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Accelerating Biological Neovascularization in Chronic Limb Threatening Ischemia

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Abstract

Chronic Limb-Threatening Ischemia (CLTI) was managed in patients who failed previous invasive revascularization. A novel non-invasive, home-based approach designed to improve blood flow by addressing eight obstacles to innate neovascularization was used. Hemodynamic evidence of improved blood flow was evident in 14 of 15 patients and was substantiated by angiographic evidence of collateral artery growth and segmental re-canalization of chronic thrombus. These substantiate our previously reported molecular evidence of endothelial activation, increase in pro-angiogenic cellular and protein mediators, low-grade fibrinolysis, and improved cellular immunity. Limb salvage for at least one year was achieved in 12 of 15 despite being "no option" CLTI patients. Recommendations for further study are made to assess this modality in CLTI as well as in other ischemic conditions

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INTRODUCTION

The hypothesis tested in this study is whether blood flow will improve by overcoming obstacles to neovascularization in ischemic vascular disease. Neovascularization (NV) via growth of collateral arteries (arteriogenesis) and capillaries, arterioles, and venules (angiogenesis) becomes impaired as ischemia from vascular disease progresses [1]. Our premise [2] is that overcoming hemodynamic and cellular obstacles to NV should yield an effective, durable, low-risk, home-based, inexpensive option to surgical or catheter revascularization [3-5].

Phase II clinical trials attempting to orchestrate NV using specific molecular or cellular strategies have yet to yield a clinically accepted option [6]. Our alternative approach is to overcome obstacles to NV by modifying the ischemic environment, rather than to address discrete NV pathways.

Endothelial shear stress initiates arteriogenesis, while hypoxia stimulates angiogenesis [7,8]. Multilevel arterial occlusions attenuate endothelial shear stress, impair delivery of oxygenated nutritive blood flow, inhibit efficient clearance of toxic metabolic by-products, hinder dissemination of protein "distress" signals and impede the arrival of monocytes and mobilized salutary progenitor cells to the distressed tissue. The result is a sub-optimal microenvironment for biochemical processes. These 5 obstacles arise directly from impaired blood flow. They were addressed by increasing blood flow with an external programmed compression pump (PCP) worn on the calf and foot in the seated position for a minimum of 3 hours daily [2].

Filgrastim (Amgen Inc) was used to overcome the sixth obstacle: diminished number and function of circulating progenitor cells reported in advanced vascular disease [9]. It is a Granulocyte Colony Stimulating Factor (G-CSF) FDA approved for stem cell mobilization. A novel dosimetry was used: 7-10 mcg/kg was injected SQ once every 72 hours, for a total of up to 10 doses. We reported [2] significant increases in the progenitor Cell population as a whole (CD34+) and the endothelial progenitor cell population in particular (VEGFR2+) the day following the 5th and 10th doses of Filgrastim versus Baseline. These cells home toward shear stress activated endothelium secreting monocyte chemoattractant protein (MCP-1). These activated cells express adhesion molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1) that capture the homing progenitor cells and monocytes to initiate Arteriogenesis.

In the initial patients treated with this approach hemodynamics improved: (increase in ankle brachial index, development of pulsatility in the forefoot and toes) [10,11]. There was abatement of ischemic rest pain, and healing of forefoot ischemic wounds. Most importantly, arteriography not only demonstrated the typical cork-screw collateral arteries of arteriogenesis, but also segmental recanalization of previously occluded infrageniculate arteries and increased rate of transit of injected contrast in the ischemic tissue. Chronic occlusive thrombus appeared to have

been partially lysed. Chronic thrombus is the seventh obstacle and has had no previous safe therapeutic solution. We reported significant ($p < 0.01$) PCP independent increases in the plasma concentration of the fibrinolytic enzyme plasmin (> 10-fold). We also reported a > 5-fold increase in the plasma level of Fibrin Degradation Products (FDP). Both plasmin and FDP were measured by ELISA one day after both the 5th and the 10th Filgrastim doses. Their increase was reported relative to their concentrations on Day 1 [2]. Gradual safe thrombolysis is a new therapeutic option for management of chronic ischemia in the extremity or elsewhere.

Patients with severe vascular disease, particularly if they are diabetic, have impaired cellular immunity. Overcoming this eighth obstacle has pro-angiogenic benefit. Filgrastim is FDA approved to improve impaired cellular immunity following cytotoxic chemotherapy. One day following the 5th and 10th doses of Filgrastim we reported the expected leukocytosis and neutrophilia compared to baseline [2]. Accompanying this was a significant ($p < 0.05$) increase in the concentration of pro-angiogenic proteins: vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), and metalloproteinase 9 (MMP-9), angiopoietin-1 (ANGPT1) and others in the serum of CLTI patients treated 24 hours earlier with 8-10 mcg/kg of Filgrastim [2]. This is in part attributable to the neutrophilia [12].

While none of these findings arose in the PCP alone group, the molecular data in our study [2] also showed that the concentration of MCP-1 increased after 2 hours of PCP, along with the concentration of serum nitrite, the break-down product of nitric oxide. Nitric oxide synthase activity is another marker of endothelial activation (see above). The effect of the pump offset the drop in MCP-1 measured in response to Filgrastim. Another marker of endothelial activation is the increase of adhesion molecules on the surface of the endothelial cell. Of note is we measured an increase in circulating CD-31, the cytokine PECAM-1.

CLINICAL CASES

Goal

The goal of this study was to prospectively test whether this novel therapy can improve blood flow in no-option CLTI patients who had failed prior vascular interventions. Blood flow was measured hemodynamically and observed angiographically.

Inclusion Criteria

CLTI patients who were not candidates for, or had failed, previous invasive revascularization procedures and were complaining of ischemic forefoot rest pain (Fontaine III), gangrene, and/or ischemic ulceration (Fontaine IV).

Criteria for this Series

Allergy to Filgrastim, intolerance of PCP, active malignancy (occult malignancy found in one patient), active vasculitis, myeloproliferative disorder, dialysis, non-salvageable extremity unless goal was to heal a below knee amputation, sickle cell disease, uncorrected significant coronary or carotid artery disease, body mass index > 34.

Population Data

Fifteen CLTI patients were treated after informed consent: 3 at University of Miami (Miami, FL), 8 at Weiss Memorial/University of Chicago (Chicago, IL), 2 at Swedish Hospital/Northshore University (Chicago, IL), 1 at Illinois Hospital (Peoria, IL), and 1 at Aventura Hospital, Miami FL. All consented to the prospective collection and publication of their data. Average age was 67+11 (8 male, 7 female). Fifteen patients had ischemic forefoot rest pain and eleven had ischemic ulcer or gangrene. All were referred in lieu of major amputation. The fifteen patients had the following co-morbidities: Hypertension (95%), Hyperlipidemia (60%), diabetes (45%), coronary artery disease (40%), Congestive heart failure (20%), Myocardial Infarction (20%) and stroke (15%). All had previous failed vascular interventions totaling 45 procedures, including bypass surgery, endarterectomy, catheter recanalization with balloon, drug eluting and non-drug eluting endografts/stents, atherectomy, laser).

Treatment

Patients were treated with a PCP named the ArtAssist Device (ACI Medical LLC, San Marcos CA, USA) and a G-CSF (Filgrastim, Amgen Inc USA). Each patient wore the PCP for a minimum of three hours daily in the sitting position and continued using the PCP until the ischemic wounds healed and the ischemic rest pain resolved. The ArtAssist applies sequential calf and foot compression in the seated position for at least 3 hours daily. The rapid inflation of the 3 pneumatic cuffs (0 to 120 mmHg in 0.3 seconds) provides an endothelial shear stress stimulus, while at the same time driving in oxygenated nutritive blood flow and facilitating venous return. The pressure is held in each of the cuffs for 3 seconds. Rapid deflation follows. Each cycle last 20 seconds to allow for restoring vascular volume. Each patient was also injected with Filgrastim 7-10 mcg/kg every 3 days. Up to 10 doses were given, especially if the CLTI was Fontaine IV. Dosing was stopped if clinical improvement occurred early, which was seen with patients Fontaine III. The first two patients were treated intravenously (IV) in the hospital; the rest were injected subcutaneously (SQ) in the abdominal wall at home.

