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1 **PLX-PAD Cell Treatment of Critical Limb Ischemia – Rationale and Design of**
2 **the PACE trial**

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48 ***What this paper adds:***

49 Placebo controlled trials of cell therapy to reduce major amputations in patients with critical
50 limb ischemia and no-option for revascularization have so far not been successful. PLX-PAD
51 cell treatment (placenta derived adherent stromal cells) has in small studies shown
52 promising results, and the phase III PACE trial is designed to evaluate the efficacy and safety
53 of two sessions of intramuscular injections, 8 weeks apart in follow up of 12-36 months.
54 Thus, the study will provide long term outcome and will collect parameters to assess
55 potential economic benefit for this kind of treatment.

56

57 **Abstract**

58 *Background:* Critical limb ischemia (CLI) is a life threatening condition with a considerable
59 risk for death and major amputation. Besides revascularization, no treatment has been
60 proven to reduce the risks. Therapeutic angiogenesis by gene or cell therapy has not
61 demonstrated definitive evidence in randomized controlled trials. PLX-PAD is an ‘off-the-
62 shelf’ allogeneic placental derived, mesenchymal-like cell therapy that in preclinical studies
63 has shown pro-angiogenic, anti-inflammatory and regenerative properties. Favorable 1-year
64 amputation free survival (AFS), and trends in reduction of pain scores and in increase of
65 tissue perfusion have been shown in two small, open-label, phase I trials.

66

67 *Study design:* The PACE study is a phase III randomized, double-blind, multicenter,
68 multinational placebo-controlled, parallel-group study to evaluate the efficacy, tolerability
69 and safety of intramuscular injections of PLX-PAD cells to treat patients with atherosclerotic
70 CLI with minor tissue loss (Rutherford Category 5) up to the ankle level, who are unsuitable
71 for revascularization or carry an unfavorable risk-benefit for that treatment. The study will

72 enroll 246 patients, who after screening are randomized in a ratio of 2:1 to treatment with
73 intramuscular injections of PLX-PAD 300X10⁶ cells or placebo at two occasions, 8 weeks
74 apart. The primary efficacy endpoint is time to major amputation or death (amputation free
75 survival), which will be assessed in follow-up of after at least 12 months and up to 36
76 months.

77

78 *Conclusions:* Based on favorable pre-clinical and initial clinical study results, the PACE phase
79 III randomized controlled trial will evaluate placenta-derived PLX-PAD cell treatment in
80 patients with critical limb ischemia, carrying an unfavorable risk-benefit for
81 revascularization.

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96 **Introduction**

97 Critical limb ischemia (CLI) constitutes the most advanced stage of chronic peripheral arterial
98 disease (PAD) and includes rest pain and ischemic foot lesions. The condition affects 1-5 % of
99 all PAD patients, which corresponds to an incidence of 500-1000 / million population per
100 year (1). Overall, the prevalence of PAD increases worldwide, most remarkably in low- and
101 middle income countries (2). Major amputation and death are the ultimate consequences of
102 CLI, and a 1-year amputation rate of 15-25% is commonly reported, while amputation &
103 mortality rate ranges 30-40%. The single evidence-based recommendation for treatment is
104 revascularization (1,3,4). Due to co-morbidities with greater risk to perform an
105 interventional procedure, or based on anatomical or technical issues, a proportion of CLI
106 patients is not reasonable to revascularize or to re-revascularize after a failed procedure.
107 Few treatments exist for such “poor-option” cases. Prostanoid therapy has been reasonably
108 well studied in randomized controlled trials, but does not carry evident effect, and is not
109 recommended in present guidelines (1,3). Since about 20 years, therapeutic angiogenesis
110 has been studied, based on either gene or cell therapy.

111

112 **Gene therapy**

113 Gene therapy utilizing growth factors, Vascular Endothelial Growth Factor (VEGF), Fibroblast
114 Growth Factor (FGF) and Hepatocyte Growth Factor (HGF), has been investigated in mostly
115 smaller clinical trials, with varying success with regard to the major efficacy endpoint,
116 amputation-free survival (AFS). Only NV1FGF has been investigated in a larger randomized
117 placebo controlled trial, TAMARIS (5) that did not show any better outcome regarding
118 survival or major amputation in the treatment group compared to placebo, despite the fact
119 that a former, smaller trial, TALISMAN (6), showed that major amputation, as a secondary

120 endpoint, was significantly less frequent among NV1FGF treated subjects. Injections of the
121 HGF plasmid have yet to prove efficacy with regard to major events, though smaller
122 randomized placebo controlled trials have shown reduced rest pain (7) and increased toe
123 brachial index (TBI) (8) at follow up. More has to be learnt both from basic and clinical
124 research to possibly adopt effective gene therapy for PAD (9), though Iver and Annex (10)
125 discuss a conceivable end of gene therapy trials, based on the lack of an evident break-
126 through.

127

128 **Cell therapy**

129 The potential benefit of cell therapy is that cell secretion is multifactorial and therefore not
130 based solely on a single growth factor. Initiated by a Japanese study (11) comparing bone-
131 marrow- and peripheral blood mononuclear cells (PBMC) injected into the limb muscles of
132 patients with PAD, several cell-based studies have been performed, specifically in CLI
133 patients with no option for revascularization. The Japanese study (11) showed improved
134 ankle-brachial index (ABI) and transcutaneous tissue oxygen pressure (TcPO₂) and reduced
135 rest pain in the bone-marrow mononuclear cell treated group. Though the majority of
136 studies have utilized intramuscular injections of the growth factor, the largest trial, Juventas,
137 treated 160 patients with intra-arterial infusions of bone-marrow mononuclear cells (BM-
138 MNC) compared to placebo (12). At 6 months there was no difference in the rate of major
139 amputations.

