Received: 18 March 2021 | Revised: 17 December 2021 | Accepted: 11 January 2022

DOI: 10.1002/term.3284

WILEY

CLINICAL CASE STUDY

Filgrastim, fibrinolysis, and neovascularization

Darwin Eton[1] | Guolin Zhou[2] | Tong-Chuan He[3] | Amelia Bartholomew[4] | Rachana Patil[5]

[1]Department of Surgery, University of Illinois Chicago, Chicago, Illinois, USA

[2]GCIS, University of Chicago, Chicago, Illinois, USA

[3]Department of Orthopedic Surgery and Rehabilitation Medicine, University of Chicago, Chicago, Illinois, USA

[4]Department of Surgery, College of Medicine Research, University of Illinois Chicago, Chicago, Illinois, USA

[5]Department of Pediatrics, Division of Stem Cell Transplant and Regenerative Medicine, Stanford University, Palo Alto, California, USA

Correspondence
Darwin Eton, Department of Surgery, University of Illinois Chicago, 2550 N Lakeview Ave, Suite N303, Chicago, IL 60614, USA.
Email: Arteriogenesis2020@gmail.com

Funding information
Warren H. and Clara Cole Foundation; Cures Within Reach

[Correction added on 15 March 2021, after first online publication: Some minor typographical errors were corrected.]

Abstract

Segmental recanalization of chronically occluded arteries was observed in patients with chronic limb-threatening ischemia (CLTI) treated with Filgrastim, a granulocyte colony stimulating factor, every 72 h for up to a month, and an infra-geniculate programmed compression pump (PCP) for 3 h daily. Molecular evidence for fibrinolysis and neovascularization was sought. CLTI patients were treated with PCP alone ($N = 19$), or with Filgrastim and PCP ($N = 8$ and $N = 6$, at two institutions). Enzyme-Linked Immunosorbent Assay was used to measure the plasma concentration of plasmin and of fibrin degradation products (FDP), and the serum concentration of proteins associated with neovascularization. In the PCP-alone group, blood was sampled on Day 1 (baseline) and after 30 days of daily PCP. In the Filgrastim and PCP group, blood was drawn on Day 1, and 1 day after the 5th and the 10th Filgrastim doses. Each blood draw occurred before and after 2 h of supervised PCP. Significant ($p < 0.01$) PCP independent increases in the plasma concentration of plasmin (>10-fold) and FDP (>5-fold) were observed 1 day after both the 5th and the 10th Filgrastim doses, compared to Day 1. Significant ($p < 0.05$) increases in the concentration of pro-angiogenic proteins (e.g., HGF, MMP-9, VEGF A) were also observed. Filgrastim at this novel dosimetry induced fibrinolysis without causing acute hemorrhage, in addition to inducing a pro-angiogenic milieu conducive to NV. Further clinical testing is warranted at this novel dosimetry in CLTI, as well as in other chronically ischemic tissue beds.

Trial registration. https://clinicaltrials.gov/ct2/show/NCT02802852.

KEYWORDS
angiogenesis, arteriogenesis, chronic limb ischemia, fibrinolysis, Filgrastim, neovascularization

1 | INTRODUCTION

In the presence of arterial occlusive disease, neovascularization (NV) is a natural process that strives to preserve tissue perfusion through growth of collateral arteries (arteriogenesis) and growth of capillaries, arterioles, and venules (angiogenesis; Heil et al., 2006). Endothelial shear stress initiates arteriogenesis, while hypoxia stimulates angiogenesis (Carmeliet, 2000; Wahlberg, 2003).

NV becomes impaired as vascular disease progresses, leading to chronic limb-threatening ischemia (CLTI). Symptoms include forefoot

ischemic rest pain, ulceration, and/or gangrene. Standard of care includes invasive revascularization by surgery or catheter-based interventions. Significant procedural risks, variable durability (Almasri et al., 2018; Conte et al., 2019; Robinson et al., 2018), and high cost (Forbes et al., 2010) have spurred efforts to develop an effective, durable, low-risk, home-based, inexpensive revascularization option.

Safely promoting NV offers the promise of durable revascularization. Phase II clinical trials (Qadura et al., 2018) attempting to orchestrate cellular or molecular aspects of NV have not yielded a solution to promoting this complex process.