Patient 1

Presentation a 56-year-old Caucasian male was treated the ArtAssist and 10 doses of Filgrastim 10 mcg/kg injected IV every 3 days over 28 days in lieu of a left below knee amputation (BKA). A femoral to peroneal artery bypass graft (BPG) was constructed a year earlier with in situ ipsilateral Greater Saphenous Vein (GSV) to allow the healing of a Trans-Metatarsal Amputation (TMA) for forefoot gangrene. Graft surveillance duplex imaging identified serial stenoses from intimal hyperplasia in the body of the conduit. Percutaneous Transluminal Balloon Angioplasty (PTA) resulted in acute graft thrombosis and embolization of the

outflow artery. The healed TMA underwent ischemic necrosis (Figure 1A). The Ankle-Brachial Index (ABI) dropped to 0 (normal = 0.95-1.1), ankle waveforms became non-pulsatile (Figure 1B), and transcutaneous oxygen tension (TcPO2) on the foot dorsum dropped to 3 mmHg.

Additional History: Bilateral carotid endarterectomy, coronary artery revascularization, and human immune-deficiency viral (HIV) infection. His anti-viral medication was hypothesized to contribute to the IH. Viral counts were undetectable. He had stopped smoking for a year. He developed an ankle extension deformity after the TMA.

Outcome: The patient's wound granulated slowly (Figure 1A). At 6 months, ABI increased to 0.43, ankle pulsatility was evident, and the foot dorsum TcPO2 increased to 24 mmHg. At 8 months, ABI increased further to 0.64 (Figure 1B), doppler waveform amplitude increased, and open dehisced TMA wound healed fully (not normally observed following graft thrombosis with ABI zero). Angiogram showed the "corkscrew" collateral growth characteristic of arteriogenesis, with previously occluded segments of larger arteries visualized (Figure 1C). Contrast flow was brisk. Achilles tendon release was performed to treat the ankle extension deformity and was complicated by calcaneal osteomyelitis. Partial calcanectomy was not feasible in a foot with a TMA. He underwent BKA one year after our treatment.

Patient 2

First Presentation: A 44-year-old Hispanic female was treated with ArtAssist and 10 doses of Filgrastim 10 mcg/kg injected IV every 3 days over 28 days in lieu of a BKA. She presented with severe ischemic forefoot rest pain, and two non-healing ankle ulcers: one exposing the Achilles tendon, and the other on the anterior ankle (Figure 2A). Previous iliac stents had been placed for ipsilateral external iliac artery stenoses. ABI on the treatment limb was 0.4 (Figure 2B), There was no metatarsal level or digital pulsatility. TcPO2 (measured from skin overlying the medial calcaneus and on the foot dorsum) were 23 mmHg and 1 mmHg respectively. Angiogram (Figure 2C) showed superficial femoral, popliteal, and tibial artery occlusive disease. Two previous femoral to tibial artery bypass grafts had failed within a month of surgery.

Additional History: Insulin Dependent Diabetes Mellitus (IDDM), renal insufficiency (Stage 3), congestive heart failure (CHF), morbid obesity, and coronary revascularization.

Outcome: Wound progress is shown in (Figure 2A). At 5 months, the anterior foot ulcer had healed. The posterior wound required temporary biologic dressings to protect the granulation tissue and exposed tendon; it took 2 years to heal. Four months after treatment, the ABI was 0.5 (Figure 2B); at 12 months, the TcPO2 increased to 46 mmHg (medial calcaneus) and 35 mmHg (foot dorsum). Metatarsal level pulsatility improved, but no toe pulsatility was seen on photoplethysmography (Figure 2B).

Second Presentation: One year later she returned to clinic with a cyanotic 5th toe after blunt trauma (Figure 2A), and slowly healing ulcer over the Achilles tendon covered with granulation tissue. ABI had increased to 0.7 and there was pulsatility in the mid-foot but none in the toes (Figure 2B). Angiogram (Figure 2C) showed corkscrew collateral growth; previously occluded

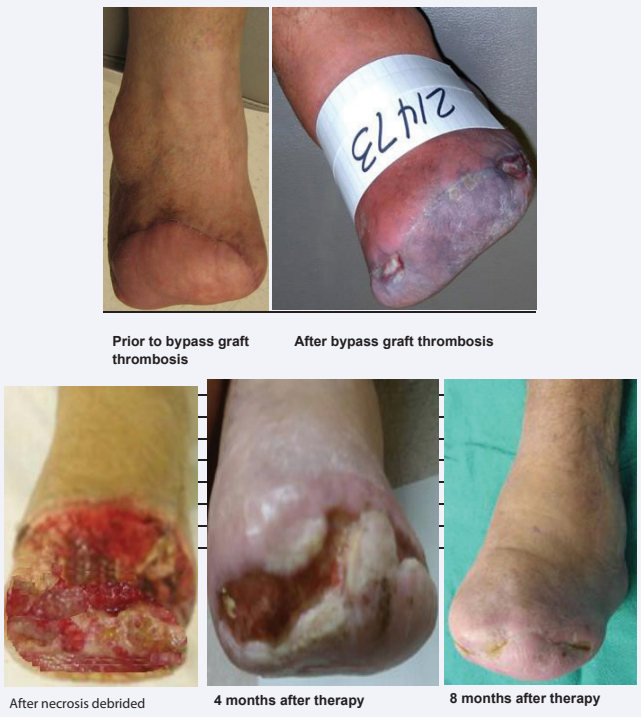


Figure 1A Patient 1: progression of Trans-Metatarsal Amputation

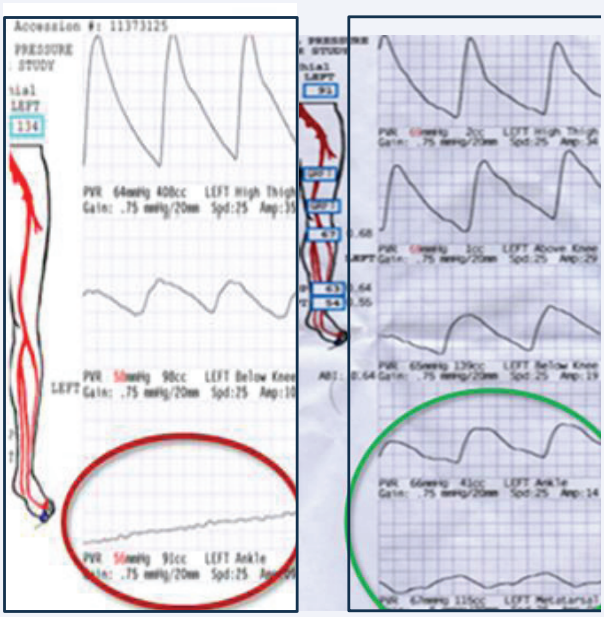


Figure 1B Patient 1: Pulse Volume Recording (PVR) and Ankle Brachial Index (ABI). The left circle shows non- pulsatile flow at the ankle just before treatment (ABI=0). The green circle shows return of pulsatility at the ankle and trans-metatarsal levels (ABI=0.64).

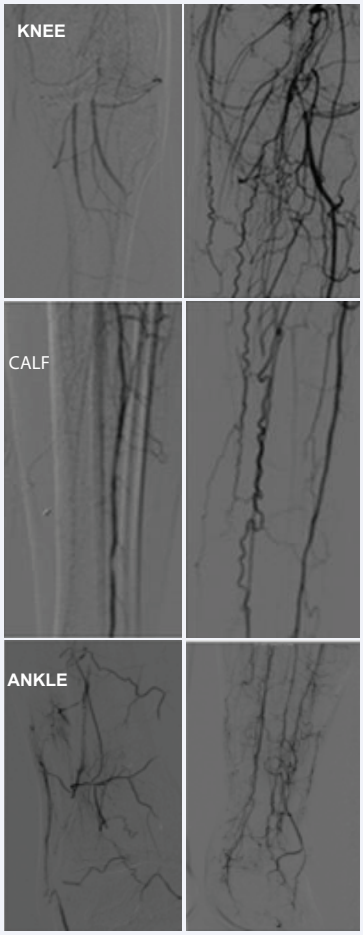


Figure 1C Patient 1: Angiogram images at start of therapy (left panels) compared to 6 months later (right panels) at the knee, calf and ankle. Note the corkscrew and other collateral growth and the segmental recanalization of a segment of the popliteal and anterior tibial artery.

Figure 2A Patient 2: Progression of 3 wounds: The top two were present initially: debrided anterior ankle ulcer, posterior ankle ulcer with exposed Achilles tendon. The third set is the 5th toe injury.