140 In a meta-analysis by Teraa et al (13), including 12 randomized controlled trials (RCT) in
141 autologous cell therapy for CLI, major amputations were significantly reduced. Most
142 importantly, when only placebo controlled RCTs were included, the major amputations were
143 no longer significantly reduced, indicating the importance of placebo controlled trials in cell

144 therapy. In a later meta-analysis by the same author group (14) only including placebo-
145 controlled RCTs, this outcome was verified. Recently this finding was also verified in another
146 meta-analysis on CD34+ mononuclear cell therapy (CD34+MCT), including 10 trials (15). Total
147 amputations and ulcer healing were reduced in comparison with findings in the placebo
148 treated groups. Major amputation and survival were, however, not significantly reduced.
149 This publication also concluded the beneficial value of a high CD34+ cell content.

150

151 **Autologous or allogeneic cell utilization**

152 From an immunological point of view, autologous cell treatment may theoretically provide
153 an immunological advantage. Nevertheless, it has been shown that cells harvested from
154 older individuals, and in particular those with cardiovascular risk factors or critical limb
155 ischemia, are reduced in number and functionality (16, 17). Furthermore, harvesting
156 autologous cells from bone marrow involves an invasive procedure, while peripheral blood
157 utilization requires granulocyte colony stimulation factor (G-CSF) treatment that potentially
158 may cause harm due to the high white blood cell content that is developed (18). Allogeneic
159 MSCs have been shown to exhibit low immunogenicity (19), thus, utilizing allogeneic
160 younger, more potent cells, rather than treatment with cells harvested from the diseased
161 patients themselves, therefore should be of benefit. In this respect PLX-PAD cells from young
162 healthy placental tissue have the potential for higher efficacy than previously seen with
163 autologous cell products.

164

165 **PLX-PAD: Allogeneic Cell Therapy**

166 PLX-PAD is a cell therapy product, composed of placental expanded adherent stromal cells.
167 While PLX-PAD cells exhibit membrane marker expression typical of classical mesenchymal

168 stromal cells (20), they have a minimal ability to differentiate in vitro into cells of
169 mesodermal lineage. Therefore, their proposed mechanism of action is a timely secretion of
170 various proteins which induce angiogenesis, immunomodulatory activities, and promotion
171 of regeneration of muscle tissue.

172 Angiogenesis, the formation of new vessels, is induced by a variety of factors released from
173 ischemic tissues, and is a critical physiological mechanism for alleviation of PAD or for
174 recovery of muscle tissue functionality after injury. The angiogenic process involves
175 migration of endothelial progenitors and pericytes towards the site of interest. *In vitro*
176 studies have shown the capacity of PLX-PAD cells to promote endothelial cell proliferation
177 (20). The cells secrete pro-angiogenic proteins including VEGF, angiopoietin-1, osteopontin,
178 MMP-1, MMP-2, HGF and angiogenin, all of which are up-regulated under hypoxic culture
179 conditions (20, 21 and unpublished data). Angiogenin further interacts with endothelial and
180 smooth muscle cells, resulting in cell migration, invasion, proliferation and formation of
181 tubular structures (22). (Fig 1, Table 1).

182 PAD is associated with an inflammatory process that leads to tissue damage and precludes
183 active repair. Oxidative stress due to endothelial dysfunction is evident in PAD and leads to
184 persistent inflammation. Proinflammatory cytokines, e.g. TNF- α , IL-6, IL-1 β , play a key role
185 in the inflammatory process, and PLX-PAD cells mitigate this process by releasing anti-
186 inflammatory and immunomodulating cytokines (i.e. GDF-15, CXCL12, TGF- β). Following
187 exposure to pro-inflammatory cytokines (such as TNF- α and IFN- γ) PLX-PAD cells further
188 upregulate some of the anti-inflammatory secretions (i.e. IDO, PD-L1, HGF, IL-11, CCL5).
189 Furthermore, when cultured with activated PBMCs, PLX-PAD induce upregulation of PBMC
190 secreted anti-inflammatory cytokines such as IL-10, and IL-1RA), also indirectly affecting
191 endothelial dysfunction and protecting endothelial cell viability (20 and unpublished data).

192 As ischemic conditions lead to muscle degeneration, muscle regeneration is of potential
193 therapeutic benefit in PAD. PLX-PAD cells have been shown to promote migration of skeletal
194 muscle cells *in vitro* and improve muscle function and accelerate muscle regeneration *in vivo*
195 (manuscript in preparation).

196 To summarize, PLX-PAD cells secrete proteins that are known to be involved in promoting
197 angiogenesis, downregulating inflammation and inducing regeneration of muscle tissue.

198

199 *In vivo*, in the mouse hind limb ischemia (HLI) model in which the femoral artery of one
200 hindlimb is cut and ligated thus inducing complete ischemia in the operated limb, (21, 23),
201 PLX-PAD cells have been shown to restore blood flow to the ischemic limb . Furthermore, it
202 was shown that PLX-PAD cells exert a systemic effect, since injection of the cells to the
203 contralateral limb exerted an almost similar restoration of blood flow, but required a larger
204 dose of cells. A second administered dose of PLX-PAD cells 21 days after the first dose
205 afforded additional efficacy in re-establishing blood flow in case the effect was declining (Fig
206 2). This study and others have also shown that PLX-PAD cells injected intramuscularly do
207 not migrate from the injection site to other tissues and do not differentiate in culture,
208 further supporting the suggested mode of action of PLX-PAD cells through secretion of
209 proteins.

210

211 **Clinical studies in PAD**

212 Two phase I open-label, dose escalation studies were conducted to assess the safety of
213 intramuscular injections of PLX-PAD cells in 27 CLI subjects (Rutherford Categories 4 and 5),
214 who were not candidates for revascularization.

