Cardiovascular Research
doi:10.1093/cvr/cvx065

Low-dose ionizing radiation induces therapeutic neovascularization in a pre-clinical model of hindlimb ischemia

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Received 23 September 2016; revised 20 January 2017; editorial decision 11 March 2017; accepted 29 March 2017

Time for primary review: 40 days

Aims We have previously shown that low-dose ionizing radiation (LDIR) induces angiogenesis but there is no evidence that it induces neovascularization in the setting of peripheral arterial disease. Here, we investigated the use of LDIR as an innovative and non-invasive strategy to stimulate therapeutic neovascularization using a model of experimentally induced hindlimb ischemia (HLI).

Methods and results After surgical induction of unilateral HLI, both hindlimbs of female C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. We demonstrate that LDIR, significantly improved blood perfusion in the murine ischemic limb by stimulating neovascularization, as assessed by laser Doppler flow, capillary density, and collateral vessel formation. LDIR significantly increased the circulating levels of VEGF, PlGF, and G-CSF, as well as the number of circulating endothelial progenitor cells (EPCs) mediating their incorporation to ischemic muscles. These effects were dependent upon LDIR exposition on the ischemic niche (thigh and shank regions). In irradiated ischemic muscles, these effects were independent of the recruitment of monocytes and macrophages. Importantly, LDIR induced a durable and simultaneous up-regulation of a repertoire of pro-angiogenic factors and their receptors in endothelial cells (ECs), as evident in ECs isolated from the irradiated gastrocnemius muscles by laser capture microdissection. This specific mechanism was mediated via vascular endothelial growth factor (VEGF) receptor signaling, since VEGF receptor inhibition abrogated the LDIR-mediated gene up-regulation and impeded the increase in capillary density. Finally, the vasculature in an irradiated non-ischemic bed was not affected and after 52 week of LDIR exposure no differences in the incidence of morbidity and mortality were seen.

Conclusions These findings disclose an innovative, non-invasive strategy to induce therapeutic neovascularization in a mouse model of HLI, emerging as a novel approach in the treatment of critical limb ischemia patients.

Keywords Neovascularization • Critical limb ischemia • Ionizing radiation • Endothelial progenitor cells • Hindlimb ischemia

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1. Introduction

Critical limb ischemia (CLI) is the end stage of peripheral arterial disease, and severe obstruction of blood flow to the affected extremity results in ischemic rest pain, ulcers, or gangrene. Surgical revascularization remains the cornerstone of therapy for limb salvage but ~30% of CLI patients require amputation in the first year, procedure associated with high morbidity and mortality. Therapeutic angiogenesis became a promising treatment for limb preservation through the revascularization of ischemic tissues by local administration of pro-angiogenic growth factors.[1–3] Several clinical trials showed that therapeutic angiogenesis could be extended to CLI patients.[4] However, the initial enthusiasm was tempered by the less successful more recent, randomized, and placebo-controlled studies with larger numbers of patients.[4] Several factors could contribute to this: (i) formation of a functional vascular network requires the concurrent use of multiple angiogenic factors, and not a monotherapy-based approach; (ii) instability of currently used factors to achieve long-term benefits; and (iii) dysfunction of endothelial cells (ECs) that may not respond.[5,6] To solve this, cell-based therapeutic strategies were developed. Although clinical trials showed that autologous bone marrow-derived mononuclear cells, including endothelial progenitor cells (EPCs), increased collateral vessel formation and had clinical benefits,[4] there are still major challenges that include determination of optimal cell phenotype, preparation protocols, dosing, route, and frequency of administration. Moreover, endothelial dysfunction is associated with a scarce viable and functional EPC population.[7]

We previously showed that low-dose ionizing radiation (LDIR) (<0.8 Gy) induces a pro-angiogenic phenotype in ECs *in vitro*, and promote angiogenesis *in vivo* during regeneration.[8] Herein, we aimed at testing an innovative non-invasive strategy, using LDIR to induce therapeutic neovascularization in CLI. Using a model of experimentally induced hindlimb ischemia (HLI), we show that LDIR improves limb reperfusion by enhancing collateral formation through EPC recruitment to sites of arteriogenesis. The effects of LDIR depend on exposure of the ischemic niche, but not on the local recruitment of myeloid cells. Moreover, LDIR induces capillary density in the gastrocnemius muscle by simultaneous activation of a repertoire of pro-angiogenic factors in a mechanism dependent of the vascular endothelial growth factor (VEGF) receptor signaling. No effects on resting vasculature were observed, disclosing the possibility of using LDIR as a non-invasive and effective therapeutic tool in lower limb vascular insufficiency.

2. Methods

Expanded method descriptions are available in Supplementary material online.

3. Study approval

All animal procedures were performed according to Directive 2010/63/EU. The procedures were approved by the institutional Animal Welfare Body, licensed by DGAV, the Portuguese competent authority for animal protection (license number 023861/2013).

3.1 *In vitro* experiments

Lung human microvascular endothelial cells (HMEC-L) were purchased from Lonza and cultured according to manufacturer's instructions. Cells were used at passages 4–6. Affymetrix GeneChip HuGene 1.0 ST Arrays were used.

3.2 *In vivo* experiments

Twenty-two-week-old female C57BL/6 mice, purchased from Charles River Laboratories, Spain, were used in all experiments. Nine-week-old female C57Bl/6-Tg(CAG-EGFP)10sb/J mice were used as a donor in bone marrow transplantation model (Instituto Gulbenkian de Ciência). Unilateral HLI was induced by surgery. Ionizing radiation was delivered using a linear accelerator operating at a dose rate of 500 MU/min. In most experiments the dose of 0.3 Gy was administered for four consecutive days, starting 12 h after ischemia induction. Blood flow was assessed by laser Doppler perfusion imaging. Capillary and collateral densities were assessed after immunohistochemistry and diaphonization, respectively. Capillaries were microdissected using a Zeiss PALM MicroBeam Laser Microdissection System. The immune cell infiltrate and EPCs were assessed by FACS. In plasma, cytokines were assessed by ELISA. RNA extraction, cDNA synthesis and qRT-PCR was performed using the primers described in the Supplementary material online. After 52 weeks post-HLI, body weights were recorded, urine, blood, and different organs collected and analysed.

3.3 Statistics

Experimental results are shown as the mean ± SEM. Data were analyzed with SPSS 20.0 software for windows. Statistical test employed are detailed described in figure legends. For GeneChip data analysis, probe sets showing differential expression were determined using one-way Analysis of Variance (ANOVA).

4. Results

4.1 LDIR increases perfusion recovery and capillary and collateral densities

We used a previously established mouse model of HLI[9] to assess the effect of LDIR in the restoration of blood flow to ischemic muscle. After surgical induction of unilateral HLI, both hindlimbs were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days (as illustrated in *Figure 1A*) and perfusion was measured overtime. As shown in *Figure 1B* and quantified in *Figure 1C*, a dramatic reduction in blood flow was observed in the ischemic limb immediately after surgery, in comparison to the contralateral limb, and as expected, a gradual improvement in perfusion was seen overtime. Strikingly, a significant improvement in blood flow recovery was seen in the LDIR group, at days 15 and 45 post-HLI, comparing with sham-irradiated mice. This demonstrates a benefit of LDIR in the setting of HLI.

Lower numbers of fractions (1 × 0.3 Gy; 2 × 0.3 Gy, or 3 × 0.3 Gy) or lower dose per fraction (4 × 0.1 Gy) were also evaluated but failed to show an effect (Supplementary material online, *Figure S1A and B*). We also evaluated the effect of the same fraction over 7 days (7 × 0.3 Gy), but no benefit was seen comparing within the 4 day (Supplementary material online, *Figure S1C*).