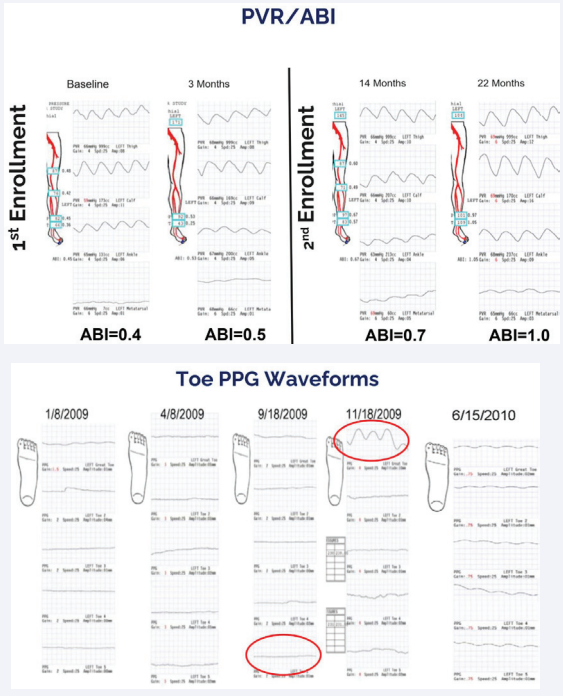


Figure 2B Patient 2: PVR and ABI over time. Note the increase in ABI from 0.4 at baseline to 0.5 at 3 months. The last ABI measured before she stubbed her toe was 0.5. After the 2nd course the ABI rose to 0.7 (14 months after initial presentation) and was 1.0 at 22 months (8 months after 2nd Filgrastim course). Note the non-pulsatile waveforms in the initial study. Note the non-pulsatile toe photoplethysmography (PPG) tracings up until 9/18/2009. The 5 toe PPG tracings start at the top with toe 1. Note 1st toe pulsatility after the 2nd course, and then in all 5 toes in the last panel (labeled 6/15/2010).

segments of larger arteries were visible. Contrast flow was brisk. The treatment regimen was repeated.

Outcome: Seven months later the ABI measured 1.0 and pulsatility returned to all 5 toes (Figure 2B). The 5th toe wound healed (Figure 2A). It took 2 years for the large ulcer over the exposed Achilles' tendon to close. Patient remained ambulatory with healed ulcers at last follow-up, 13 years later. The posterior ulcer required subsequent intermittent wound care when the thin overlying epithelium was traumatized (diabetic neuropathy).

Patient 3

Presentation: A 65-year-old Hispanic female was treated with ArtAssist and 10 doses of Filgrastim 600 mcg (8.7 mcg/kg) injected SQ every 3 days over 28 days in lieu of BKA for severe ischemic forefoot rest pain, a non-healing dorsal wound ulcer (Figure 3A) and gangrene of toes 1 and 2. She had had two femoral distal bypass grafts that failed acutely. A popliteal artery stent was placed via the dorsalis pedis artery (DP) and immediately thrombosed. The skin puncture wound over the DP became gangrenous. ABI was 0, TcPO2 was 2mmHg. No bypass targets or conduit were identified. Angiogram showed occlusion of the superficial femoral artery and the popliteal artery, an occluded popliteal artery stent, and tibial artery occlusion (Figure 3B).

Additional History: Hypercoagulability identified: anticardiolipin antibody (lupus anticoagulant) and thrombocytosis.

Outcome: Ischemic rest pain improved in the first month. At 3 months, angiogram showed corkscrew collateral formation extending down to the foot; named vessels became apparent (Figure 3B). Over 8 months, wounds healed, and gangrene sloughed (Figure 3A). Toe pulsatility returned and remained stable. At 8 years ABI was 0.65 (Figure 3A). She was ambulatory for 11 years. She developed renal failure at 9 years. She was a Jehovah's witness who did not accept transfusion. She passed away during her third year on dialysis.

Patient 4

Presentation: A 67-year-old African American male was treated with ArtAssist and 5 doses of Filgrastim 600mcg (7.4 mcg/kg) injected SQ every 3 days over 14 days in lieu of BKA for severe ischemic forefoot rest pain. He had 7 previous vascular interventions in each leg.

Additional History: NIDDM, CHF, stroke. Tobacco use had stopped 3 months earlier. Two months of ARTASSIST alone yielded no symptomatic benefit or improvement in blood flow. The ABI was 0.17 on the left and 0.49 on the right. The Toe-Brachial index (TBI) was 0 bilaterally. Patient had had an epidural implant placed for pain management, without benefit in walking distance or ABI.

Outcome: Filgrastim was stopped after 5 doses when he reported the ischemic rest pain had resolved and the pain free walking distance improved, with no claudication unless he walked rapidly or took the stairs. Three months later he developed a cyanotic right first toe after blunt injury. Toe pressure was 0 mmHg. A second course of 5 Filgrastim doses over 2 weeks led to healing of the toe by the 6th week. The ABI increased to 0.55 on the left and 0.78 on the right. The injured right great toe TBI increased to 0.31. The ARTASSIST device was used for 3 to 8 hours daily throughout this time.

He was subsequently ambulatory for 5 years until thrombosis of an old left iliofemoral bypass. During thrombectomy 4 chronically occluded grafts were removed from the left groin. A

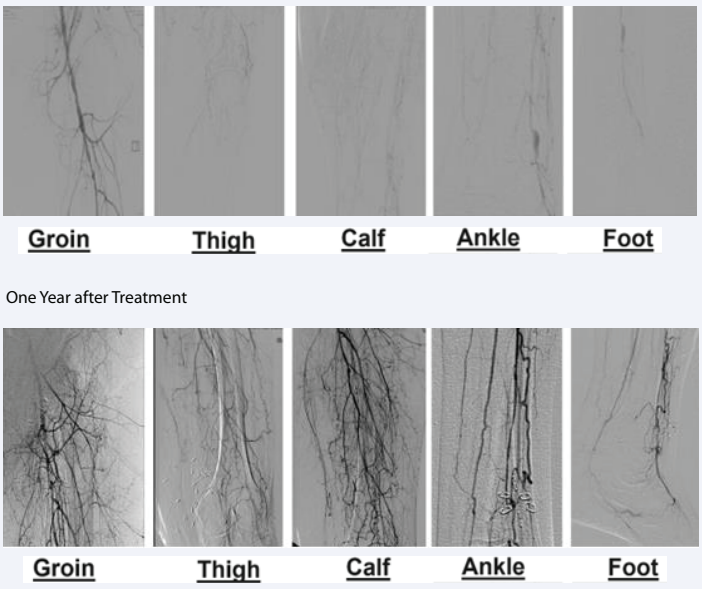


Figure 2C Patient 2: Angiogram of the left leg from groin to foot before (top panel), and one year after treatment. Note the collateral growth and the segmental recanalization of previously occluded arteries. Also note the improved contrast filling of the second angiogram

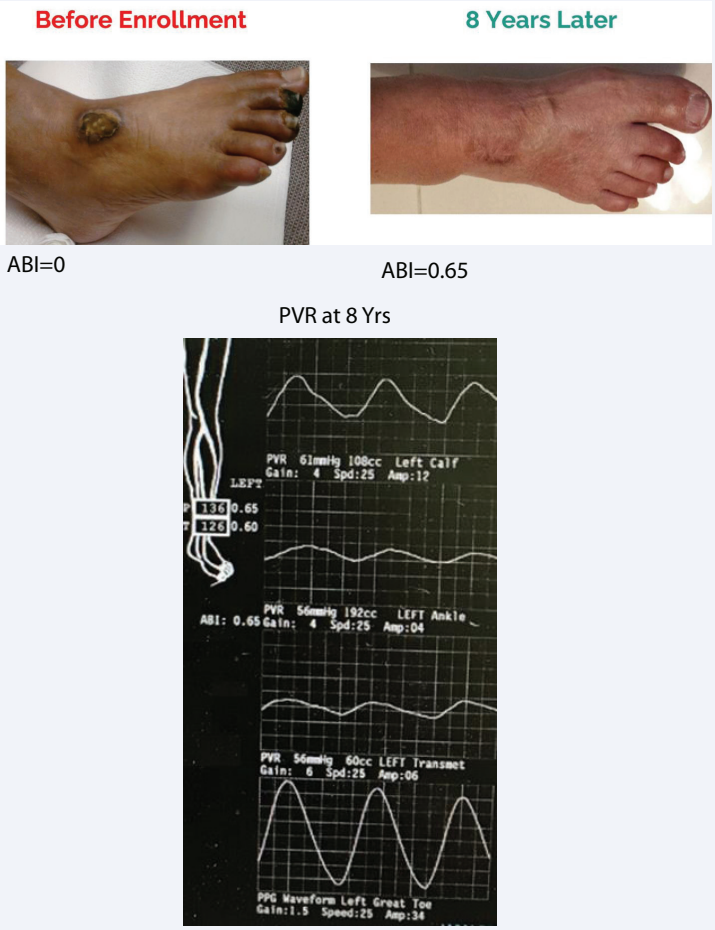


Figure 3A Patient 3: Note the gangrenous ulcer on the foot dorsum. This is where the dorsalis pedis artery was accessed to place a stent placement into the popliteal artery. Also note gangrene of the tips of toes 2 and 3. The ABI was 0 at that time. The next panel shows the healed wounds. The picture was taken 8 years after treatment; the ABI measured 0.65 at that time.