215 Study 1202-1 was conducted in Germany and assessed three single doses of 175 million cells
216 (low dose, n=3), 315 million cells (intermediate dose, n=6) and 595 million cells (high dose,
217 n=6). Study 1202-2 was conducted in the United States (US) and assessed a single versus 2
218 doses (2 weeks apart) of 280 million cells, the first group included 7 patients, the latter
219 included 5 patients. PLX-PAD cells were administered intramuscularly into the affected leg
220 via 30 to 50 injections.

221 Overall, the safety of this process in CLI subjects was found to be acceptable, and it was
222 confirmed that HLA-matching is not required. Adverse events included mostly injection-sites
223 reactions such as pain, muscle contractions/fasciculations, pruritus, hematoma, etc. (mostly
224 transient and of mild/moderate intensity), transient allergic reactions, and bad breath due to
225 the DMSO (dimethyl sulfoxide) content.

226 These phase I studies were not powered to demonstrate clinical efficacy, however, some
227 parameters have indicated a positive clinical effect. The pooled amputation free survival rate
228 at 6 months and 1 year across the two studies was 96% and 85% respectively, which is
229 higher than the rates described in similar patient populations (24, 25). Pain scores, as
230 assessed by the Visual Analog Scale (VAS), showed a trend of decrease after treatment with
231 PLX-PAD in all dose groups, up to a decrease of 2.5 units in the patients treated at the dose
232 of 315 million cells. TcPO₂, which is considered an indicator of tissue perfusion,
233 demonstrated a trend of increase over time in all study groups with the greatest increase of
234 up to 15 mmHg in the repeated-dose group (Fig 3). (data on file)

235 In summary, based on the pro-angiogenic, immunomodulatory, and muscle regeneration
236 capacities of PLX-PAD, as well as the results from animal experiments and outcome of the
237 clinical studies in PAD patients, a phase III trial was designed.

238

239 ***PACE trial design***

240 The PACE study (*A randomized, double-blind, multicenter, placebo-controlled, parallel-group*
241 *Phase III study to evaluate the efficacy, tolerability and safety of intramuscular injections of*
242 *PLX-PAD for the treatment of subjects with critical limb ischemia (CLI) with minor tissue loss*
243 *who are unsuitable for revascularization*) was designed to investigate time to major
244 amputation or death (AFS) after up to 36 months. The study is planned to enroll a total of
245 246 patients with minor foot lesions (Rutherford Category 5) up to the ankle level. Patients
246 should be unsuitable for revascularization or carry an unfavorable risk- benefit to
247 revascularization. Ineligibility for revascularization is determined by either severe co-
248 morbidity, anatomical or technical challenges (e.g. lack of vein for a bypass or inadequate
249 target vessels for an endovascular procedure) or failed revascularization procedures with
250 persistence of CLI after the procedure. Only patients with atherosclerotic disease are
251 included, those with thrombangitis obliterans (Buerger's disease) are excluded. Table 2
252 shows the main inclusion and exclusion criteria.

253 Subjects are screened up to 5 weeks before randomization. If found eligible, patients are
254 randomized in a ratio of 2:1 to treatment with PLX-PAD 300×10^6 cells or with placebo.
255 Treatment is administered at two time points, 8 weeks apart. At each occasion, thirty
256 intramuscular injections, 0.5 mL each, are administered in the index leg along its length,
257 anteriorly and posteriorly, according to a standard injection-sites scheme. A strict procedure
258 is applied for cell preparation and administration in order to maintain study blinding. Dosage
259 and timing of injections are based on preclinical and accumulated clinical data.

260 Each subject will be followed-up for at least 12 months post randomization or until the 12
261 months visit of the last patient randomized. Maximal follow up allowed by protocol is 36

262 months post randomization, hence all subjects will be followed-up for 12-36 months. The
263 study design is presented in Fig. 4.
264 The primary efficacy endpoint of the study is time-to occurrence of major amputation or
265 death, i.e. amputation-free survival up to 36 months after randomization. Safety and
266 tolerability are to be evaluated as well as other secondary and exploratory endpoints (Table
267 3). The study will also assess a potential economic benefit of this regenerative treatment
268 approach by applying a health-economic evaluation, taking into account relevant parameters
269 as days of hospitalization and patient reported quality of life.

270 The study will be performed in 50 sites in Europe and the USA

271

272 ***Statistical considerations***

273 The sample size of 246 subjects provides a power of 89.7%, and is based on the 2:1 ratio
274 randomization to treatment, an estimated AFS of 65% in the placebo group at the end of the
275 first year, and a risk reduction of approximately 50% for the PLX-PAD group during the first
276 year, using the the log-rank test. The primary endpoint will be analyzed using the Cox
277 Proportional Hazards model. The study randomization is stratified for the presence of
278 diabetes mellitus, for the extent of ischemic lesions, and for geographical region, which will
279 be covariates in the statistical model.

280

281 ***Discussion***

282 Although critical limb ischemia affects a small proportion of patients with PAD, and an
283 increasing part of them are offered revascularization (26), other treatments are required for
284 some patients in order to possibly increase survival and reduce major amputations. The fact
285 that trials have had problems with slow recruitment of no-option patients, e.g. the TAMARIS

286 trial (5) and the AGILITY HGF trial, that had to be canceled for that reason (10) might be
287 interpreted in a way that few patients do require alternative treatments. However, in
288 addition to no-option cases, revascularizations may fail or only partly reduce CLI symptoms,
289 and poor option subjects for revascularization due to co-morbidity or for technical reasons
290 will still be a reality. In a recent paper, Martinez et al (27) discussed predictive factors of
291 poor short-term outcome (mortality and major amputation) following revascularization,
292 including age, low hemoglobin values, acute myocardial infarction, ischemic ulcers and
293 infrapopliteal revascularization. For such groups of fragile CLI patients, therapeutic
294 angiogenesis may be an alternative.