Subsequently, we quantified capillary density and collateral vessel development in hindlimb muscles, since blood flow recovery depends on both angiogenesis and arteriogenesis. Consistently, HLI increases capillary density *per se*, assessed through quantification of CD31-positive capillaries on histological sections of gastrocnemius muscle. Importantly, this effect is further amplified after LDIR exposure and a significant

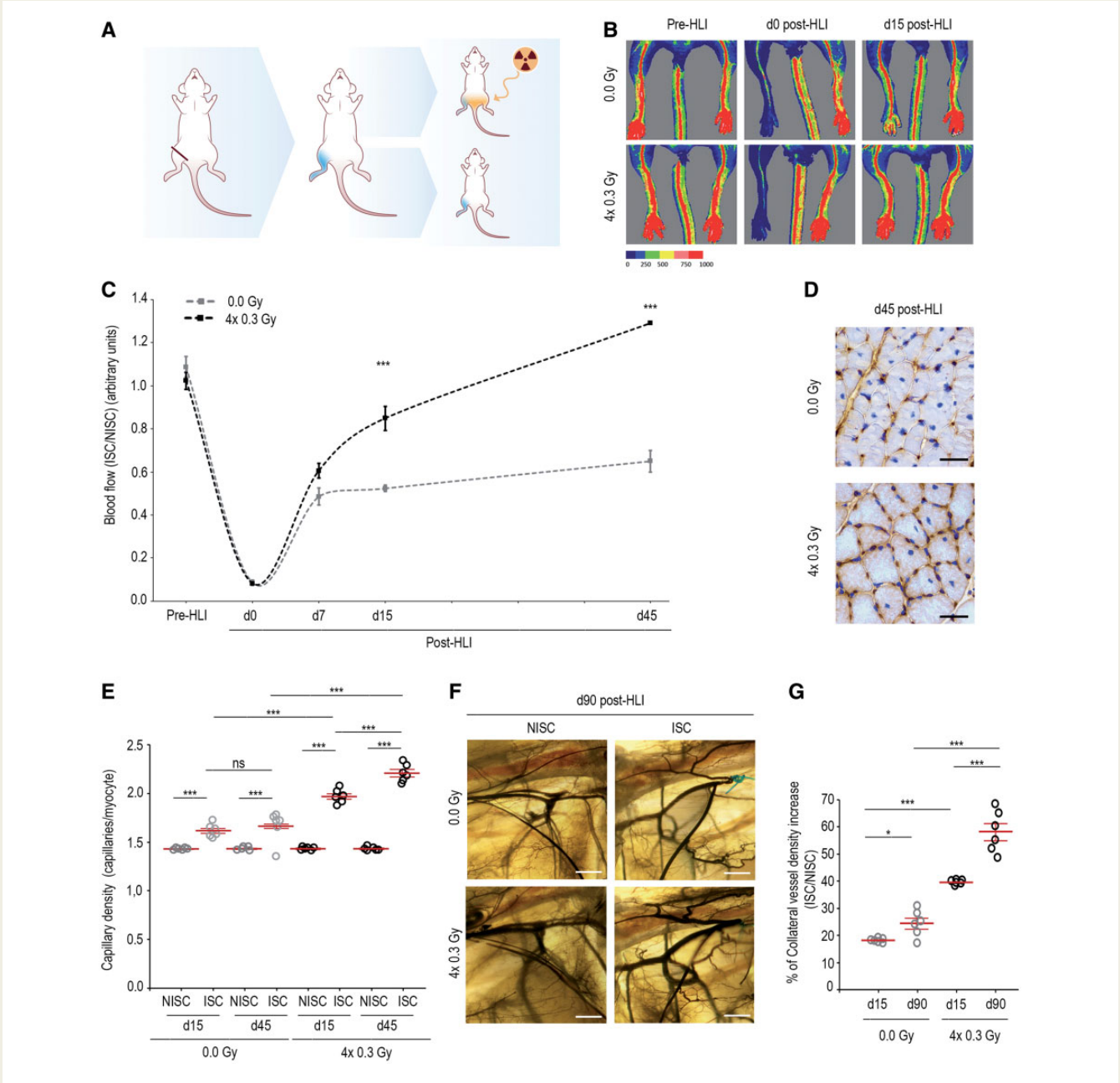

Figure 1 LDIR increases perfusion recovery, capillary and collateral densities. After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. (*A*) A schematic illustration of our experimental design. After unilateral HLI (represented by a thick brown line), the flow to the ischemic limb is dramatically decreased (in blue). Both hindlimbs are irradiated (in orange) or sham-irradiated. (*B*) Representative laser Doppler flow images pre-HLI, and at days 0 (d0) and 15 (d15) post-HLI induction. (*C*) Quantitative evaluation of blood flow expressed as a ratio of ISC to NISC limb demonstrated significantly enhanced limb blood perfusion in irradiated mice vs. sham-irradiated ones both at days 15 (d15) and 45 (d45) post-HLI. Between-group changes were assessed by two-way repeated measurements ANOVA followed by Bonferroni post-hoc test (n = 12 mice per group). Means ± SEM are shown. (*D*) Representative sections from sham-irradiated and irradiated ischemic gastrocnemius muscles at day 45 post-HLI. Capillaries and myocytes were identified by CD31 immunohistochemistry and haematoxylin, respectively. Scale bar, 150 μm. (*E*) Quantitative analysis revealed increased capillary density (capillaries/myocyte) in irradiated ischemic gastrocnemius muscles compared to sham-irradiated ischemic ones at days 15 and 45 post-HLI. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factors of day and irradiation (n = 6 mice per group). (*F*) Illustrative images of selected regions of interest (ROI) for sham-irradiated and irradiated mice. ISC and NISC limbs at day 90 post-HLI are shown. Scale bar, 300 μm. (*G*) Data are represented as the percentage of CVD increase of the ISC limb relatively to the NISC one. At days 15 and 90 post-HLI, irradiated mice presented significantly higher CVD increase (%) vs. sham-irradiated mice. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation (n = 6 mice per group). (E, G) Individual data and means ± SEM (in red) are shown. *P < 0.05; *** P < 0.001; ns, non-significant. HLI, hindlimb ischemia; ISC, ischemic; NISC, non-ischemic; Pre-HLI, before hindlimb ischemia.

increase in capillary density is observed in irradiated ischemic muscle vs. the sham-irradiated ischemic ones at days 15 and 45 post-HLI (*Figure 1D and 1E*). Of note, while the capillary density did not significantly increase between days 15 and 45 in the sham-irradiated ischemic muscles, a significant increase is observed for the irradiated ones.

The collateral vessel density (CVD) was evaluated, at days 15 and 90 post-HLI. Mice were diaphonized and an equivalent ROI, corresponding to 20% of the limb area, was selected for CVD quantification (*Figure 1F*). A greater increase in CVD was observed for the ischemic limbs of LDIR mice, vs. the sham-irradiated ones (*Figure 1G*).

Noteworthy, no difference in these parameters was seen in non-ischemic muscle, LDIR and sham-irradiated, showing that irradiation *per se* does not have an effect on resting vasculature.

4.2 LDIR modulates the expression of endothelial genes involved in an angiogenic response *in vitro*

Previously, using human lung microvascular ECs (HMVEC-L) we showed that 0.3 Gy leads to rapid phosphorylation of the VEGF receptor 2 (VEGFR2),[8] a key signal transduction mediator in the angiogenic process. Consequently, signaling pathways such as PI3K/AKT and ERK/MAPK are activated and gene expression modulated. To assess the effect of LDIR on the EC gene expression profile, HMVEC-L were exposed to a single dose of 0.3 Gy, RNA was extracted at 4 h post-LDIR and a global gene expression analysis was performed; sham-irradiated HMVEC-L were used as control. Principal component analysis (PCA) revealed a separation of all HMVEC-L based on the irradiation status (*Figure 2A*). Two thousand three hundred and seventy-four genes were differentially expressed in LDIR vs. control HMVEC-L, at a cutoff corresponding to a *P* value <0.03 (*Figure 2B*).