A novel approach has been tested clinically that seeks to restore innate NV by overcoming obstacles that arise due to the multilevel arterial occlusive disease and progenitor cell deficits (Fadini et al., 2005) characteristic of CLTI (Table 1). This outpatient cell therapy uses a programmed pneumatic compression pump (PCP) and Filgrastim (FDA approved for stem cell mobilization). In the first patients so treated, there was abatement of ischemic rest pain and healing of forefoot ischemic wounds (Eton, 2012; Eton & Yu, 2010). Hemodynamics improved (increase in ankle brachial index, development of photoplethysmographic pulsatility in the forefoot and toes). Angiography showed corkscrew collateral growth and improved contrast transit. Surprisingly, segmental recanalization of previously occluded infrageniculate arteries (Figures 1 and 2) was also observed. Lysis of chronic thrombus was suspected. This study reports the

TABLE 1 Rationale for a novel neovascularization strategy [Colour figure can be viewed at wileyonlinelibrary.com]

Restoring Neovascularization in Chronic Limb-Threatening Ischemia			
8 Obstacles	**Problems**	**2 Solutions**	**Rational**
Multi-level arterial occlusion	Decreases Endothelial Shear Stress Required to initiate Arteriogenesis	**Cyclic External Calf and Foot Compression (PCP)**	Arteriogenesis is initiated when shear stress from PCP activates the endothelium (increases NOS activity, MCP-1 expression, surface adhesion molecules)
	Diminished oygenated nutritive blood flow impairs oxidative phosphorylation and increases glycolysis		PCP drives in oxygenated nutitive blood flow, improving cellular protein function
	Diminished clearance of toxic metabolic by-products increases acidosis leading to protein/enzyme conformational change and impaired synthesis		PCP helps drive out the toxic metabolic by-products, improving cellular function
	"Distress" proteins produced in response to ischemia are ineffectively delivered into the circulation		PCP helps transport these signals out of the ischemic tissue to recruit salutory progenitor cells from the remote bone marrow niches
	Diminished arrival of monocytes and mobilized progenitor cells back to the ischemic tissue		PCP drives these pro-angiogenic cells back to the ischemic tissue
Circulating Progenitor cell deficit	Decreased number and function of these cells in CLTI and diabetes	**Filgrastim**	Filgrastim hyper mobilizes these cells into the circulation from their bone marrow niches
Chronic Thrombus	Amplifies the hemodynamic failure		Filgrastim promotes physiologic fibrinolysis systemically, re-establishing flow in occluded vessels of all sizes
Sub optimal angiogenic environment	Vessel assembly is impaired		Filgrastim increases the concentration of pro-angiogenic proteins (HGF, VEGF-A, MMP-9...)

Note: Neovascularization is a natural response to arterial occlusive disease. CLTI arises when neovascularization fails. Our strategy (Column 3) was developed to overcome the obstacles listed in this table. The goal of this strategy is to help restore this natural process.

Abbreviations: CLTI, chronic limb-threatening ischemia; HGF, Hepatocyte Growth Factor; MCP-1, Monocyte Chemoattractant Protein; MMP-9, Matrix Metalloproteinase-9; NOS, nitric oxide synthase; PCP, programmed compression pump; VEGF-A, Vascular Endothelial Growth Factor A.

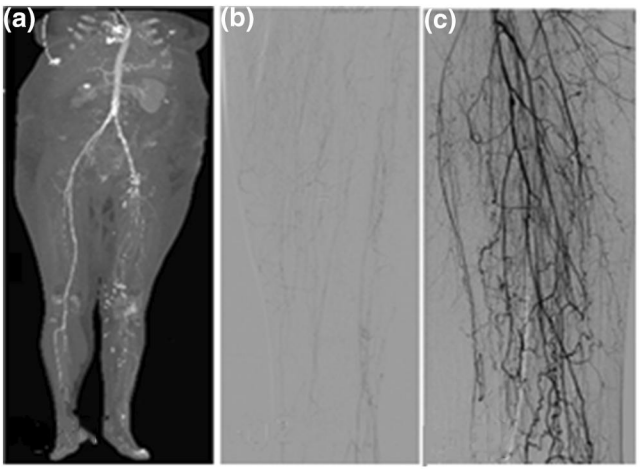


FIGURE 1 (a) CT angiogram of an ischemic left leg in a diabetic lady after two failed bypasses and 5-cm ulcers on anterior and posterior ankle. The bright signals in the left leg are surgical hemoclips. (b) The pre-treatment catheter angiogram showing attenuated left infrageniculate flow. (c) An angiogram 1 year after treatment showing recanalization of tibial arteries and cork-screw collaterals expected with neovascularization. Her foot wounds healed. She still has her limb 12 years later

impact of this therapy on plasma and serum levels of proteins associated with fibrinolysis and with NV measured using Enzyme Linked Immunosorbent Assay (ELISA) on CLTI patients treated at two independent institutions.