295 As larger gene therapy trials have failed, although there is still an interest in the evaluation
296 of HGF (9), and doubts exist with regard to cell therapy (14,15), no such treatment has yet
297 been approved for clinical use. It could be interpreted that single growth factor trials may
298 not be able to provide the complete array of factors that the patients in this population
299 require. Therefore, precursor cell therapy would potentially provide a more complete array
300 of factors. It is reasonable to assume that the age and condition of cells, harvested from the
301 potential patients, are crucial. It has been shown that CLI patients produce lower levels of
302 progenitor cells (17) and an increasing cardiovascular risk is also related to a lower number
303 of progenitor cells (16). In addition, cells harvested and injected at the point of service, are
304 not by their nature able to be characterized nor quantified before being injected, therefore
305 bringing into question their very nature. Furthermore, it has been shown that growth of
306 isolated mesenchymal stem cells is significantly related to the age of the donor (28), and
307 thus young allogeneic placental cells may be most relevant for the purpose of treatment as
308 they come from a young healthy donor.

309 Most importantly, PLX-PAD cells, being of a placental source, known for its immune-
310 privileged characteristics, have been shown to not exert an immunological effect neither in
311 vitro nor in vivo in animal models and humans, requiring no immunosuppression prior to PLX-
312 PAD administration (29).

313 The PACE trial only includes patients with ischemic lesions and does not enroll Rutherford
314 Category 4 cases with just rest pain, due to the inobjectivity of evaluating pain. In practice,
315 CLI patients with rest pain may also be those who most frequently will be offered
316 revascularization. Hence, pain is not included in the composite primary efficacy endpoint in
317 Rutherford Category 5 patients. Furthermore, these patients are at higher risk of major
318 amputation, thus providing the best evidence on the effect on AFS.

319 The trial design takes into account the greater efficacy of two cell administrations rather
320 than one as shown in both animal models and human subjects, and therefore a second
321 administration session is given two months after the first session. Some patients will be
322 followed up to 36 months, which will enable collection of highly important information on
323 long-term effects of the treatment. and will also increase knowledge on the natural course
324 of severe CLI . The primary efficacy endpoint, amputation free survival is selected as the
325 strictest endpoint to be evaluated. Disease progression, wound healing, ischemic pain,
326 quality of life, TcPO₂, ABI/TBI measures and hospitalization days data are included as
327 secondary and exploratory endpoints.

328 The term therapeutic angiogenesis may be interpreted as the mode of action by which new
329 vessels are formed, thus potentially increasing perfusion. In human studies, however,
330 present imaging technology is only occasionally able to show newly developed vessels
331 despite the fact that subjects may be improved. It is evident that other pathophysiological
332 events are affected as well, primarily the inflammatory process. PLX-PAD cells exert effects

333 on both angiogenesis and tissue inflammation, but also on regeneration of muscle cells.
334 Whether the latter is a mechanism of value for improvement of function and symptoms in
335 CLI patients should be further investigated.
336 In summary, cell therapy works in a multifactorial way, PLX-PAD cells are young and potent,
337 they secrete relevant factors, are easily accessible in required quantity without harvesting
338 from fragile patients putting those at additional risk and have shown pre-clinical and initial
339 clinical evidence of efficacy. The design of the PACE trial, including only patients with
340 ischemic foot lesions, dual injections along the whole limb, follow-up up to 36 months, and
341 with a primary efficacy endpoint based on long term time-to-event regarding amputation-
342 free survival may allow for better understanding of perfusion enhancement and change of
343 inflammatory response and improved outcome for patients with severe critical limb
344 ischemia.

345
346

347 **Declaration of interest:** L Norgren, N Weiss, S Nikol, RJ Hinchliffe, JC Lantis, MR Patel and H
348 Reinecke are members of the Steering Committee for the PACE trial. R Ofir, Y Rosen, D Peres
349 and Z Aberman are employees of Pluristem Ltd

350
351

352 **Conflicts of Interest**

353

354 L Norgren: Consultations, advisory boards and/or research grants: AnGes, AstraZeneca,
355 Bayer, CESCA, Mitsubishi, Pluristem

356

357 N Weiss: Consultations, advisory boards and/or research grants: Amgen, Bard, Bayer,
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359

360 S Nikol: Consultations: Pluristem

361

362 RJ Hinchliffe: Nothing to declare

363

364 JC Lantis: Consultations: Pluristem

365

366 MR Patel: Advisory boards: Bayer, Jansen. Research grants: Pluristem, Bayer, Jansen,
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369 H Reinecke : Consultations: BMS, MedUpdate, NephroUpdate, Pfizer, Pluristem. Grants:
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373 **References**

374 1.Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG. Inter-Society
375 Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc
376 Endovasc Surg 2007;33:S1-S75.

377 2.Fowkes FG, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM et al.
378 Comparison of global estimates of prevalence and risk factors for peripheral artery disease in
379 2000 and 2010: a systematic review and analysis. Lancet 2013; 382:1329-40

380
381 3.Gerhard-Herman MD, Gornik HL, Barrett C, Barshes NR, Corriere MA, Drachman DE, et
382 al. 2016 AHA/ACC Guideline on the Management of Patients With Lower Extremity
383 Peripheral Artery Disease: A Report of the American College of Cardiology/American Heart
384 Association Task Force on Clinical Practice Guidelines. Circulation 2017;135:e726-e779.
385
386
387
388

389 4.Aboyans V, Ricco J-B, Bartelink EL, Bjorck M, Brodmann M, Cohnert T et al. 2017 ESC
390 guidelines on the diagnosis and treatment of peripheral arterial diseases, in collaboration with
391 the European Society for Vascular Surgery (ESVS). Eur J Vasc Endovasc Surg. 2018; 55:305-
392 368