Particular attention was paid to growth factors and receptors associated with angiogenesis, VEGF receptor 1 (VEGFR1) and VEGFR2, angiopoietin-2 (ANGPT2), transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2), and their expression was validated by quantitative RT-PCR. Hepatocyte growth factor (HGF) and its receptor, MET, were also validated, as the use of HGF has been proposed in the setting of therapeutic angiogenesis, and clinical trials with HGF gene therapy are ongoing.[4] HMVEC-L were irradiated with a single dose of 0.3 Gy and screened at 4, 8, and 12 h post-LDIR. Gene expression increased at 4 h post-LDIR, compared with sham-irradiated HMVEC-L, with exception of *Tgfb2* whose increase was significant at 8 h post-LDIR. Expression levels of all genes returned to baseline at 12 h post-LDIR (*Figure 2C*). We tried to modulate gene expression by increasing total dose (or number of fractions), using daily 0.3 Gy fractions during 2, 3, and 4 consecutive days; and cells were screened at 4, 8, and 12 h after the last irradiation. Regardless of total dose, gene expression pattern, and magnitude were similar to that observed after the single irradiation dose experiment (*Figure 2D–F*).

4.3 LDIR induces the expression of pro-angiogenic genes in ECs isolated from irradiated ischemic gastrocnemius muscles

LDIR modulates the expression of angiogenic genes in a resting endothelial *in vitro* monoculture, not exposed to injury. Next, we evaluated the gene expression levels in ECs isolated from gastrocnemius muscle of mice subjected to HLI and exposed to LDIR in daily fractions of 0.3 Gy for 4 days or sham-irradiated. Forty-five days post-LDIR mice were killed and gastrocnemius muscle sections stained for CD31 and visualized using a laser capture microdissection microscope (LCM). CD31-positive cells from ischemic and non-ischemic gastrocnemius muscles were dissected and isolated. First, we validated that these CD31-positive cells consisted primarily of ECs and not myeloid cells nor perivascular cells. We assessed the gene expression of surface and transcription markers *Pecam1* encoding CD31, *Erg* and *Etv2* that are specific for ECs; *Itgam* encoding CD11b and *Spi1* encoding PU-1 for myeloid cells and *Des* encoding Desmin, *Pdgfrb* and *Acta2* encoding smooth muscle alpha-actin for perivascular cells. The CD31+ cells isolated by LCM expressed high levels of endothelial-specific transcripts but negligible amounts (more than 10 000 times less) of myeloid or perivascular-specific transcripts (Supplementary material online, *Figure S2*). Next, the same ECs were assessed by quantitative RT-PCR for the expression of *Vegfr2, Vegfr1, Fgf2, Angpt2, Pdgfc, Tgfb2, Hgf*, and *Met*. Transcripts for all these genes were clearly up-regulated in ECs isolated from muscle of the ischemic limb, comparing with the contralateral limb, exclusively in mice exposed to LDIR (*Figure 3A*). Sham-irradiated mice show the opposite, down-regulating the expression of the angiogenic genes repertoire in endothelium from the ischemic limb, comparing with the contralateral limb. Then, we questioned whether the increase of the capillary density conferred by LDIR and observed in the ischemic gastrocnemius muscle could be correlated with the increase of the expression levels of these pro-angiogenic genes. To address this, we used the adductor muscle that does not present an increase of the capillary density at day 45 post-HLI neither in response to ischemia *per se*, nor after LDIR exposure (Supplementary material online, *Figure S3A*). Thus, the adductor muscle provides a control subjected to HLI and irradiation without increase in the capillary density. We isolated the ECs from the adductor muscles and the levels of *Vegfr2, Vegfr1, Fgf2, Angpt2, Pdgfc, Tgfb2, Hgf*, and *Met* mRNA were measured. We confirmed that the transcripts are down-regulated in the irradiated ischemic limb when compared with the contralateral one (Supplementary material online, *Figure S3B*). We have previously demonstrated that VEGFR tyrosine kinase inhibition impairs the LDIR-induced pro-angiogenic response.[8] To assess the functional and clinical relevance of VEGF signaling induced by LDIR in the setting of HLI, VEGFR tyrosine kinase inhibition was achieved through oral gavage of PTK/ZK (100 mg/kg), after HLI and 2 h before each LDIR exposure. VEGFR inhibition abrogated the LDIR-mediated gene up-regulation of pro-angiogenic factors and receptors (*Figure 3B*). Moreover, the capillary density induced by LDIR, but not by HLI, was denied by treatment with PTK/ZK (*Figure 3C*). Conversely, the collateral density induced after LDIR exposure was not affected PTK/ZK (*Figure 3D*).

Thus, 0.3 Gy administered during four consecutive days might act through VEGFR-signaling for angiogenesis, but not arteriogenesis in the ischemic gastrocnemius limb.

4.4 LDIR does not mobilize myeloid cells to the ischemic tissue

Given that, in response to ischemia, the myeloid cells play an important role in the collateral formation[10] we questioned whether LDIR controlled the migration of myeloid cells in the ischemic tissue. Note that this process was already found for higher doses of ionizing radiation[11] (daily therapeutic doses ex: 2.0 Gy). Thus, after unilateral HLI, both hindlimbs were sham-irradiated or irradiated with four daily fractions of 0.3 or 2.0 Gy as additional control. The myeloid infiltration was assessed day 4 post-HLI in ischemic and non-ischemic adductor muscles by flow cytometry (*Figure 4A*). As expected, the number of CD45+ cells increased significantly in

