ,  $p=0.018$  and  $p=0.032$ ). The blood flow of the ischemic hindlimb is expressed as the ratio between perfusion of the ischemic limb versus uninjured limb.

Online-Figure 4

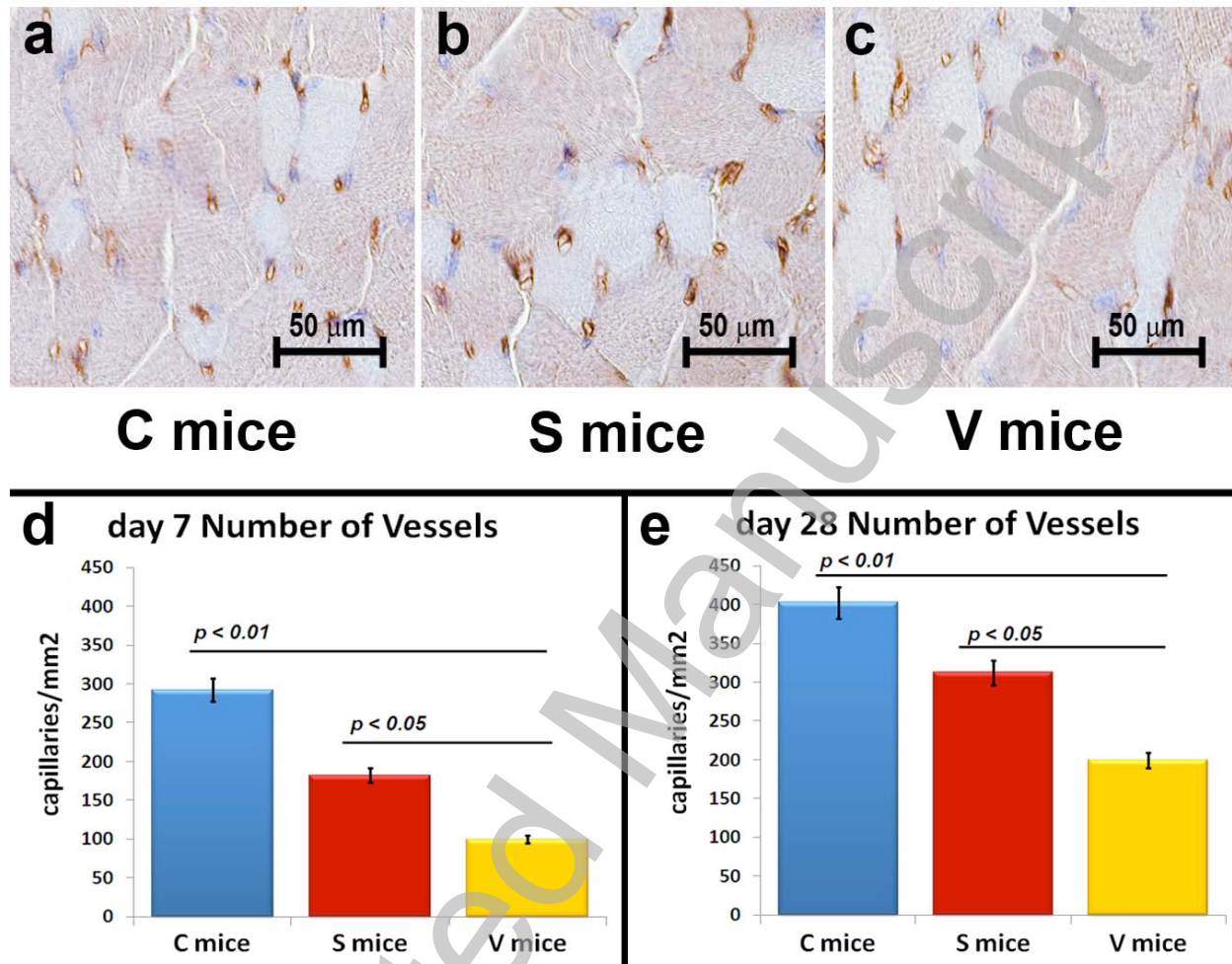


Representative photomicrographs of ischemic muscle sections from S and V mice stained with antibody directed against Ki67, with TUNEL, and with antibody directed against CD45, 7 days after surgery. Magnification  $\times 40$ , positive staining appears in brown (a). Evaluation of proliferation, apoptosis and leukocyte infiltration in the ischemic muscle sections from S and V mice. There are no differences between S and V mice according to all three aspects.  $p = n.s.$  vs. V mice.