393 5.Belch J, Hiatt WR, Baumgartner I, Driver IV, Nikol S, Norgren L et al. Effect of fibroblast
394 growth factor NV1FGF on amputation and death: a randomized placebo-controlled trial of
395 gene therapy in critical limb ischemia. Lancet 2011; 377:1929-37

396 6.Nikol S, Baumgartner I, Van Belle E, Diehm C, Visona A, Capogrossi MC et al.
397 Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in

398 patients with critical limb ischemia. *Mol Ther* 2008; 16:972-8

399 7. Shigematsu H, Yasuda K, Iwai T, Sasajima T, Ishimaru S, Ohashi Y et al. Randomized,
400 double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical
401 limb ischemia. *Gene Ther* 2010; 17:1152-61

402 8. Powell RJ, Goodney P, Mendelsohn FO, Moen EK, Annex BH, HGF-205 Trial
403 Investigators. Safety and efficacy of patient specific intramuscular injection of HGF plasmid
404 gene therapy on limb perfusion and wound healing in patients with ischemic lower extremity
405 ulceration: results of the HGF-0205 trial. *J Vasc Surg* 2010; 52:1525-30

406 9. Sanada F, Taniyama Y, Muratsu J, Otsu R, Shimitzu H, Rakugi H et al. Gene-therapeutic
407 strategies targeting angiogenesis in peripheral artery disease. *Medicines* 2018; 30: 5(2). doi:
408 10.3390/medicines5020031

409 10. Iver SR, Annex BH. Therapeutic angiogenesis for peripheral artery disease. *JACC Basic*
410 *Trans Sci* 2017; 2:503-12

411 11. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H et al.
412 Therapeutic angiogenesis for patients with critical limb ischaemia by autologous
413 transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*
414 2002; 360:427-35

415 12. Teraa M, Sprengers RW, Schutgens RE, Slaper-Cortenbach I, van der Graaf Y, Algra A et
416 al. Effect of repetitive intra-arterial infusion of bone marrow mononuclear cells in patients
417 with no-option limb ischemia. The randomized, double-blind, placebo-controlled rejuvenating
418 endothelial progenitor cells via transcutaneous intra-arterial supplementation. *Circulation*
419 2015; 131:851-60

420 13. Teraa M, Sprengers RW, van der Graaf Y, Peters CE, Moll FL, Verhaar MC. Autologous
421 bone marrow derived cell therapy in patients with critical limb ischemia: a meta-analysis of
422 randomized controlled clinical trials. *Ann Surg* 2013; 258:922-9

- 423 14. Peeters Weem SM, Teraa M, De Borst GJ, Verhaar MC, Moll FL. Bone marrow derived
424 cell therapy in critical limb ischemia: a meta-analysis of randomized placebo controlled trials.
425 Eur J Vasc Endovasc Surg 2015; 50:775-83
- 426 15. Pan T, Wei Z, Fang Y, Dong Z, Fu W. Therapeutic efficacy of CD34+ cell-involved
427 mononuclear cell therapy for no-option critical limb ischemia: a meta-analysis of randomized
428 controlled clinical trials. Vasc Med 2018; doi: 10.1177/1358863X17752556 (Epub ahead of
429 print)
- 430 16. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA et al.
431 Circulating endothelial progenitor cells, vascular function and cardiovascular risk. New Eng J
432 Med 2003; 348:593-600
- 433 17. Teraa M, Sprengers RW, Westerweel PE, Gremmels H, Goumans MJ, Teerlink T et al.
434 Bone marrow alteration and lower endothelial progenitor cell numbers in critical limb
435 ischemia patients.
436 PLoS One 2013; 8: e55592 doi:10.1371/journal.pone.0055592. Epub 2013
437
438
439
440
- 441 18. Jonsson TB, Larzon T, Arfvidsson B, Tidefeldt U, Axelsson CG, Jurstrand M et al.
442 Adverse events during treatment of critical limb ischemia with autologous peripheral blood
443 mononuclear cell implant. Int Angiol 2012; 31:77-84
- 444 19. Zhang J, Huang X, Wang H, Liu X, Zhang T, Wang Y, Hu D. The challenges and
445 promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. Stem Cell
446 Res Ther. 2015 Dec 1;6:234.
- 447 20. Roy R, Brodarac A, Kukucka M, Kurtz A, Becher PM, Jülke K et al. Cardioprotection by
448 placenta-derived stromal cells in a murine myocardial infarction model. J Surg Res. 2013
449 Nov;185(1):70-83.
- 450 21 Zahavi-Goldstein E, Blumenfeld M, Fuchs-Telem D, Pinzur L, Rubin S, Aberman Z, Sher
451 N, Ofir R. Placenta-derived PLX-PAD mesenchymal-like stromal cells are efficacious in

452 rescuing blood flow in hind limb ischemia mouse model by a dose- and site-dependent
453 mechanism of action. *Cytotherapy*. 2017 Dec;19(12):1438-1446.

454 22. Gao X, Xu Z. Mechanisms of action of angiogenin. *Acta Biochim Biophys Sin(Shanghai)*.
455 2008 Jul;40(7):619-24.

456 23 Prather WR, Toren A, Meiron M, Ofir R, Tschöpe C, Horwitz EM. The role of placenta
457 derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia.
458 *Cytotherapy*. 2009;11(4):427-34.

459 24 Van Belle E, Nikol S, Norgren L, Baumgartner I, Driver V, Hiatt WR, Belch J. Insights on
460 the role of diabetes and geographic variation in patients with critical limb ischaemia.
461 *Eur J Vasc Endovasc Surg*. 2011 Sep;42(3):365-73.