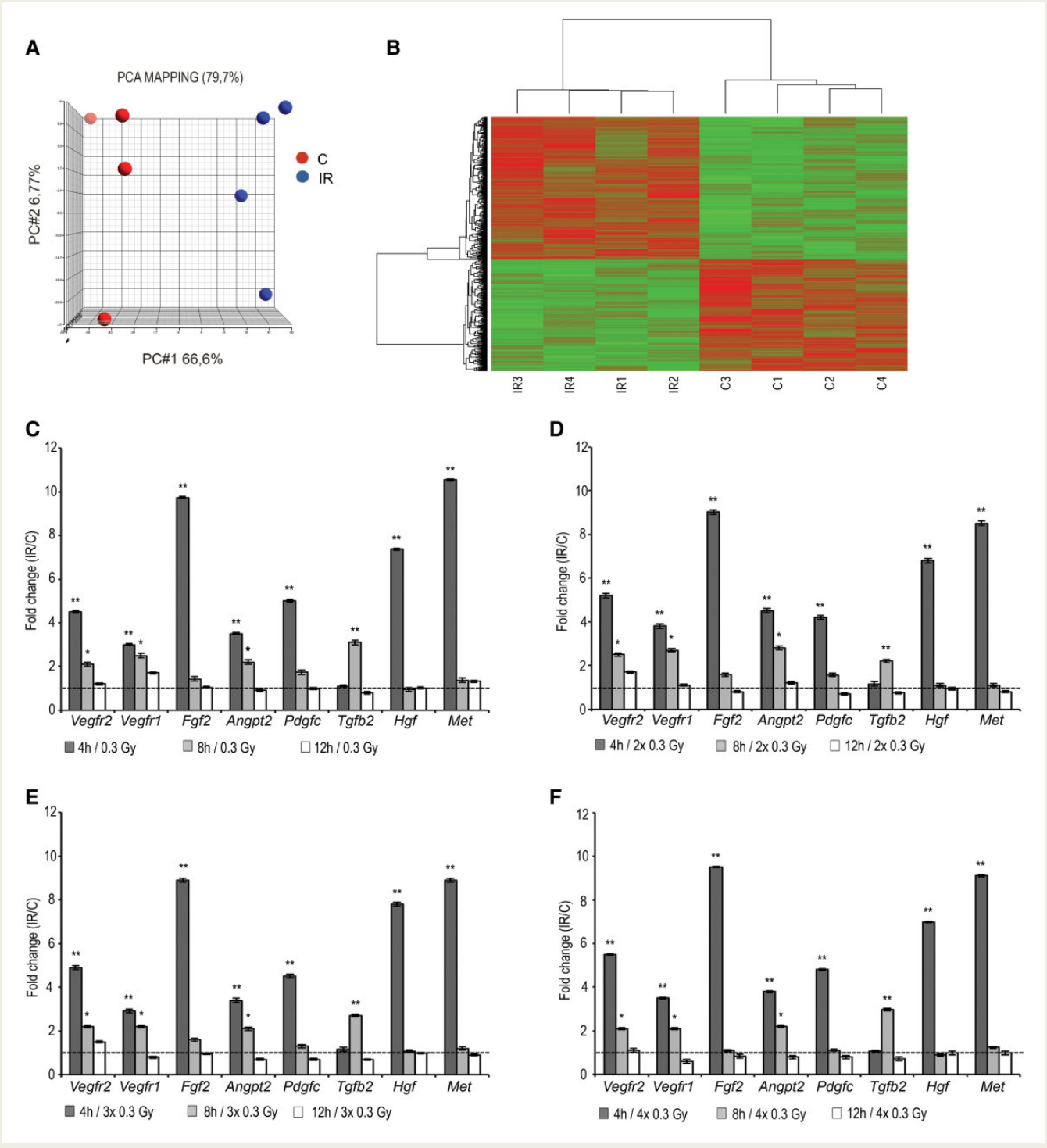

Figure 2 LDIR modulates the expression of endothelial genes involved in an angiogenic response. Four RNA samples of irradiated (0.3 Gy) or sham-irradiated HMVEC-L were processed for hybridization to Affymetrix Human Gene 1.0 ST arrays. (*A*) Three-dimensional PCA plot. The red and blue points represent control (sham-irradiated) and irradiated samples, respectively, indicating a separation of samples based on the ionizing radiation stimulus. (*B*) Heatmap for the 2374 genes differentially expressed in LDIR vs. control [one-way analysis of variance (ANOVA test) $P < 0.03$]. Columns and rows and represent biological replicates and individual genes, respectively. Red and green indicate genes up- or down-regulated compared with control cells (sham-irradiated), respectively. (C–F) HMVEC-L sham-irradiated or irradiated with 0.3 Gy (*C*) once; (*D*) twice, (*E*) three, or (*F*) four consecutive days. (C–F) Data (means ± SEM) represent the fold change in gene expression relative to the internal calibrator (sham-irradiated) in triplicate measurements and are representative of four independent experiments. Data demonstrated a significant increase in the relative expression of *Vegfr2, Vegfr1*, *Fgf2*, *Angpt2*, *Pdgfc*, *Hgf* and *Met*, at 4 h, and of *Tgfb2* at 8 hours post-irradiation, when compared to sham-irradiated cells (dashed line). Values assumed normal distribution, equal variance and independent two-tailed *t* test was used; $*P < 0.05$; $**P < 0.002$. C, sham-irradiated; IR, irradiated.

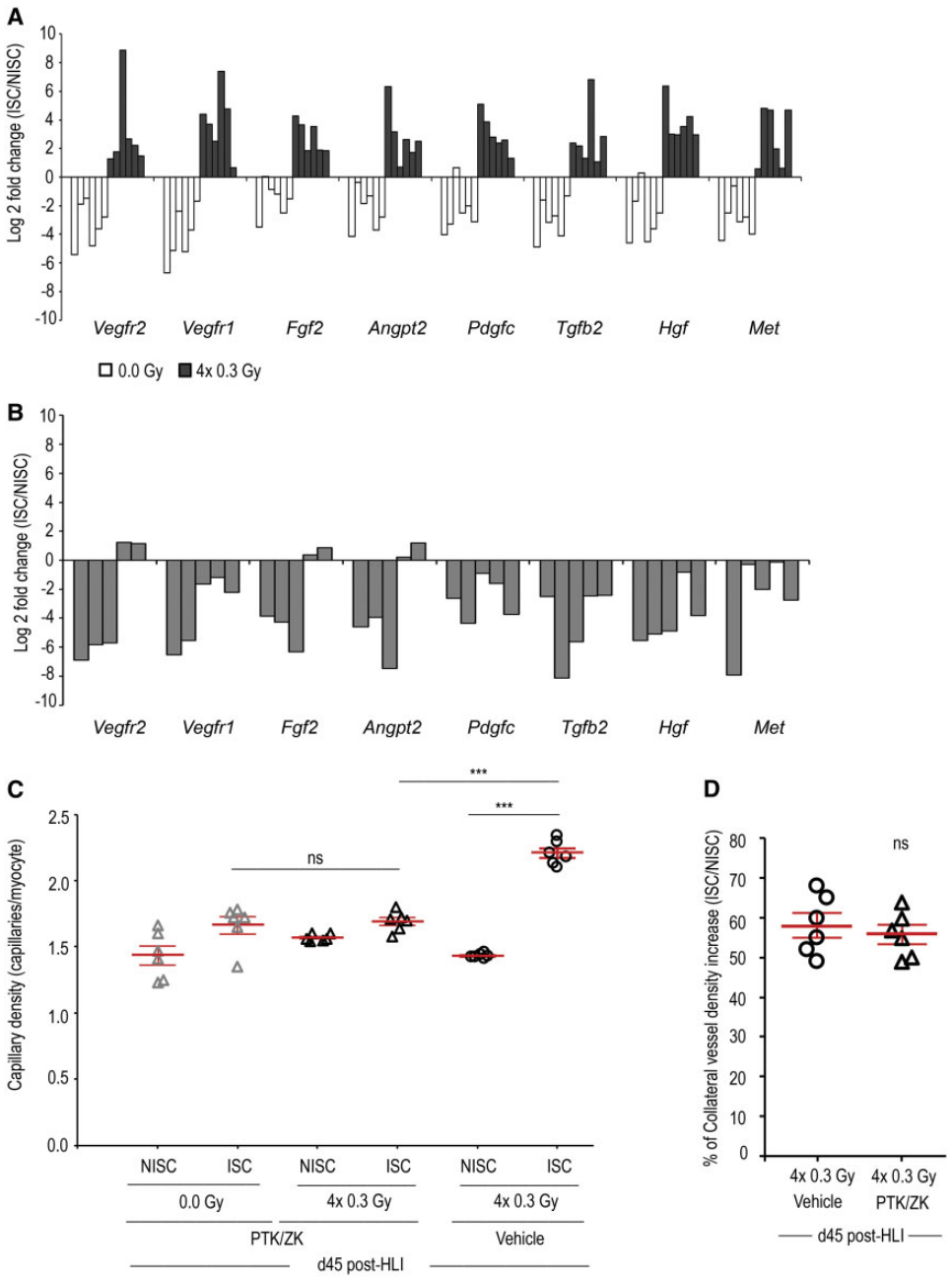

Figure 3 LDIR upregulates the expression of angiogenic genes in ECs isolated from irradiated ischemic gastrocnemius muscles. After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. (*A*, *B*) At day 45 post-HLI, the expression of pro-angiogenic factors and their receptors was evaluated by qRT-PCR exclusively on ECs. Gastrocnemius muscle sections were stained for CD31. Individual endothelial CD31+ cells were visualized, dissected, and isolated using an LCM. (*A*) Each bar represents the relative gene expression in one animal. White and gray bars represent sham-irradiated and irradiated mice, respectively. Values were normalized to 18S to obtain relative expression levels. Results expressed as log2 fold changes between ISC and NISC samples demonstrated relative abundance of the transcripts in irradiated mice; in contrast, a down-regulation is observed in sham-irradiated mice. (*B–D*) Two hours before each irradiation, ischemic mice were pretreated with PTK/ZK (100 mg/kg). (*B*) Light grey bars represent irradiated mice pretreated with PTK/ZK. A down-regulation in relative gene expression is found in irradiated mice treated with PTK/ZK. (*C*) Quantitative analysis, at day 45 post-HLI, revealed no difference in capillary density (capillaries/myocyte) between ISC irradiated vs. ISC sham-irradiated gastrocnemius muscles, both treated with PTK/ZK. As expected a significant increase is observed between ISC irradiated and PTK/ZK treated vs. ISC irradiated and treated with the control vehicle. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factors of irradiation and PTK/ZK treatment. (*D*) Data are represented as the percentage of CVD increase of the ISC limb relatively to the NISC one. At day 45 post-HLI, no difference was observed in CVD between irradiated mice treated with PTK/ZK vs. irradiated mice treated with the control vehicle. Independent two-tailed *t* test was used. (*C*, *D*) Individual data and means ± SEM (in red) are shown from $n = 6$ mice per group; ***$P < 0.001$; ns, non-significant. ISC, ischemic; NISC, non-ischemic.