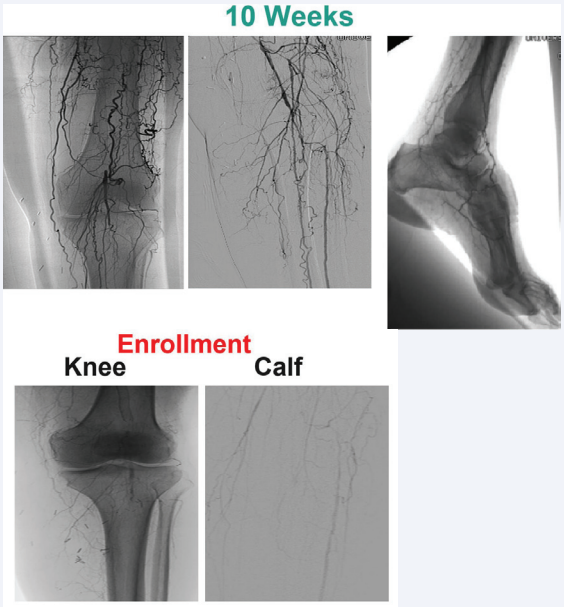


Figure 3B Patient 3: The top panels show the knee and upper calf with occluded stent and non-visualization of the popliteal and tibial arteries. Ten weeks after onset of our therapy repeat angiogram showed collateral arteries and pedal blood flow.

lymphocele formed and became infected, necessitating placement of a cryopreserved iliofemoral graft. This thrombosed several weeks later, leading to above-knee amputation (AKA). Patient died two years later.

Patient 5

Presentation: A 79-year-old Caucasian female was treated with ArtAssist and 5 doses of Filgrastim 600mcg (10.2 mcg/kg) injected SQ every 3 days over 14 days in lieu of BKA for severe ischemic forefoot rest pain after a second femoral tibial bypass graft occluded in her right leg. Right ABI was 0.16 and TBI was 0. The contralateral (non-symptomatic) limb DP and PT ABI were 0.8 and 0 respectively. The left toe TBI was 0.35. Angiogram (first image in Figures 4 A-C) showed occlusion of grafts and native femoral/popliteal and tibial arteries. To detect collateral flow (Second image in Figures 4 A-C), a power injector was used to inject full strength contrast with the vasodilator Nitroglycerine (NTG). The transient vasodilation briefly demonstrated the potential rich collateral network that may arise with arteriogenesis. The foot briefly turned red and warm, then reverted back to baseline in minutes.

Outcome: Rest pain resolved after 5 doses. At 6 months the right ABI had increased to 0.39. The contralateral (non-symptomatic) limb ABI rose to 1.06. The third image in Figures 4 A-C shows the maturation of the collateral network at 5 months after our treatment was initiated: no NTG was required to obtain these images. The third images in Figures 4A and B were obtained by antegrade hand injection of 10 ml Ten milliliters (ml) of half-strength contrast into a 4 French catheter positioned within the common femoral artery. Full-strength contrast (12 ml) was used to obtain the 3rd and 4th ankle images in Figure 4C. All of these images are firm evidence of collateral growth.

Nine months after starting the treatment she sustained a traumatic deep soft tissue injury to her right mid foot, heel, and ankle. She was thin (45 kg) with paucity of soft tissue ("skin

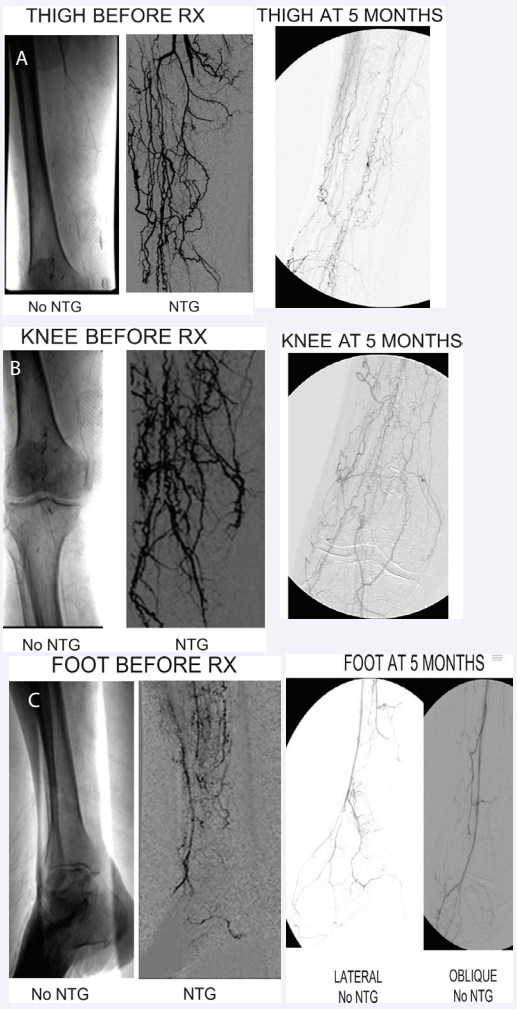


Figure 4A-C Patient 5: The top left image in each is a standard angiogram showing occlusion of the superficial femoral artery (4A), the popliteal artery (4B), and the tibial arteries (C). The top right images were obtained during a nitroglycerin (NTG) angiogram done with full strength contrast and a power injector. The NTG transiently dilated the collaterals to make them visible. The lower images were obtained five months later after hand injection of Angiovist antegrade into a 14 French catheter inserted in the femoral artery. Ten ml of 50% dilute contrast was used in the thigh(4A) and calf (4B), and full strength for the ankle and foot (4C). This is direct evidence of the desired collateral artery growth.

on bone") in this region. The tarsal bones and midfoot tendons were exposed. Urgent vascular bypass failed, followed by failed revision. She underwent BKA 286 days after initiating the therapy.

Patient 6

Presentation: 74-year-old Caucasian male was treated with ArtAssist and two 5 dose regimens of tbo-Filgrastim 900 mcg (Granix 10 mcg/kg) injected SQ every 3 days each over 14 days (separated by 1 week) in lieu of repeat revascularization for left forefoot rest pain and numbness causing inability to sleep. He had two recent failed left femoral Drug-Coated Balloon angioplasties. Prior vascular stents in both extremities were occluded. His left ABI was 0.3 and his left toe pressure was 0, with monophasic flattened toe photoplethysmographic (PPG) waveforms.

Additional history: Stage III renal insufficiency, abnormal fasting glucose, COPD. He quit tobacco 18 years earlier.

Outcome: Ischemic rest pain resolved within six weeks shortly after the last dose. At nine months he was able to ambulate half a mile. His left ABI was 0.6 at 1 year follow-up. His left toe pressure was 37 mmHg, with pulsatility in all 5 toes. He resumed playing golf until he developed right calf claudication at 300 feet two years later. He had been able to walk one mile prior to this without left calf claudication. He underwent a successful right iliofemoral endarterectomy and patch angioplasty. At 5 years he remains ambulatory at 0.25 miles, limited by right calf claudication only.

Patient 7

Presentation: 75 year old Caucasian male was treated with ArtAssist and 10 doses of Filgrastim 960mcg (10.9 mcg/kg) injected SQ every 3 days over 39 days (11 day delay while waiting for dose 6) in lieu of BKA amputation presented with wet gangrene of toes 1 and 2 (Figure 5A) after failed laser recanalization of the DP and anterior tibial arteries. Medial sclerosis and incompressible extremity arteries limited accuracy of hemodynamic measurements. Infected toes were amputated acutely, the wound thoroughly irrigated and debrided, and opposing tissue planes loosely approximated. Antibiotics were given IV. A week later the wound looked unhealthy and was reopened,debrided (Figure 5A), and therapy was initiated.

Additional History: IDDM, Stage III renal insufficiency, contralateral BKA. Ambulatory with a prosthesis.

Outcome: At 3 months DP Doppler waveforms became biphasic. PT Doppler waveforms also improved. The wound eventually healed. Cork-screw collaterals and improved contrast transit were observed (Figure 5B). Patient remained ambulatory with prosthesis on contra-lateral limb and was lost to follow-up at 3 years.