462 25. Reinecke H, Unrath M, Freisinger E, Bunzemeier H, Meyborg M, Lüders F, Gebauer K,
463 Roeder N, Berger K, Malyar NM. Peripheral arterial disease and critical limb ischaemia: still
464 poor outcomes and lack of guideline adherence. *Eur Heart J*. 2015 Apr 14;36(15):932-8.

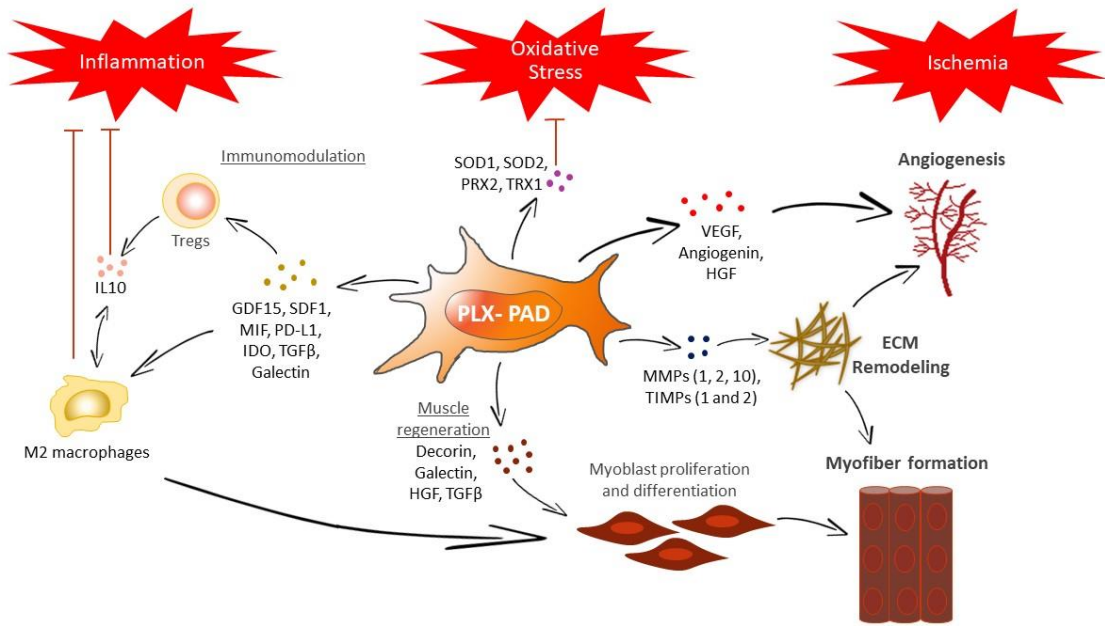
465 26.Hong MS, Beck AW,Nelson PR. Emerging national trends in the management and
466 outcomes of lower extremity peripheral arterial disease. *Ann Vasc Surg* 2011; 25:44-54

467 27.Martinez M, Sosa C, Velescu A, Llorca C, Elosus R Clara A. Predictive factors of a poor
468 outcome following revascularization for critical limb ischemia: implications for practice.
469 *Int Angiol* 2018; doi:10.23736/S0392-9590.18.03986-X (E-pub ahead of print)

470 28. Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM. Impact of aging on the
471 regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal
472 stem/stromal cells. *PLoS One*. 2014 Dec 26;9(12):e115963.