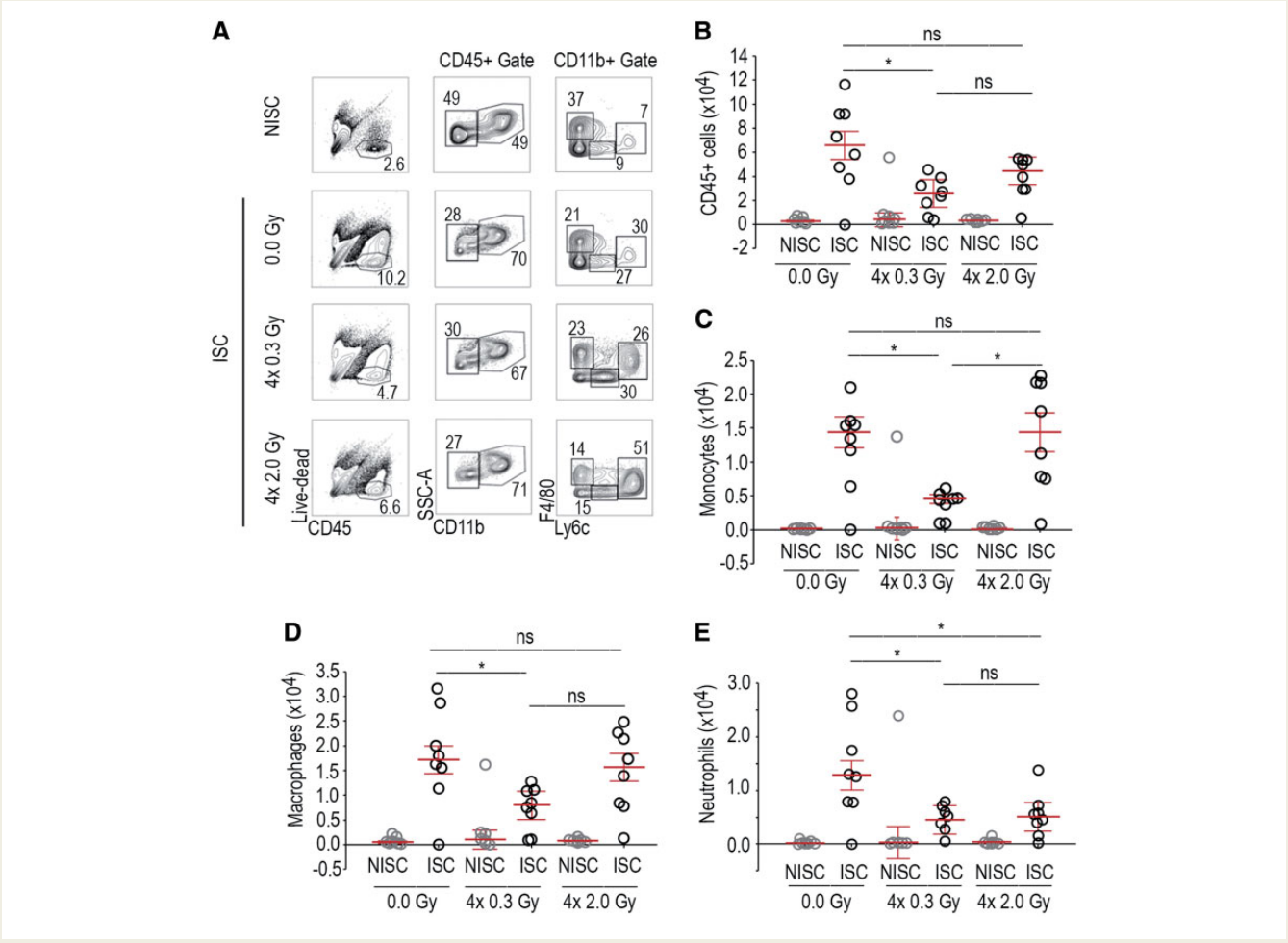

Figure 4 Profiles of leukocytes mobilized to ischemic muscles upon LDIR. After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 or 2.0 Gy in consecutive days. At day 4 post-HLI, analysis of CD45+ immune cells that infiltrate ischemic adductor muscles was assessed. (*A*) Representative analysis of hematopoietic CD45+ cells present in ischemic muscle as assessed by flow cytometry. Analysis of the accumulation of myeloid CD11b+ cells, and in particular macrophages (CD45+CD11b+F4/80+ cells), monocytes (CD45+CD11b+LY6C+F4/80int cells), and neutrophils (CD45+CD11b+Ly6Cint). The graphs show numbers of (*B*) total CD45+ cells; (*C*) monocytes; (*D*) macrophages, and (*E*) neutrophils, isolated from ISC and NISC adductor muscles and represent the data derived from two independent experiments. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factor of irradiation. Individual data and means ± SEM are shown (in red) from $n = 8$ mice per group; $^*P < 0.05$; ns, non-significant. ISC, ischemic; NISC, non-ischemic.

response to HLI. Strikingly, exposure with 0.3 or 2.0 Gy inhibited the CD45+ cell accumulation (*Figure 4B*). Then we went on to assess which myeloid cells were modulated in the ischemic muscles. Monocyte and macrophage numbers increased significantly after HLI in sham-irradiated ischemic muscles. A similar response was observed in ischemic muscles upon 2.0 Gy exposure. In stark contrast, low 0.3 Gy irradiation dose inhibited this monocyte and macrophage accumulation. This shows that different doses of ionizing radiation differently modulate myeloid cell infiltration in response to ischemia (*Figure 4C and D*). Moreover, while neutrophil numbers increased substantially in response to HLI, both 0.3 and 2 Gy irradiation dosages impaired this accumulation (*Figure 4E*). We also assessed whether at later time point myeloid cells could account for the increase in collateral density in response to LDIR. Fifteen days post-HLI the numbers of CD45+ cells, monocytes, macrophages, and neutrophils were similar in the ischemic muscles in 0.3 Gy; 2 Gy or sham-irradiated groups (Supplementary material online, *Figure S4*).

4.5 LDIR enhances collateral formation through EPC recruitment in a process that is dependent of the ischemic niche irradiation

We next assessed the effect of LDIR on EPC mobilization from the bone marrow into the circulation. Total blood was collected at days 4, 5, 6, and 7 post-HLI and EPCs were identified by flow cytometry as mononuclear cells being VEGFR2+/Sca-1+/CD117+ cells. Consistent with the literature,[12] significant increase of the percentage of EPCs occurred at day 6 in sham-irradiated mice in response to HLI (*Figure 5A*). Surprisingly,