Patient 8

Presentation: 77-year-old Caucasian male was treated with ArtAssist and 5 doses of Filgrastim 600 mcg (7.9 mcg/kg) injected SQ every 3 days over 14 days in lieu of repeat attempt at bypass for ischemic forefoot rest pain and disabling claudication. A femoral popliteal bypass failed 3 years before; diffuse infrageniculate arterial occlusive disease was not conducive to revascularization. He was treated for 3 years with the ArtAssist device alone, during which ABI did not improve and he developed ischemic rest pain. Left DP and PT ABIs were 0.34 and 0.38 respectively; left great toe TBI was 0.2.

Outcome: After 5 doses the ischemic forefoot rest pain resolved. Ambulation distance increased over 6 weeks. At 6 months the left DP and PT ABI rose to 0.50 and 0.46 respectively; the left great toe TBI increased to 0.38 (Figure 8A). Follow up angiogram (Figure 8B) showed collaterals that mimicked a bypass graft. He remained ambulatory at last follow-up at 4 years.

Patient 9: Presentation: A 26-year-old Caucasian male was treated with ArtAssist and 10 doses of Filgrastim 960mcg (8.8mcg/kg) injected SQ every 3 days over 28 days in lieu of AKA for severe ischemic rest pain and short distance calf and thigh claudication as result of recurrent occlusion of multiple bypass grafts spanning the common iliac artery to the infrageniculate arterial circulation. The profunda artery was also occluded. ABI was 0. Angiogram showed multi-level arterial occlusive disease from the common iliac artery origin to the lower calf where the posterior tibial reconstituted and perfused the plantar artery.

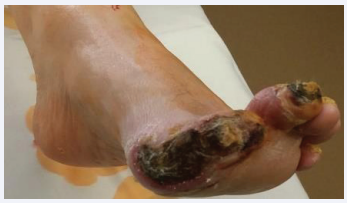
Additional History: Juvenile onset IDDM, obesity, and hypercoagulability.

Outcome: After one month, rest pain resolved, and patient could walk one block. Improvement continued after patient entered a weight loss program and exercised, reaching 4 blocks of pain free walking before onset of thigh and calf claudication. He was lost to follow-up at 4.5 years.

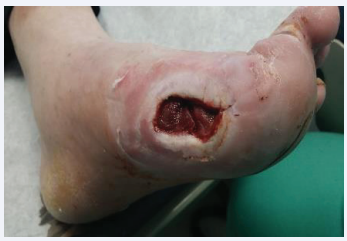
Patient 10: Presentation: A 63-year-old African American female was treated with ArtAssist and 7 doses of Filgrastim 600 mcg (6.6mcg/kg) injected SQ every 3 days over 21 days in lieu of AKA following emergent surgical ligation of the common femoral, profunda femoral, superficial femoral arteries for recurrent mycotic rupture, and explantation of a bypass graft. The goal of ArtAssist and 10 doses of Filgrastim 10 mcg/kg SC injected SQ every 3 days over 28 days was to heal a below knee amputation. The lower leg was gangrenous. The ArtAssist device was placed on the thigh.

Outcome: By week 2 warmth developed in the popliteal fossa. By week 3 warmth in the upper calf suggesting improved blood flow. A doppler was obtained in the popliteal artery at 2 weeks. A successful BKA was performed before the last 3 doses of Filgrastim were given.

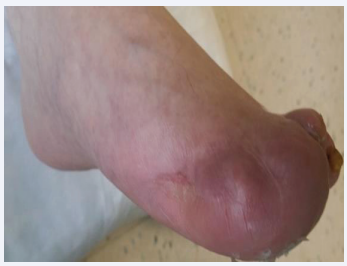
Patient 11: Presentation: A 70-year-old Caucasian male was treated with ArtAssist and 10 doses of Filgrastim 600mcg (7.0 mcg/kg) injected SQ every 3 days over 48 days (20 day delay waiting for the 6th dose) in lieu of BKA for right toe gangrene



Before Enrollment

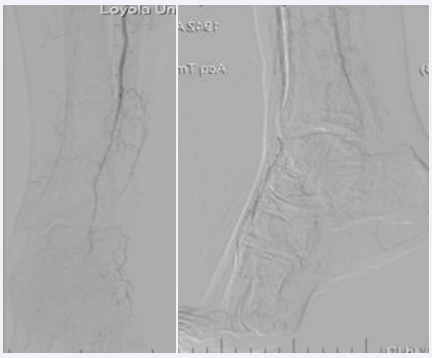


Enrollment

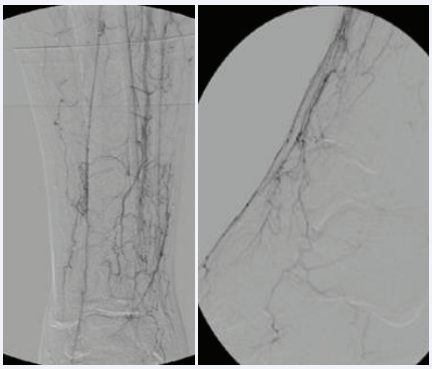


11 Months Later

Figure 5A Patient 7: The top image shows wet gangrene, which had to be surgically debrided prior to treatment, including amputation of toe 1 and 2. The Filgrastim and ArtAssist strategy was implemented when the loosely closed amputation incision was opened, debrided further, and packed with a biological dressing. Further toe amputations were needed until the forefoot finally healed.



Before treatment CALF to ANKLE FOOT



9 Months after treatment CALF to ANKLE FOOT

Figure 5B Patient 7: Angiogram before and 9 months after treatment. Note the posterior tibial artery and dorsalis pedis artery occlusion following laser angioplasty in the before treatment images. Also note the cork screw collateral growth on the 9 month after treatment images.

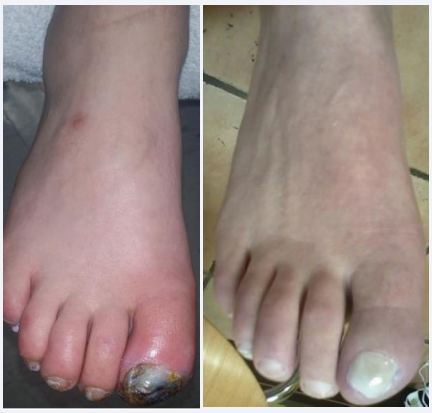


Figure 6A Patient 14: First image is gangrene of toe 1 and dependent rubor at conclusion of the 30 days of Filgrastim. The Art Asssist was continued for 9 months until the gangrene sloughed (Rght image)

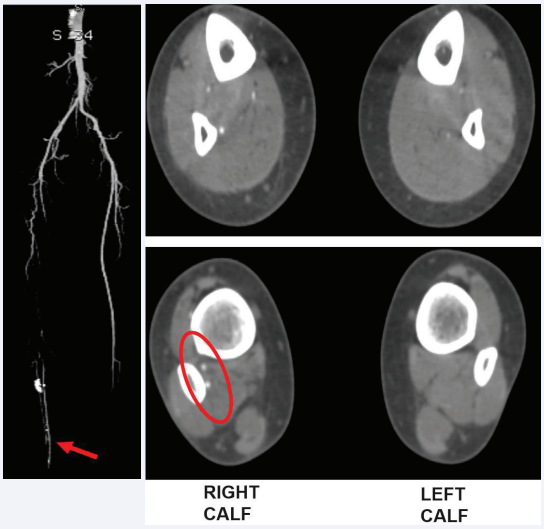


Figure 6B Patient 14: CT Angiogram showing the right superficial- and profunda- femoral and popliteal and tibial artery occlusions. The lateral artery in the groin is a profunda collateral artery, but this modality is not suitable to seeing the smaller collaterals. However they must be substantial in number for the contrast to reach the mid tibial artery on the right before the asymptomatic left side. The right panel shows 2 transverse sections through the calf highlighting the bright contrast in the right tibial arteries. These arteries were directly under the ArtAssist device. All three right tibial arteries are visible in the upper image, and the right anterior tibial and peroneal arteries are circled in the lower image

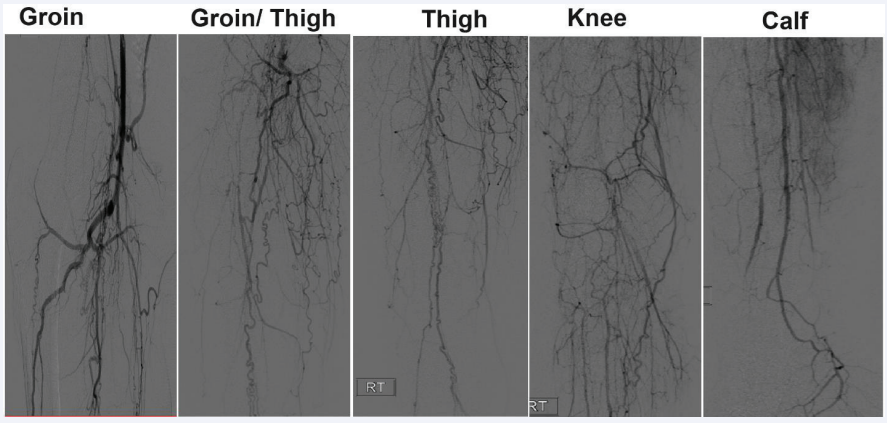


Figure 6C Patient 14: Aagiogram at 1 year showing the rich collateral network and segmental arterial recanalization