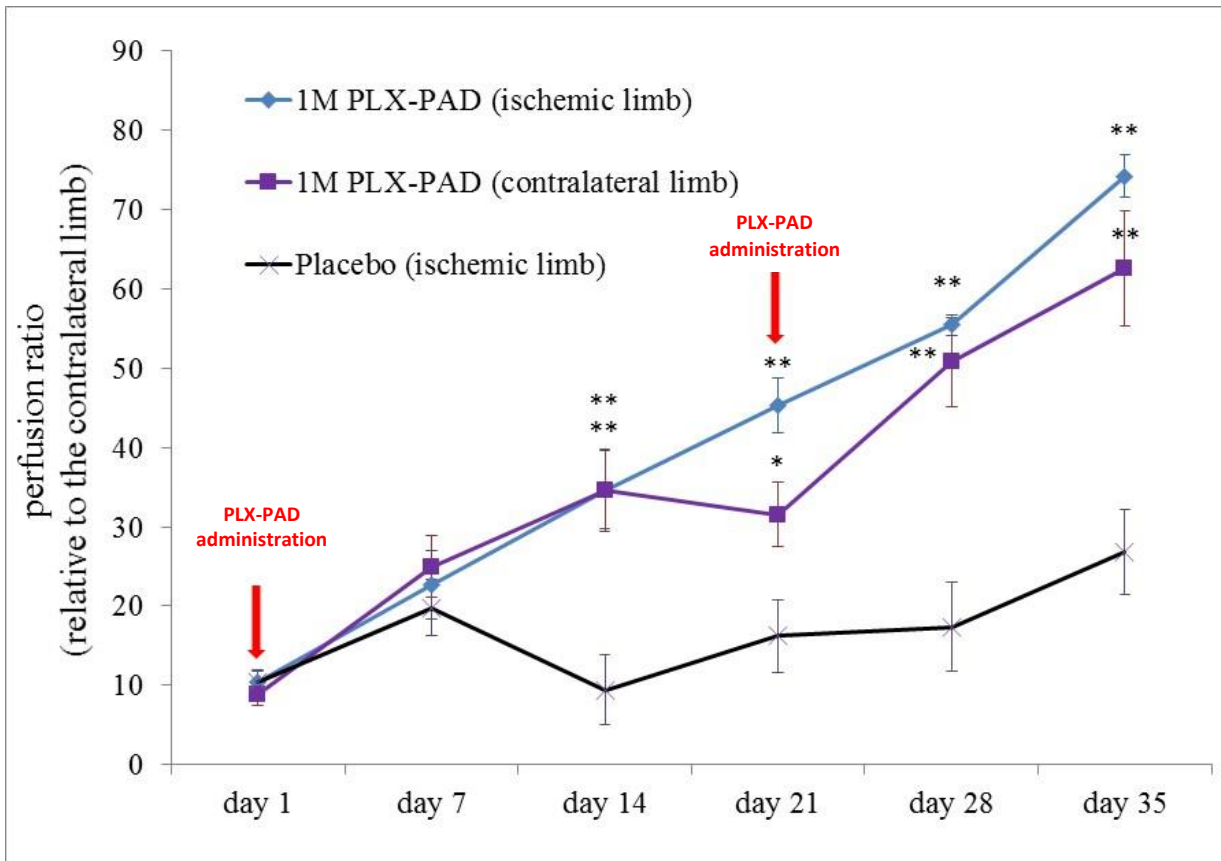
473 29. Consentius C, Akyüz L, Schmidt-Lucke JA, Tschöpe C, Pinzur L, .Ofir R et.al
474 Mesenchymal Stromal Cells Prevent Allostimulation In Vivo and Control Checkpoints of Th1
475 Priming: Migration of Human DC to Lymph Nodes and NK Cell Activation.
476 *Stem Cells*. 2015 Oct;33(10):3087-99. doi: 10.1002/stem.2104.
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478 Figure 1
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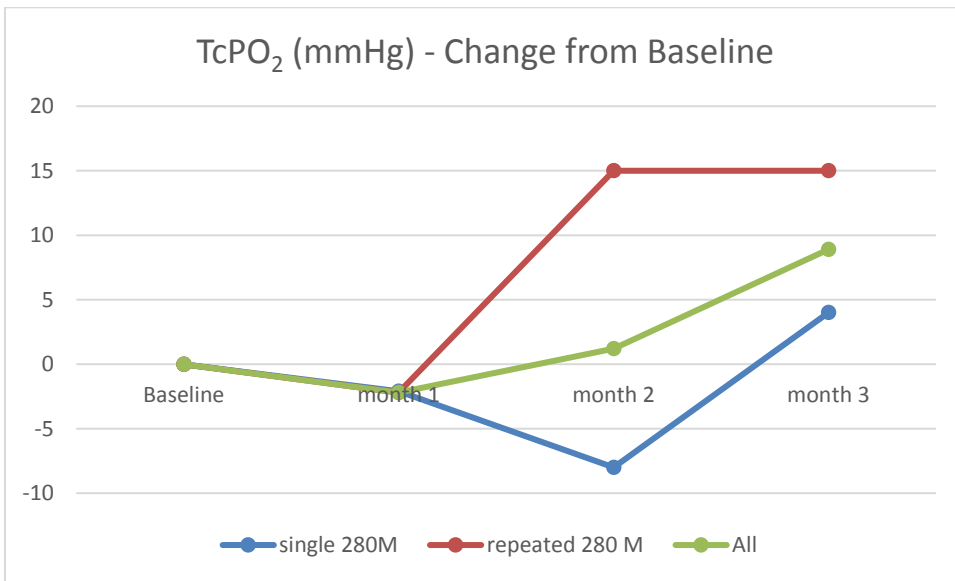
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509 Figure 2



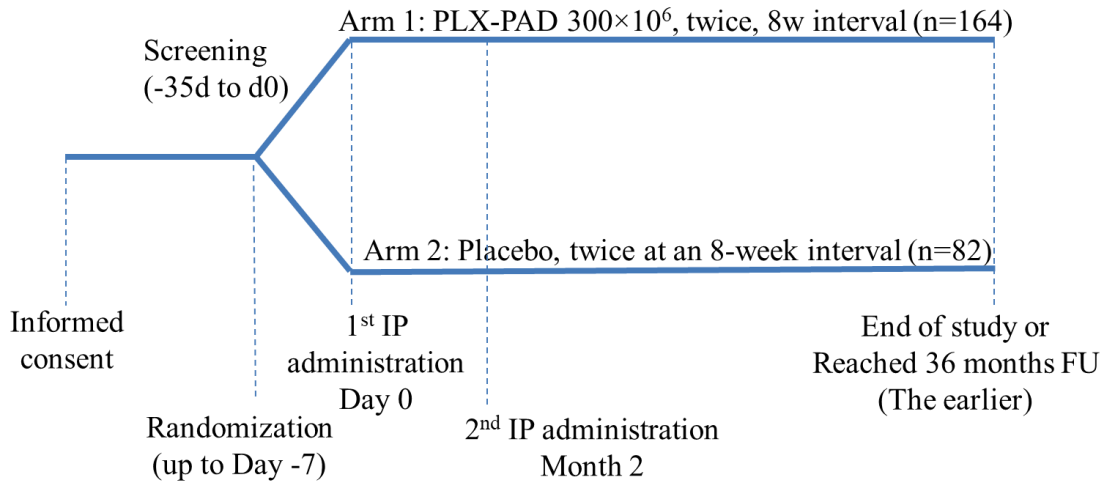
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Figure 3



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591 Figure 4
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619 **Table 1**

620 Cytokines secreted by PLX-PAD and their function

621

622 Angiogenesis

VEGF (Vascular Endothelial Growth Factor)

623

Angiogenin

624

Angiopoietin 1

625

HGF (Hepatocyte Growth Factor)

626

Osteopontin

627

MMP-1 (matrix metalloproteinase 1)

628

MMP-2

629 Immunomodulation

Osteopontin

630

CXCL12 /SDF 1 (Stromal Cell-derived Factor 1)

631

GDF 15 (Growth Differentiation Factor 15)

632

MIF (Macrophage Migration Inhibition Factor)

633

IDO (Indoleamine 2,3-dioxygenase)