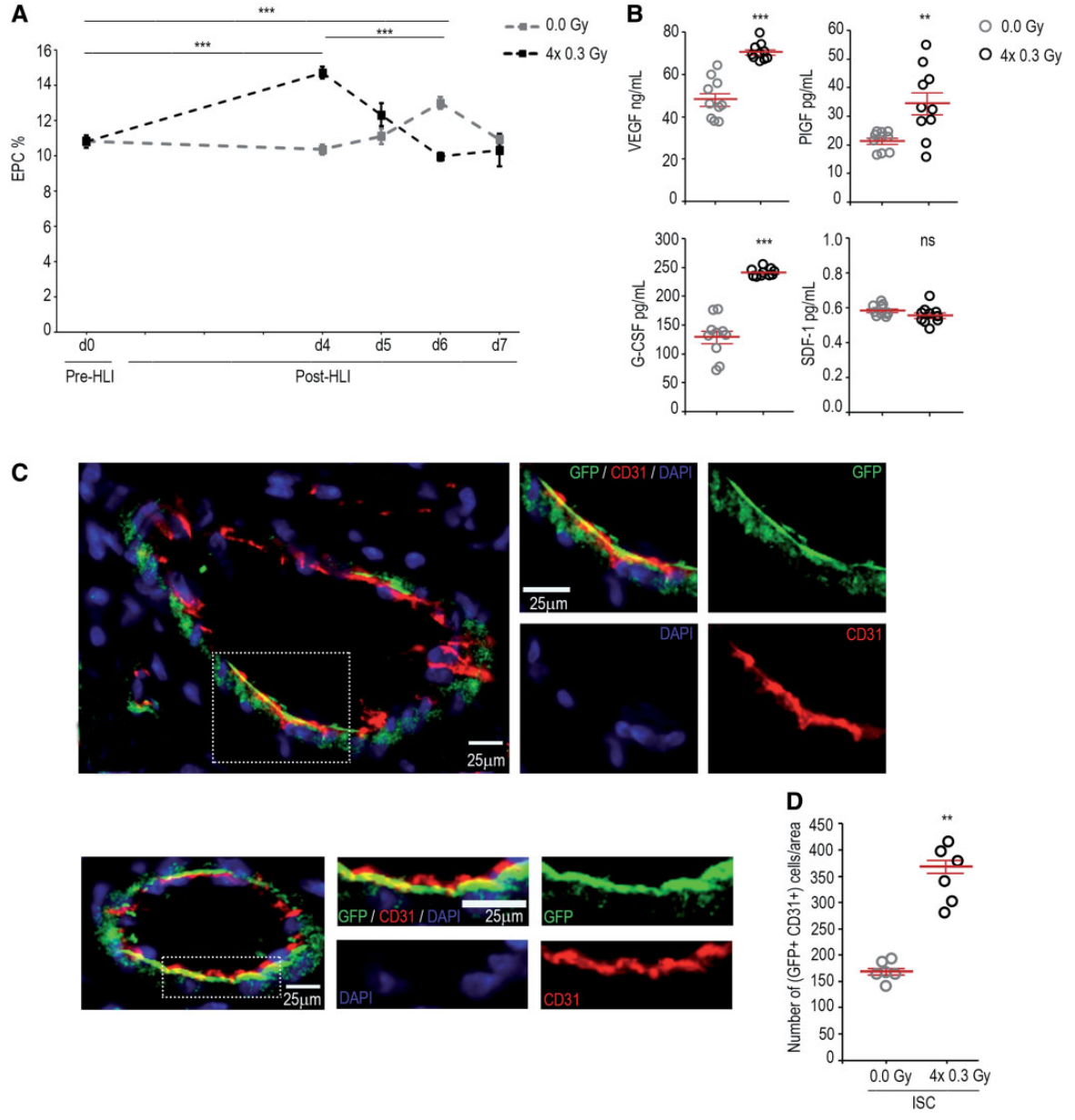

Figure 5 Post-HLI induction, LDIR increases the number of circulating EPCs, the levels of VEGF, PlFG and G-CSF and mediate EPC recruitment in ischemia. After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days. (*A*) Quantitative analysis of EPCs in peripheral blood demonstrated a significant increase of the percentage of EPCs in irradiated mice, at day 4 post-HLI and in sham-irradiated mice, at day 6 post-HLI when compared with the percentage before HLI induction (d0 pre-HLI). Interestingly, the increase conferred by LDIR at day 4 in response to HLI is significantly higher to the one found at day 6 post-HLI in sham-irradiated mice. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation; $n = 6$ mice per group. Means ± SEM are shown. (*B*) The concentrations of VEGF, PlGF, G-CSF, and SDF-1α were measured in the plasma, at day 4 post-HLI. LDIR significantly increases the VEGF, PlGF, and G-CSF concentrations after HLI induction vs. sham-irradiation [$n = 10$ mice per group; values assumed normal distribution and equal or unequal (G-CSF) variance and independent two-tailed *t* test was used]. (*C, D*) Eight weeks after bone marrow transplantation, HLI was performed in C57BL/6 mice. Mice were sham-irradiated or irradiated with 0.3 Gy during four consecutive days and at day 15 post-HLI the adductor muscles were collected. (*C*) At left, two representative images of incorporated EPCs identified by double-fluorescent labeling (green/red) in collateral vessels. Transplanted GFP:β-actin cells were identified by green fluorescence; vasculature by red fluorescence (CD31 staining) and nucleus by blue fluorescence (DAPI). Scale bar, 25 µm. At right, single and merged channels after digital zoomed of the boxed areas are shown. (*D*) Quantitative analysis revealed a significant increase of EPCs incorporation (identified as GFP+/CD31+ cells) into irradiated ischemic muscles compared with sham-irradiated ischemic ones ($n = 6$ mice per group; values assumed normal distribution, unequal variance and independent two-tailed *t* test was used). (*B, D*) Individual data and means ± SEM (in red) are shown; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significant. ISC, ischemic; Pre-HLI, before HLI.

Table 1 Clinical, hematological, biochemical, and histopathological data from sham-irradiated and LDIR mice

End point	0.0 Gy	4 × 0.3 Gy
BW gain, % (n = 140; mean ± SD)	14.2 ± 3.5	21.4 ± 5.3
Mortality (n = 140)	2*/140	1†/140
Blood cell counts (n = 24)	WNR[A]	WNR
Clinical chemistry (n = 24)	WNR	WNR
Coagulation tests (n = 24)	WNR	WNR
Urinalysis (n = 24)	WNR	WNR
Bone Marrow cytology: abnormalities (n = 6)	0/6	0/6
Histopathological analysis (liver; kidney; lung; bone marrow; spleen; thymus) (n = 6)	0/6	0/6

*Found dead, from unknown cause.
†Killed due to abscess formation after surgical procedure (HLI).
AWNR, within normal range.

the percentage of circulating EPCs significantly increased already at day 4 post-HLI in irradiated mice, and to a level noticeably higher than in sham-irradiated mice at day 6 (*Figure 5A*). This suggests LDIR synergized with ischemia to increase EPCs in peripheral blood, a process that is not inhibited after PTK/ZK treatment (Supplementary material online, *Figure S5A*).

In addition, we confirmed that LDIR increases the circulating EPCs percentage in a process dependent of ischemia induction (Supplementary material online, *Figure S6*).

As EPC mobilization involves a complex network of migratory factors,[2,13,14] we assessed whether LDIR modulates cytokine or chemokine concentrations synergistically with ischemia that could generate gradients that guided EPCs to areas of ischemia or/and locally induce arteriogenesis. We previously showed that *in vitro* and under hypoxia-mimicking conditions LDIR enhances VEGF expression in ECs.[8]

Using a similar approach, we found that in the presence of cobalt chloride (CoCl2), which mimics hypoxic conditions, ECs significantly increased *Pgf* mRNA [encoding placental growth factor (PlGF)] expression, in a way that synergized with exposure to 0.3 Gy (Supplementary material online, *Figure S7*). However, hypoxia and irradiation do not always synergize to regulate expression of migration factors. While *Cxcl12* mRNA [encoding stroma-derived factor-1α (SDF-1α)] expression was increased by hypoxia but not by LDIR, conversely, *Csf3* mRNA [encoding granulocyte-colony stimulating factor (G-CSF)] expression was induced by LDIR but not by hypoxia (Supplementary material online, *Figure S7*). These findings were confirmed *in vivo* by ELISA. VEGF, PlGF, and G-CSF concentrations in the plasma were significantly increased in the plasma of irradiated mice at day 4 post-HLI when compared with sham-irradiated ones (*Figure 5B*). In contrast and consistent with our *in vitro* data, the levels of SDF-1α are not modulated by LDIR at least at day 4 post-HLI. Moreover, the levels of VEGF, PlGF and G-CSF were not changed in irradiated mice treated with PTK/ZK after HLI induction (Supplementary material online, *Figure S5B*).