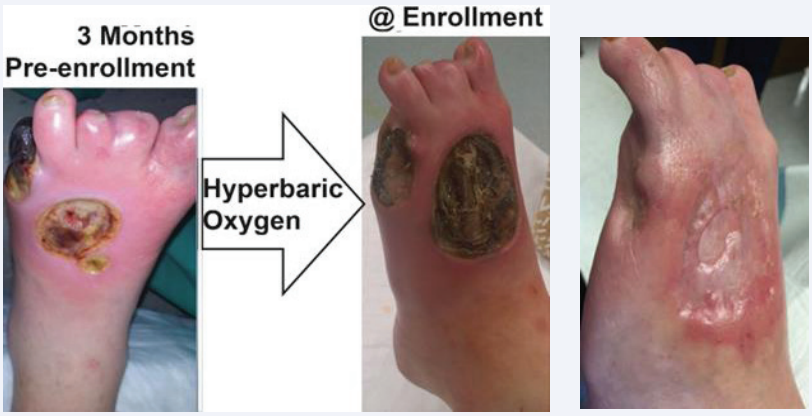


Figure 7A Patient 15: The top two images show the progression of foot necrosis despite wound care antibiotics and hyperbaric oxygen therapy. The fifth toe gangrene autoamputated. All devitablized tissue was removed anassiduous wound care followed. The lower panel image was obtained 18 months after the Filgrastim was given. The ArtAssist was continued daily until the wound healed. The healing phase could have been significantly accelerated if the patient allowed skin graft coverage of the granulation bed that completely covered the wound at 5 months

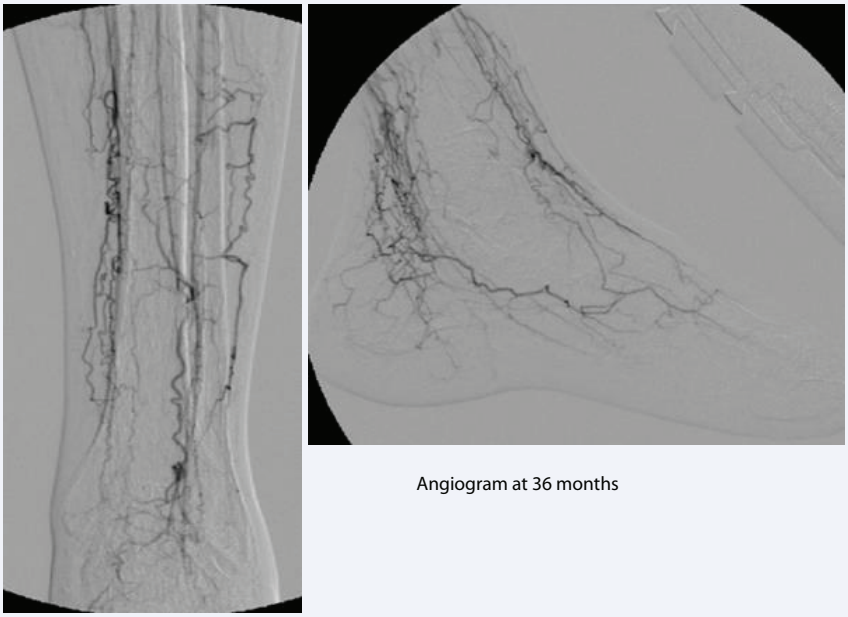


Figure 7B Patient 15: Follow up angiogram at 36 months showing arteriogenesis in the calf ankle and foot.

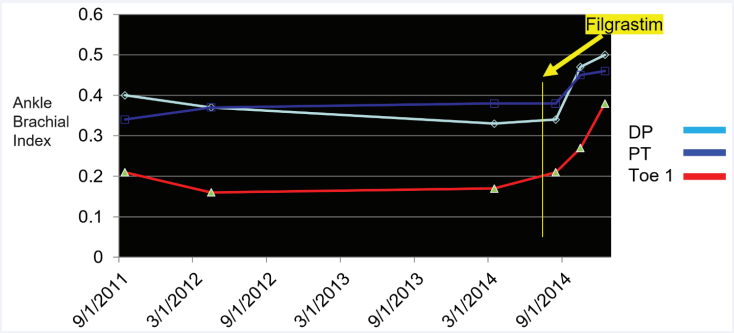


Figure 8A Patient 8: For the first 3 years this patient was treated with the ArtAssist alone, with no benefit to symptoms or impact on the ABI. However his symptoms improved and his ABI increased in 9/2014 shortly after Filgrastim was added to the regimen.

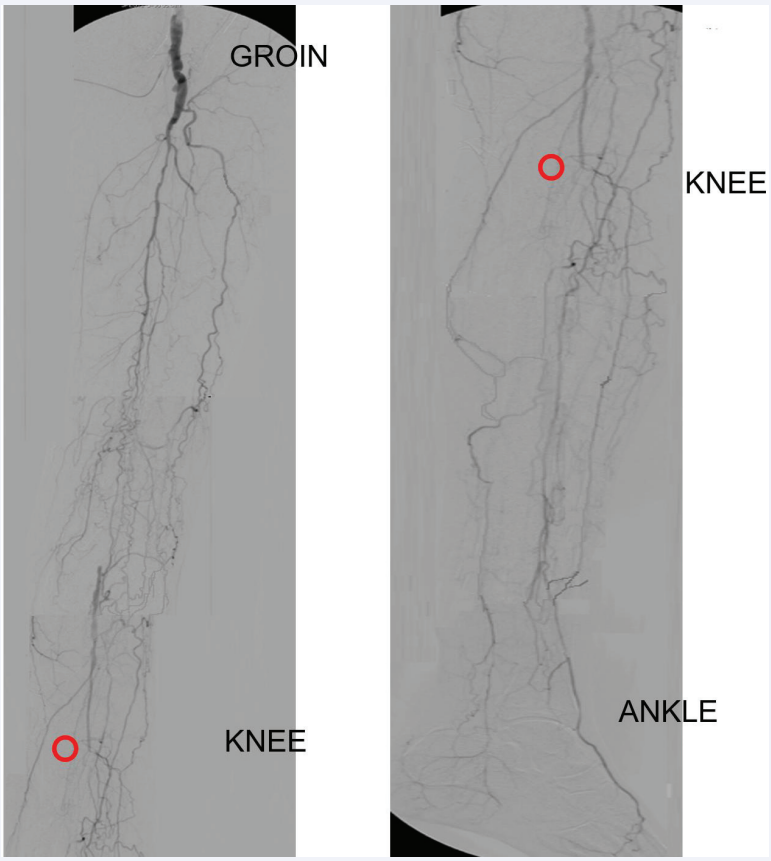


Figure 8B Patient 8: This angiogram shows the collateral growth from his groin to his foot. There is a circular marker at the bottom left of the first panel that matches up with the location in the top center of the right panel.

and a 2 month history of forefoot rest pain, toe cyanosis. Patient was unsuitable for invasive revascularization due to extensive evidence of pedal and distal tibial artery occlusive disease. He was referred after a negative hypercoaguable work-up by a hematologist-oncologist. He had deep venous thrombosis (DVT). The toe pathology was suggestive of blue toe syndrome though an embolic source was not apparent.

Additional History: DVT is a contraindication for ArtAssist use. A retrievable vena cava temporary filter was placed to protect from pulmonary embolism.

Outcome: Within 2 weeks, skin on the toes looked better perfused and rest pain improved. In the next 2 weeks, toes looked more ischemic, and patient reported no symptomatic benefit. On the day after the last filgrastim dose patient complained of feeling lethargic. CBC revealed a 2-point drop in hemoglobin compared to at the start of treatment. Stool guaiac was positive for occult blood. Upper endoscopy led to diagnosis of Stage 4 stomach adenocarcinoma. Patient was started on Folinic acid (leucovorin), Fluorouracil (5-FU), and Oxaliplatin (Eloxatin) but the tumor did not respond. He was then placed on Taxol and Cyramza (anti-angiogenic). Follow-up imaging showed complete remission (no detectable tumor). Meanwhile, the ischemia worsened in both limbs. Gangrene progressed to involve the whole right forefoot, necessitating BKA. The left limb recovered after chemotherapy stopped. He died one year later from brisk tumor recurrence.