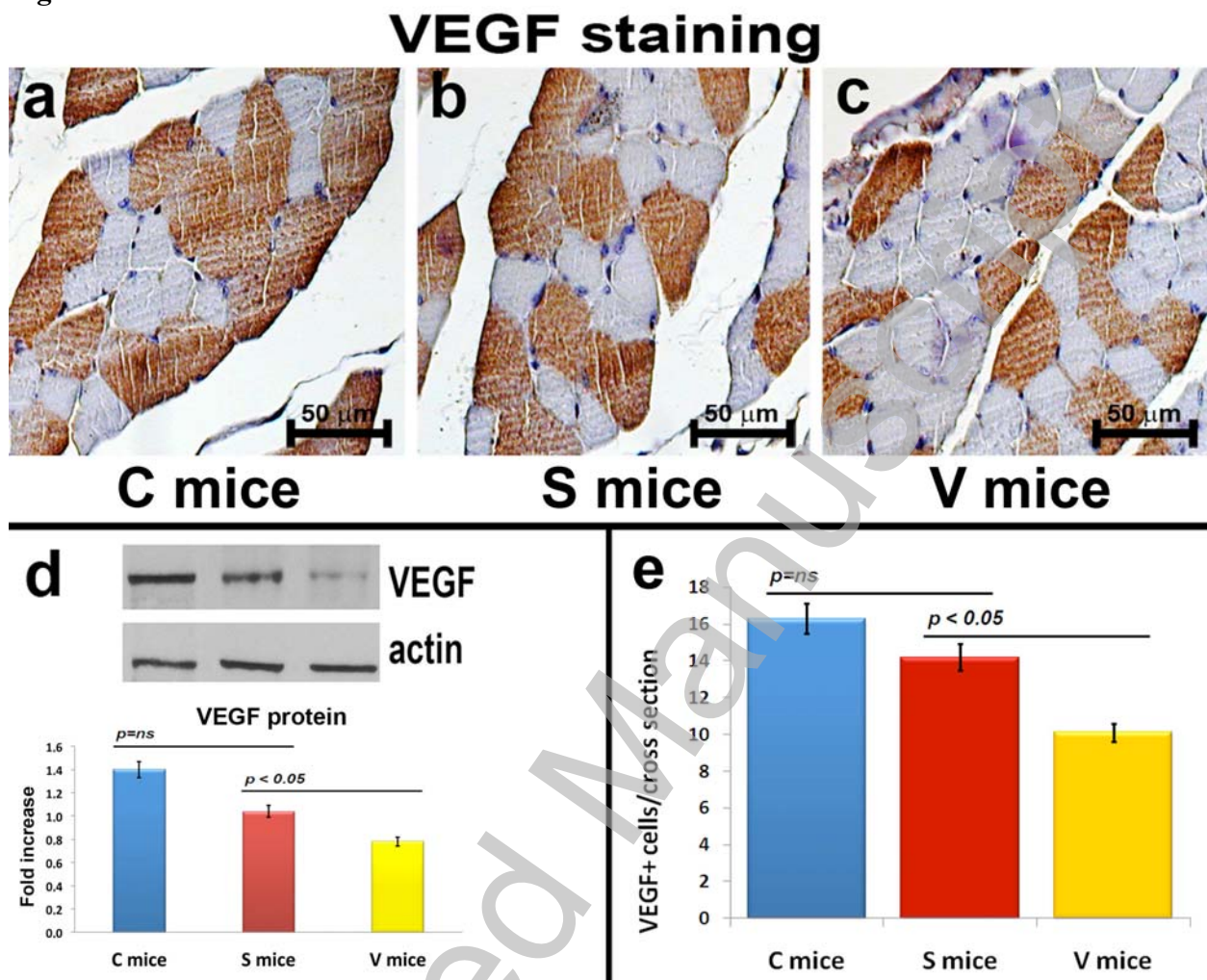
Figure 5

## CD 31 staining



Representative photomicrographs of ischemic muscle sections from C (a), S (b) and V mice (c) stained with antibody directed against CD-31, 28 days after surgery (10 mice each group). Positive staining appears in brown. Magnification  $\times 40$ . Number of vessels per cross section is significantly reduced in V mice respect to control and to S mice on postoperative days 7 (d) and 28 (e).

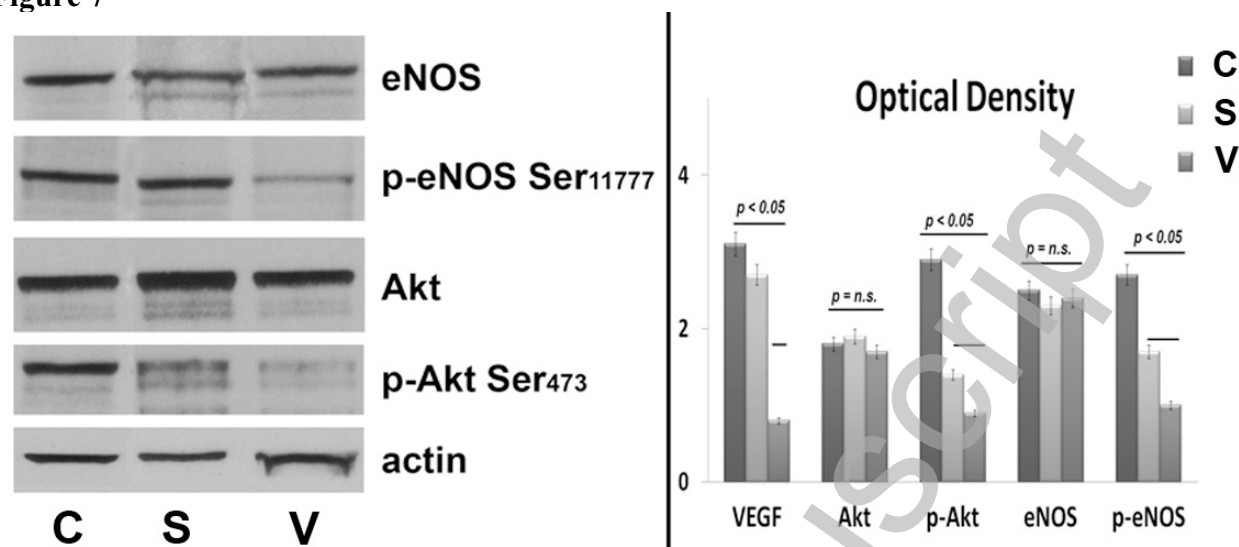
Figure 6



Representative photomicrographs of ischemic muscle sections from C (a), S (b) and V mice (c) stained with antibody directed against VEGF, 7 days after surgery (10 mice each group). Positive staining appears in brown. Magnification  $\times 40$ . Representative Western blotting and ELISA quantitative evaluation of VEGF content in the ischemic legs of C, S and V mice (d). VEGF positive cells in ischemic tissue were significantly impaired in V mice compared to C and to S mice 7 days after surgery (e).



**Figure 7**



Representative Western blotting and relative Optical Density evaluation of VEGF, eNOS, phospho-eNOS, Akt and phospho-Akt content in the ischemic legs of C, S and V mice group mice (10 mice each group). Phosphorylated eNOS and phosphorylated Akt concentrations in ischemic tissue were significantly impaired in V mice compared to C and to S mice 7 days after surgery.

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