To confirm that this process is dependent of the effect of LDIR on the ischemic/hypoxic niche, we irradiated mice outside the ischemic niche. Since technically, it is not possible to irradiate only the non-ischemic hindlimb assuring that the contralateral ischemic one was not exposed to LDIR, we irradiated the upper (above hip) part of the mouse body (Supplementary material online, *Figure S8A*). Upper body LDIR exposition was not sufficient to increase the proportion of circulating EPC (Supplementary material online, *Figure S8B*). Consistently, the VEGF, PlGF, G-CSF, and SDF-1α levels did not increase upon LDIR exposure (Supplementary material online, *Figure S8C*). Importantly, the increase by LDIR of collateral density is not achieved (Supplementary material online, *Figure S8D*). This strongly suggests that the exposure of the ischemic niche to LDIR is critical for the increase of cytokines, mobilization of EPCs and collateral formation.

Next, we aimed to show that LDIR-induced circulating EPCs are functionally relevant for enhancement of their recruitment and incorporation into ischemic tissues. A bone marrow transplantation using C57Bl/6-Tg(CAG-EGFP)10sb/J donor was performed in C57Bl/6 mice and 8 weeks after, HLI was induced. Mice were sham-irradiated or irradiated with 0.3 Gy during four consecutive days and at day 15 post-HLI the adductor muscles were collected. Our results show that at day 15 post-HLI EPCs are recruited in the large vessels as identified by double-fluorescent labeling (green and red), in response to HLI (*Figure 5C*). To confirm the wide-field data, confocal images of the same 10 µm thick section were acquired to show that green and red fluorescent signals belong to the same cells (Supplementary material online, *Figure S9*). Importantly, a quantitative evaluation of the histological sections revealed a significantly increased number of GFP+/CD31+ cells per area into irradiated ischemic thigh muscles when compared with the sham-irradiated ones (*Figure 5D*).

4.6 LDIR exposure is not associated with increased morbidity or mortality

To check for a possible effect of LDIR in the health status of the animals, a 52 week study was performed in a group of sham-irradiated and LDIR mice (n = 140), throughout which mice were assessed for clinical signs of disease. There was no increased incidence of morbidity or mortality in the LDIR mice, compared with sham-irradiated, and there was no difference in body weight gain (at weeks 24, 36, 48, and 52 post-HLI). Fifty-two weeks post-HLI mice were killed and no significant difference was observed in organ weight, serum biochemistry (n = 24), urinalysis or hematological parameters (*Table 1*); and the histological analysis revealed no neoplastic lesions or major changes in the liver, lung, spleen, thymus, or bone marrow of these mice (n = 6) (Supplementary material online, *Figure S10*).

5. Discussion

Therapeutic neovascularization aims to stimulate new blood vessel growth. Current strategies using proteins, genes or stem cells have demonstrated efficacy in animal models however, clinical translation remains challenging. We show that LDIR synergized with HLI and significantly enhance blood perfusion, capillary density in gastrocnemius muscle and collateral vessel development, tilting the angiogenic balance toward an even more pro-angiogenic phenotype, and suggesting that LDIR may favor the functional recovery of ischemic tissues. In contrast, resting vasculature, not subjected to ischemia, are unaffected by LDIR since capillary density and CVD are similar in non-ischemic muscles exposed or not to LDIR. This is in agreement with our previous work, where interray capillary density remained unchanged after LDIR of non-amputated zebrafish caudal fin.[8] Our data also show that the maximal efficacy in perfusion recovery, capillary, and collateral densities involves the administration of 1.2 Gy in four daily fractions of 0.3 Gy per fraction. A global gene expression analysis revealed that 2374 genes were modulated by LDIR and from those, 1344, many of which with a role in angiogenesis, were upregulated in LDIR vs. control HMVEC-L. As soon as 4 h after exposure to 0.3 Gy the expression of the majority of the pro-angiogenic molecules were increased, and returned to baseline12 h post-LDIR. This acute short-term effect of LDIR on ECs is independent from dose fractionation since cells exposed to 0.3 Gy administered 2, 3, or 4 consecutive days presented similar gene expression pattern and magnitude. The evaluation of expression of angiogenic genes in ECs isolated from gastrocnemius muscle of mice subjected to HLI revealed that LDIR modulates the expression of angiogenic genes in the endothelium and, thus, suggested a link for the long-term advantage in blood perfusion, capillary density, and collaterals in HLI. LDIR induced a sustained and prolonged pro-angiogenic response in ECs, still evident 45 days after irradiation. Because this contrasts with the transient *in vitro* response, one may hypothesize either that endothelium itself could be differently modulated by LDIR in a hypoxic microenvironment created by ischemia; some cells (e.g. adipocytes) could contribute to perpetuate the effect(s) of irradiation in ways that *in vitro* cultures cannot mimic.

There is evidence that ionizing radiation can affect a variety of inflammatory processes and the composition of responding immune cells.[15] However, this highly depends on the dose, for low doses (e.g. ≤1 Gy) promote anti-inflammatory responses,[16] while high doses (e.g. ≥2 Gy) exert pro-inflammatory effects.[17] Therapeutic applicability was further demonstrated on inflammatory disease as symptomatic improvement of rheumatoid arthritis was observed when mice were irradiated with 0.5 Gy in five fractions within 1 week.[18] The hematopoietic infiltrate was monitored from inflamed and ischemic tissues to assess a potential role of immune cells upon LDIR. Ischemia *per se* induced about 20-folds increase in the immune CD45+ cell infiltrate recruited to the injured muscle at day 4 post-HLI. Exposure with 4× 0.3 Gy significantly inhibited the CD45+ cell accumulation with particular effects on monocytes, macrophages, and neutrophils. In contrast, with 4× 2.0 Gy the total CD45+ accumulation in ischemic muscle was still reduced, and although numbers of monocytes and macrophages were restored, neutrophils were not. This is consistent with the fact that high irradiation doses have opposing effects on certain myeloid subsets, for they activate macrophages[19] while they are reported to induce rapid, but transient, neutropenia.[20] Importantly, effect of irradiation was short-lasted. Fifteen days post-HLI the profiles of myeloid cells that infiltrated non-irradiated and irradiated ischemic muscles were similar. Altogether these data pointed for a mechanism of LDIR-induced arteriogenesis independent of local myeloid cell recruitment.

In the setting of HLI we showed that LDIR boosts the induction of a sustained VEGFR-mediated pro-angiogenic program in ECs from ischemic gastrocnemius muscle. These results corroborate our previous findings[8] and suggest a new mechanism: LDIR under HLI induces capillary density. Consistently, the capillary density induced by LDIR, but not by HLI, was abrogated by treatment with PTK/ZK. Conversely, the enhancement promoted in collateral density by LDIR was not affected by PTK/ZK, suggesting that this process is regulated by a mechanism independent of the VEGF receptor signaling.

Consistent with our previous results showing that in hypoxic mimicking conditions, LDIR increases the expression of VEGF in ECs,[8] here we found that the expression of *Pgf* and *Csf3* is also increased in ECs. Importantly, these results were confirmed *in vivo* as VEGF, PlGF and G-CSF concentrations significantly increase in the plasma at day 4, upon LDIR exposure. Of note, beside VEGF, the upregulation of other cytokines (such as PlGF and G-CSF), might explain why circulating EPCs are insensitive to PTK/ZK inhibition. It is plausible that the effects of VEGF, PlGF, and G-CSF are redundant on EPCs, and may explain why PTK/ZK does not affect the enhancement promoted in collateral density by LDIR. These cytokines were reported as being involved in the guidance of EPC to ischemic tissue.[2,13,14] In line with this, we observed EPC mobilization and recruitment to the ischemic tissue upon LDIR. In the absence of ischemia, LDIR *per se* does not induce that effect and notably the irradiation of the ischemic tissue is critical for the mobilization of EPCs and collateral formation. Of note, in our transplantation model, all hematopoietic cells are GFP+ (including circulating and extravasated/tissue leucocytes, erythrocytes, and platelets), but EPC, forming the inner lining of blood vessels, exhibit both green (GFP+) and red (CD31+). Thus, our results suggest that LDIR increases these growth factor concentrations synergistically with HLI and given the fact that this happens only if the ischemic tissue is irradiated, we hypothesize that a hypoxic niche is critical for this process. Although we cannot exclude that other cells could modulate the levels of these cytokines upon LDIR, our results strongly suggest the involvement of ECs.