Laboratory data: One day after the 5th Filgrastim dose the ELISA concentration of plasmin and Fibrin Degradation Products (FDP) increased by 4300% and 722% respectively compared to baseline. One day after the 10th dose the concentration of each were 6733% and 678% respectively higher than baseline. The gastric tumor may have bled because of this lytic state. VEGF165B was detected by ELISA after the 10th dose (52+13 pg/ml). This anti-angiogenic molecule may explain why after two weeks his ischemic status worsened.

Patient 12

Presentation: A 68-year-old African American female was treated with ArtAssist and 10 doses of Filgrastim 960 mcg (8.6mcg/kg) injected SQ every 3 days over 28 days in lieu of BKA for ischemic rest pain and short distance calf claudication after 2 failed femoral popliteal bypass grafts and significant infrageniculate soft tissue scarring from previous surgeries. ABI was 0.3; toe pressure 0.17. Dorsalis pedis waveform was monophasic.

Additional History: IDDM, obesity, unable to refrain from tobacco use.

G-CSF Treatment: Her initial 10 dose course was stopped

when it was discovered that the doses were not refrigerated. Lab values confirmed no change in her cell blood count after her 5th dose. A second course of 10 doses 960mcg every 3 days was given, starting 6 weeks after the first course.

Clinical Result: ABI increased to 0.68 and toe pressure increased to 0.43 seven weeks after concluding the 2nd course. Dorsalis pedis waveform became biphasic. Symptoms improved over 6 months, with claudication occurring at 2 blocks rather than at less than half a block. At 9 months the dorsum of her mid foot was impaled by a metal walker. The wound became infected. Blood flow was insufficient to prevent progressive necrosis. A BKA was performed nearly a year after her treatment.

Patient 13: Presentation: A 71-year-old African American male was treated with ArtAssist and 5 doses of Filgrastim 600 mcg (8.3mcg/kg) injected SQ every 3 days over 14 days in lieu of BKA for forefoot gangrene. Failure of two femoral to tibial artery bypasses, a laser recanalization, an atherectomy, and failed popliteal and tibial artery angioplasties were documented, He developed forefoot dry gangrene from recurrent injury. ABI was 0.28, too low to heal a TMA without our revascularization strategy.

Additional information: Tobacco addiction, altered sensorium from alcoholism, vascular dementia, non-compliance. Contralateral AKA after multiple revascularization attempts. Living in VA assisted living facility, he used his remaining leg to help get into a wheelchair.

Outcome: He was treated in in-patient rehab a day after his TMA with primary closure. After the 5th dose he was transferred to the VA nursing facility where he stopped using the ArtAssist device and became total care dependent. He resumed tobacco use. At 2 months, the ABI was increased to 0.53 and the TMA wound edges were observed to be healing slowly, but dry gangrene was evident. He used his foot to stabilize himself in the wheelchair, causing recurrent trauma to the TMA. There was insufficient blood flow to avert a BKA for TMA stump necrosis. In retrospect, the patient was a poor treatment candidate due to dementia, lack of compliance, and failure to thrive.

Patient 14

Presentation: A 36-year-old Caucasian female was treated with ArtAssist and 10 doses of Filgrastim 600mcg (9.4mcg/kg) injected SQ every 3 days over 28 days in lieu of AKA for profound ischemic rest pain and gangrene of toe 1 (Figure 6A). Intra-arterial thrombolysis of the superficial femoral, popliteal and proximal tibial arteries failed to successfully manage acute ischemia and led to profunda artery occlusion. An emergency thrombectomy and femoral popliteal bypass failed immediately, as did an emergency operative thrombectomy and femoral to tibial bypass. A detailed hypercoagulable work up was negative. No embolic source was found. Subsequent profundoplasty and 15cm vein patch angioplasty failed in the recovery room despite intraoperative completion duplex imaging showing normal profunda artery hemodynamics. History was positive for marijuana use, and acute vasculitis was believed to be responsible for the adverse clinical course. ABI was 0.2, and pedal waveforms were non-pulsatile. The ArtAssist device was used alone until her C-reactive-protein (CRP) and erythrocyte sediment rate (ESR) were confirmed to be normal.

Outcome: Patient improved progressively. Rest pain abated over the first 2 weeks. By six months her toe gangrene sloughed (Figure 6A). Her ambulation distance increased to over 3 blocks; she could walk up one flight of stairs. ABI increased to 0.58 at 1 year follow-up; TBI increased to 0.32. Toe pulsatility returned. CTA at 6 months (Figure 6B) showed flow into the right tibial arteries earlier than in the contralateral asymptomatic left leg, despite the profunda femoral and superficial femoral artery occlusion. This implies significant collateral growth. To optimally visualize these collaterals an angiogram was performed at 1 year and showed the rich network of corkscrew collaterals (Figure 6C).

Five years after treatment she presented with acute ischemia of the left upper arm shortly after resuming smoking tobacco and marijuana. Marijuana associated vasculitis was suspected. Catheter directed thrombolysis failed, during which time ischemia worsened. Two subsequent emergent revascularizations (bypasses) failed immediately. Catheter directed thrombolysis was re-attempted during which patient sustained a left cerebral infarct in the middle and posterior circulation distribution resulting in thromboembolic posterior cerebrovascular stroke. The left arm remained ischemic. Her fingertips developed dry gangrene. Her right upper and lower arms had flaccid paralysis. Intraventricular drainage was performed to prevent herniation. Metabolic brain activity was pharmacologically lowered (induced coma). MRI showed infarcts in brain tissue perfused by the middle and posterior circulation. Acute catheter directed intracerebral thrombectomy was attempted. Multiple sessions of hyperbaric oxygen therapy were provided. CT scan showed evolution of a cerebellar infarct. On advocacy of the father, 600 mcg filgrastim was again given SQ every 3 days for 14 days. She woke up 2 weeks later. She was transferred to the in-patient rehabilitation unit with flaccid paralysis in the right upper and lower limbs and left arm weakness and ischemia. Filgrastim 600 mcg filgrastim was again administered every 3 days for 28 days (3rd course), this time with the ArtAssist device placed on the left arm. The left arm ischemia improved. Fingertip gangrene sloughed eventually. Function returned in the right hand and leg. She now lives independently and has 4 out of 5 strength in the previously flaccid right upper and lower extremities. She has indistinct sensory deficit in both hands. Her left arm strength is normal. She has residual right peripheral visual field deficit and left hearing is 50%. She exercises for 2 hours each morning and does not use a walker, though her gait is unsteady at times and she limps. She does not have right calf claudication unless she walks fast or walks uphill. Her right lower and left upper extremities were salvaged following profound ischemic events 10 and 5 years ago respectively. Any role filgrastim has in her better-than-expected neurological outcome is hypothetical [13-15].

Patient 15

Presentation: A 40-year-old Caucasian female was treated with ArtAssist and 10 doses of Filgrastim 600mcg (9.4mcg/kg) injected SQ every 3 days over 28 days in lieu of BKA for progressive enlarging ischemic necrosis on the foot dorsum (Figure 7A), and severe ischemic rest pain. She had thromboangitis obliterans (Buerger's vasculitis) and was initially directed to stop smoking. She was managed in wound care clinic with antibiotics, debridement, and a course of hyperbaric oxygen. However, necrosis progressed (Figure 7A), Auto-amputation of the 5th toe occurred, leaving a gangrenous ulcer. Angiogram showed SFA and popliteal atherosclerosis and distal tibial and plantar arch arterial occlusive disease with diminutive cork-screw collaterals consistent with thromboangiitis obliterans. ABI was 0.7. Toe pressures were unmeasurable. Her CRP and ESR were normal when treated with the protocol.

Outcome: The dorsal foot ulceration and 5th toe amputation healed with assiduous wound care (Figure 7A). At 5 months healthy granulation covered the wound. She refused skin grafting. At 10 months her ABI was 1.13. By 18 months the wound healed completely. At 3 years angiogram (Figure 7B) showed collateral flow into the foot. No named arteries were visible from the distal third of the calf distally. Her limb has remained functional for 9 years as of this report.

DISCUSSION

A-Clinical course

Ischemic rest pain, coolness, and numbness typically begin to improve in the second week. Filgrastim period was 30 days. PCP was continued until rest pain resolved and wounds healed. The larger forefoot ischemic wounds required over 12 months to heal. There is a race between the rate NV and fibrinolysis occur and the rate of progression of the destructive effect of severe tissue ischemia. Whether filgrastim or a long-acting form of filgrastim (e.g. peg-filgrastim) should continue for more than a month will need further investigation. As with all vascular interventions, the earlier the intervention the better.