634

TGF- β (Transforming growth factor beta)

635

PD-L1 (Programmed death ligand 1)

636

HGF

637

IL-11 (Interleukin 11)

638

CCL5 (RANTES- regulated on activation, normal T cell

639

expressed and secreted)

640

641 Muscle regeneration

Decorin

642

MMP 1

643

HGF

644

TGF β

645

Galectin 1

646

IGFBP-3 (Insulin growth factor binding protein 3)

647

FLRG (FSTL3- Follistatin-related protein 3)

648

Osteopontin

649

CXCL12 /SDF 1

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656 **Table 2**

657

658 Main inclusion criteria:

- 659 • Age 45-99 years.
- 660 • CLI due to atherosclerosis with minor tissue loss (Rutherford 5) up to the ankle level.
- 661 • Ankle pressure ≤ 70 mmHg or toe pressure ≤ 50 mmHg.
- 662 • Subject unsuitable for revascularization (by any method) in the index leg, based on
- 663 unfavorable risk-benefit assessment.
- 664 • Ischemic lesions neither healing, nor significantly worsening (within 2 weeks during
- 665 screening)
- 666 • Ischemic lesions without tendon or bone exposure (unless secondary to a minor
- 667 amputation).

668

669 Main Exclusion criteria:

- 670 • Non-atherosclerotic PAD (e.g. Buerger's disease).
- 671 • CLI with major tissue loss (Rutherford Category 6) in either leg.
- 672 • Evidence of active infection (e.g., cellulitis, osteomyelitis).
- 673 • Subject having undergone surgical revascularization <1 month prior to study, or
- 674 endovascular revascularization/minor amputation <2 weeks prior.
- 675 • Planned or potential need for major/minor amputation or revascularization within 1 month
- 676 of study entry.
- 677 • Aorto-iliac stenosis or common femoral artery stenosis $\geq 70\%$.
- 678 • Use of hyperbaric oxygen therapy, prostanoids, spinal cord stimulation, lumbar
- 679 sympathectomy, wound dressing containing cells or growth factors, or topical platelet
- 680 derived growth factor.
- 681 • Stroke or acute myocardial infarction/unstable angina within 3 months prior to screening.
- 682 • Severe congestive heart failure symptoms (New York Heart Association [NYHA] Stage
- 683 IV).
- 684 • Uncontrolled severe hypertension.
- 685 • Diabetes mellitus with HbA1c $> 10\%$.
- 686 • Subject on renal replacement therapy or with eGFR < 15 mL/min/1.73m².
- 687 • Pulmonary disease requiring supplemental oxygen treatment on a daily basis.
- 688 • Active malignancy or history of malignancy within 5 years prior to study entry.

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- 703 **Table 3**
- 704 Primary Efficacy Endpoint:
- 705 • Time to occurrence of major amputation or death (amputation-free survival).
- 706
- 707 Main Secondary and Exploratory Endpoints:
- 708 • Time to first occurrence of any of the following single events:
- 709 o Major amputation of the index leg.
- 710 o Revascularization due to worsening of CLI in the index leg.
- 711 o Doubling of total ulcer area from baseline in the index leg.
- 712 o De novo necrosis in the index leg.
- 713 o All-cause mortality.
- 714 • Time to major amputation of the index leg.
- 715 • Complete healing of all ischemic lesions at 12 months.
- 716 • Change from baseline in ischemic pain (Numerical rating scale (NRS)) at 6 months.
- 717 • Time to death or major amputation or adjudicated major amputation of the index leg.
- 718 • Time to all cause death.
- 719 • Decrease of 50% or more in total ulcer area at 6 months.
- 720 • Complete healing of all ischemic lesions in the contralateral leg.
- 721 • Time to occurrence of major amputation of the contralateral leg.
- 722 • Change in health- and disease-related Quality of Life at 12 months.
- 723 • Changes in tcPO₂, ankle-brachial index (ABI), toe-brachial index (TBI).
- 724 • Revascularization procedure in the index leg within 12 months from treatment.
- 725 • Hospitalization days.
- 726 • Change from baseline in plasma cytokine levels after PLX-PAD administration.
- 727 • Change from baseline in mRNA expression profile after PLX-PAD administration.

749 **Legends to figures**

750

751 **Figure 1**

752

753 Suggested mechanism of PLX-PAD effect in CLI. PLX-PAD secretions can mitigate CLI
754 pathology by simultaneously affecting several disease associated pathways. PLX-PAD secrete
755 immunomodulatory cytokines which support the induction of M2 macrophages and elevate
756 the level of circulating regulatory T cells, leading to elevation in IL-10 and resolution of
757 inflammation. In addition, PLX-PAD secrete factors which directly support angiogenesis and
758 muscle regeneration. These processes are further supported by the PLX-PAD secretion of
759 enzymes with antioxidant activity, which can protect blood vessels from oxidative damage,
760 and the secretion of ECM (extracellular matrix) remodeling enzymes which enable
761 regeneration.

762

763 **Figure 2**

764

765 PLX-PAD cells are effective in re-establishing blood flow in the HLI mouse model.
766 Intramuscular (IM) administration to the ischemic or contralateral limb, were effective in
767 rescuing blood flow to the ischemic limb compared to placebo control. PLX-PAD were
768 administered 1 and 21 days (depicted by arrows on graph) following induction of HLI. n=10
769 for each PLX-PAD treated group and n=5 for placebo group. $F(39,70)=30.82$, $p<0.0001$. Blood
770 flow is measured as perfusion ratio relative to the contralateral limb. * $p<0.05$; ***
771 $p < 0.0001$, compared to placebo control.

772

773 **Figure 3**

774

775 Change of TcPO2 in Study 1202-2. M=million cells

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780 **Figure 4**

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782 Study design, timing of injections and follow up.

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