We propose a model of enhanced and sustained angiogenesis induction by *in situ* LDIR administration as a promising therapeutic approach for ischemic diseases (*Figure 6*). LDIR applied as one daily irradiation of 0.3 Gy, administered for four consecutive days, synergistically act with the ischemic insult, exacerbating the local pro-angiogenic response. Our results suggest that this is achieved through (i) increased capillary density accompanied by an up-regulation of several pro-angiogenic target genes in ECs localized in gastrocnemius muscles, a process that is dependent of VEGF signaling and (ii) the mobilization and recruitment of EPCs by increasing the concentrations of VEGF, PlGF, and G-CSF that may explain the collateral density increase in the ischemic limb leading to blood perfusion improvement.

One important concern when addressing ionizing radiation is its toxic effect. According to the linear no-threshold (LNT) hypothesis, the dose–response is linear and no threshold exists where damage begins to show. Recent advances in radiobiology challenge the validity of the LNT suggesting that it overestimates radiation risks.[21] We performed a 52 week study and LDIR had no significant impact in the morbidity and mortality of the mice, although the possibility of LDIR long-term toxicity cannot be ruled out. Importantly, the LDIR proposed herein is usually absorbed by healthy tissues during radiotherapy, in areas where no adverse effects were found during the follow-up of the patient for several years.

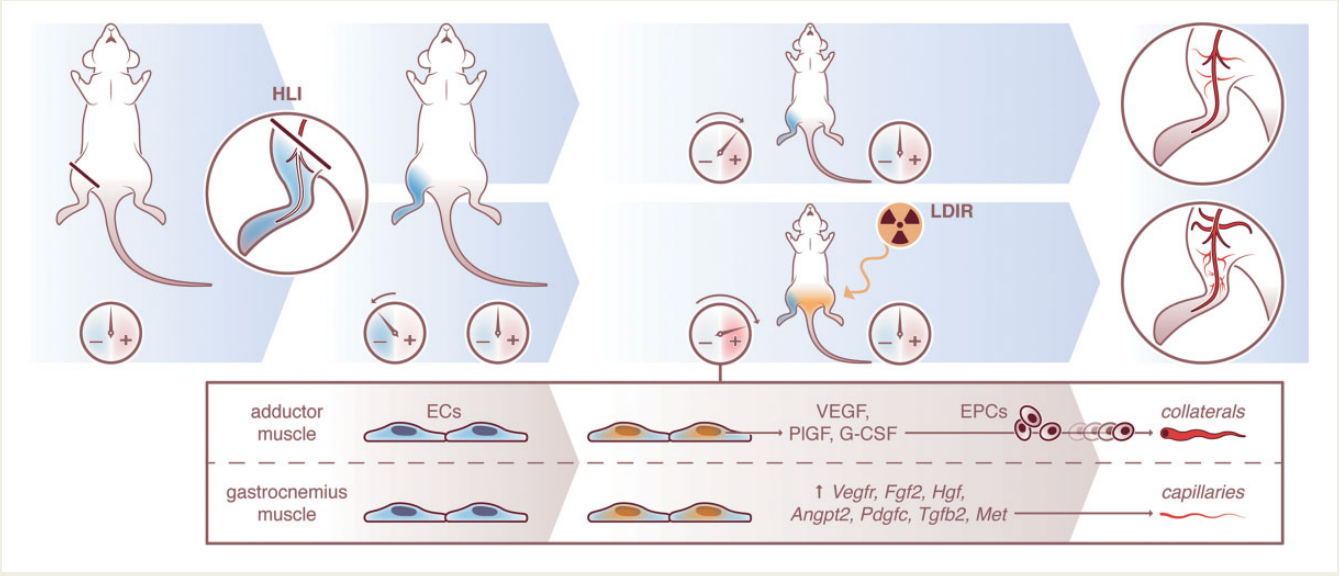

Figure 6 Proposed model of LDIR effect after HLI. After unilateral HLI (represented by a thick brown line), the flow to the ischemic limb is dramatically decreased (in blue) and vascular function is compromised as represented by a reduction in the dial. After LDIR exposure of both hindlimbs (in orange), we propose a synergistic effect occurs with the ischemic insult, tilting the dial towards an improvement of the vascular function by stimulating neovascularization. Then, ECs in a hypoxic microenvironment secrete VEGF, PlGF and G-CSF leading to the recruitment of EPCs and consequently to collateral density increase. Simultaneously, the up-regulation of several pro-angiogenic target genes namely *Vegfr (Vegfr-2 and -1)*, *Fgf2*, *Angpt2*, *Pdgfc, Tgfb2, Hgf* and *Met* in ECs induces capillary density. The vascular function of non-ischemic hindlimbs was not affected by LDIR exposure as represented by the dial in an equilibrium status before, during and after ischemia recovery. (©Diogo Guerra. 2016).

The angiogenic potential of ionizing radiation has already been shown.[22] We and others have shown that LDIR favors angiogenesis by promoting EC proliferation and migration, accelerating wound healing.[8,22] However, there is no consensus about the doses described as pro-angiogenic, as different radiation schemes are used. Herein, ionizing radiation was delivered through a linear accelerator producing photon beams, currently used in the clinical practice. The use of conventional radiotherapy dose (2–10 Gy, administered once, Caesium-137 source) has been shown to induce neovascularization in HLI through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization; however, potential adverse effects were seen[11] and so, to the best of our knowledge, to date the use of those high doses has not been proposed for therapeutic neovascularization.

Our data support the use of LDIR in enhancing ischemia-induced neovascularization *in vivo*, which is achieved through the increase of cytokines, mobilization, and recruitment of EPC to the ischemic tissue and simultaneous activation of a repertoire of pro-angiogenic factors and resulting in enhanced recovery of blood flow. LDIR may therefore have a clinical significant impact in the treatment of peripheral arterial disease that represents a growing health problem worldwide, with high economic burden and limited therapeutic options. We currently have an ongoing exploratory clinical trial to determine the clinical and molecular effects in "non-option" CLI patients. The success of this clinical trial will lead to the development of new trials to propose a novel and effective therapeutic tool with worldwide impact to peripheral arterial disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We thank the Department of Radiotherapy, Centro Hospitalar Lisboa Norte, Lisbon, Portugal, particularly to I. Diegues, C. Raimundo, A. Borges, D. Parreira, M. Pereira, C. Moreira, A. Curto, C. Mestre, A. Momede, H. Silva, I. Lima, T. Mouro, and V. Quintino for help in irradiation delivery. We also thank A. Duarte and R. Malveiro for help in the treatment planning and dosimetry; and I. Monteiro Grillo and M. Jorge in the quality of ex-directors of the department of radiotherapy for opening the doors when this work started, for their unconditional support and for all the discussions we had. We gratefully acknowledge A. Portêlo for statistical advice and D. Guerra for the illustration of the proposed model.

Conflict of interest: none declared.

Funding

PO is supported by a fellowship (SFRH/BD/80483/2011) from Fundação para a Ciência e Tecnologia. KS received a Postdoctoral fellowship (SFRH/BPD/78039/2011) and an Investigator Program (IF/00004/2014) from Fundação para a Ciência e Tecnologia.

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