B- Blood Flow

Our hypothesis was that overcoming obstacles to innate neovascularization promotes improved blood flow. This was supported in 14 of the 15 patients. The one exception is patient 11 with the occult malignancy (discussed below). Our data refute the established CLTI dogma that invasive vascular intervention is needed to improve arterial hemodynamic measurements and restore pulsatility when none is present. In our study ABI increased 50% ($p = 0.004$, $N = 15$) from baseline. Angiographic evidence corroborates the hemodynamic data, as does the clinical data and the previously published molecular and cellular data [2]. Rapid contrast transit, higher contrast density, cork-screw collaterals, and segmental arterial recanalization were all evident on angiogram, and support therapeutic arteriogenesis.
Advanced CLTI is characterized by multi-level arterial occlusive disease which attenuates endothelial shear stress and the ischemia inhibits metabolic and cellular processes. Neovascularization is a natural response to arterial occlusive disease. Our combination therapy helps when the obstacles become severe, as in advanced CLTI, when progressive deterioration is evident.

C-Limb salvage

Improved blood flow is fundamental to achieving limb salvage. However, limb salvage was not the primary endpoint of this evaluation due to the spectrum of adjunctive requirements, some of which are difficult to control for. The latter include meticulous wound care, management of atherosclerotic risk factors (tobacco cessation, management of hyperlipidemia, diabetes, and hypertension), good nutrition, control of infection, avoidance of trauma, physical therapy and compliance. Greater than one year limb salvage was achieved in 9 patients (the longest being 13 years) who would otherwise have had definitive amputation. Of these, patient number 4 had an AKA at 5 years after a series of complications related to an old iliofemoral bypass graft. Until this he had stable blood flow and claudication at > 6 blocks.

The earliest BKA occurred in patient 11 during anti-angiogenic chemotherapy. Patient 1 had a BKA at 1 year due to post-surgical calcaneal osteomyelitis. Patients 5,12, and 13 had post traumatic BKA. These four BKAs illustrate the fact that improved blood flow is only part of limb salvage. All 4 complications were preventable. Patient 10's BKA was planned in the setting of a gangrenous distal calf and foot after acute ligation of the arteries in the thigh. If the blood flow had not increased, AKA would have been the preferred treatment.

D-Fibrinolysis

An association between filgrastim and fibrinolysis was previously reported [16-18]. but was never evaluated clinically. Filgrastim, has a half-life of 3.5 hours. Our measurements were a day after the Filgrastim dose. Whether the increased plasmin is due to the increase in neutrophils, or to a specific change in the thrombostatic milieu will need further investigation. Furthermore, confirmation of the plasmin effect will require measuring the plasma concentrations of plasmin-alpha 2 antiplasmin [19] and of tissue plasminogen activator- plasmin activator inhibitor complexes (tPA-PAI1) [20] . Also, any contribution to the protein concentrations from the local tissue response to subcutaneous injection will require future placebo comparison.

Spontaneous recanalization of chronic thrombus is rare in CLTI. Moreover, it is unlikely to be achieved with prolonged intravascular infusion of potent fibrinolytic agents without the risk of hemorrhage. An agent that can safely lyse chronic obstructive thrombus over a period of weeks at a physiologic level is a potentially transformative therapy. Thrombolysis likely accounts for a significant component of the increase in ABI. Improved flow would accelerate collateral growth downstream. Such a novel strategy would facilitate management of chronic ischemia not only in CLTI, but also in other vascular tissue beds (heart, brain, lung, kidney, etc.). Unlike in the leg, PCP would not

be necessary in central ischemic beds due to proximity to the force of the pumping heart.

E-Neovascularization

Activated human neutrophils release angiogenic proteins including VEGF-A, ANGPT1, CXCL8, HGF, and MMP-9, and form neutrophil extracellular traps (NETs) [12]. ANGPTs released by endothelial and peri-endothelial mural cells induce platelet-activating factor synthesis and neutrophil adhesion to endothelial cells. NETs directly exert proangiogenic activities in human endothelial cells. NETs induced by ANGPTs and PAF have a pro-angiogenic effect in vitro and in vivo. [12] Neutrophil elastase does not directly inhibit angiogenesis but is reported to disrupt vascular endothelial integrity and stabilization, which can affect angiogenesis [21]. This disruption affects the expression of angiopoietins, important to vascular stabilization. A specific neutrophil elastase inhibitor (sivelestat sodium) was administered after spinal cord injury upregulates angiopoietin-1 via the AKT pathway, preventing tight junction protein degradation. This suggests that inhibiting neutrophil elastase can promote vascular stabilization and potentially enhance angiogenesis [21].

F- Filgrastim and VEGF 165b

The VEGF 165b isoform inhibits VEGFR2 signaling by inducing differential phosphorylation and has been reported to block angiogenesis in vivo [22]. A monoclonal isoform-specific antibody against VEGF 165b was recently reported to promote nitric oxide independent therapeutic angiogenesis in a preclinical ischemia model [23]. To assess the effect of Filgrastim on VEGF-165b expression in CLTI, its ELISA serum concentration was measured on Day 0, and then one day after the 5th and 10th doses in 8 CLTI patients. It was not detected in the 8 patients at baseline, nor after the 5th dose. In only one patient, Patient 11, was it detected after the 10th dose (52+13 pg/ml). The required hematology/oncology screens for hypercoaguability and for neoplasm prior to management of CLTI with Filgrastim were insufficient to make the oncologic diagnosis (exclusion criterion). The Filgrastim fibrinolytic effect may have contributed to patient 11's melena, which led to detection of this virulent tumor. Whether VEGF165B expression by the tumor was induced by Filgrastim, and contributed to the recurrence of ischemia is plausible.

Filgrastim has been used in the oncologic population for decades, and has not been associated with promoting tumor growth, despite the pro-neovascularization and pro-fibrinolysis properties we observed. Whether Filgrastim induction of VEGF-165b tumor expression is a reason for this requires further investigation.

G-Filgrastim Resistance

Stem cell mobilization with G-CSF is not effective in 15%–20% of patients, particularly in diabetics [24]. Diabetics are also prone to accelerated atherosclerosis leading to CLTI. The concentration of proteins associated with fibrinolysis and NV in patients with this G-CSF resistance is yet to be delineated. Our patient cohort was too small to accurately observe differential influence of diabetes [25]. Plerixafor (Mozobil, Genzyme) binds to CXCR4 and blocks the binding of its cognate ligand CXCL12 (Stroma-derived factor 1 alpha) [26]. The combination of G- CSF with plerixafor showed promise in overcoming ineffective hematopoietic stem cell mobilization and may be a solution to this problem if it arises in CLTI.

H-Role after resolution of Vasculitis

Vasculitis in Patients 14 and 15 led to CLTI. They had dramatic resolution of their ischemic symptoms (amputation free survival at 9 years) despite tissue loss and progression prior to treatment. Both were treated after smoking cessation and vasculitis resolution. Whether our findings related to neovascularization and fibrinolysis by Filgrastim can be used to manage pulmonary hypertension following cessation of the vasculitis from COVID (long COVID) needs to be ascertained.

I- Immunologic Deficit and Wound Healing

Filgrastim amplifies cellular immunity after cytotoxic chemotherapy. Diabetes impairs the immune system. In a 2013 Cochrane review of diabetic foot infections (not CLTI), Filgrastim was reported to reduce the need for surgical interventions, especially amputations, as well as the duration of hospitalization [27]. The role of Filgrastim in assisting in the management of infection in CLTI wounds will need further investigation at the novel dosimetry we used.

J-Future Investigation

With these favorable preliminary data, a controlled clinical trial is indicated. Previous trials of Filgrastim in the leg and in other circulatory beds (e.g., coronary) will need to be revisited at a dosimetry that capitalizes on both fibrinolysis and NV. In the lower extremity, increasing endothelial shear stress (for example with a PCP) to activate the endothelium and initiate arteriogenesis is important to overcome the hemodynamic obstacles to NV. Our reported observation [2] that the serum concentration of MCP-1 is decreased one day after Filgrastim, but returns to baseline after two hours of PCP illustrates the utility of the ArtAssist device remote from the heart in the extremities.

CONCLUSION

This is the first clinical report of a cell therapy for vascular disease that documents increased blood flow hemodynamically and angiographically with a strategy that overcomes specific obstacles to neovascularization. It is supported by our report of the proteomic and cytometry data [2].

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