2 | METHODS

2.1 | Population

CLTI patients who were not candidates for, or had failed, previous invasive revascularization procedures.

2.2 | Inclusion criteria

Patients complaining of ischemic forefoot rest pain, gangrene, and/or ischemic ulceration were enrolled if the ankle brachial arterial index was <0.45.

2.3 | Exclusion criteria

Acute limb ischemia, non-salvageable extremity, untreated hypercoagulable disorder, sickle cell disease, myeloproliferative disorder, dialysis, creatinine above 3.5 mg/dl, active cancer, dementia, noncompliance, intolerance of PCP, body mass index over 34, venous stasis ulcer, history of lymphoma or leukemia, uncorrected symptomatic coronary artery disease, severe carotid stenosis, sepsis proximal to the forefoot, allergy to Filgrastim.

2.4 | Patient groups

Table 2 lists the patient characteristics. Blood assays were performed on two patient groups. The first group was treated at University of Chicago (UC, 2012–2013) with PCP alone ($N = 19$). The second group ($N = 14$) was treated with PCP and Filgrastim: six at University of Illinois at Chicago (UIC, 2016–2019) and eight at Weiss Memorial Hospital (2014–2015).

2.5 | Filgrastim

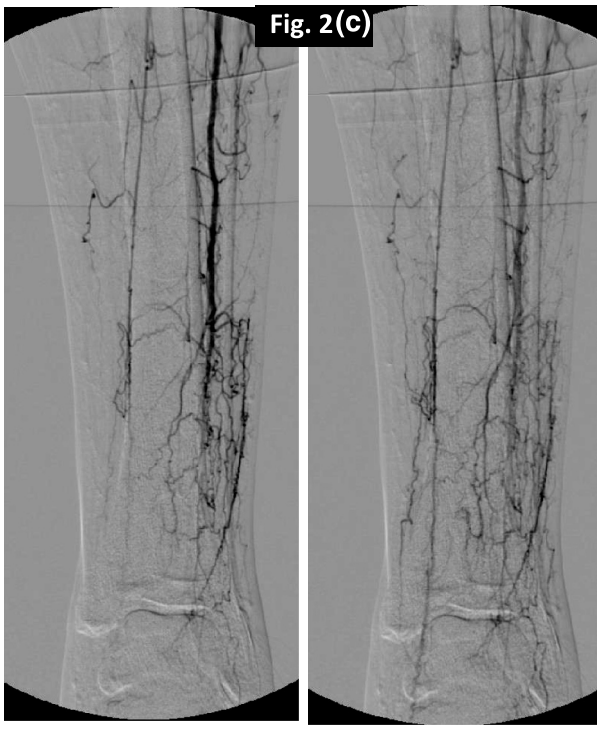
Each patient was to receive Filgrastim subcutaneous injections, one every 72 h at approximately 10 mcg/kg for up to 10 doses. Filgrastim (Neupogen) was purchased from Amgen Inc. in 480- and 300-mcg vials. These were kept refrigerated between 2 and 8°C and stored in the dark. Small losses of agent occurred during administration, which typically required two separate injections in the subcutaneous tissue in the lower abdomen. Fourteen patients received five doses. Ten of the 14 received all 10 doses. Mobilized progenitor cells circulated until either being engrafted or otherwise cleared from the circulation. The leukocyte count returned to normal approximately every 72 h. The product label for Filgrastim lists progenitor cell mobilization as an indication (not CLTI). The FDA granted a waiver to use Filgrastim at the novel dosimetry in CLTI patients at both institutions. Filgrastim has been safely used in healthy patients for the purpose of bone marrow harvest of mobilized progenitor cells (Shaw et al., 2015). Oncologists have nearly 3 decades of experience with Filgrastim. Initial concerns over promoting tumor angiogenesis were not substantiated (Okazaki et al., 2006). While severe complications are uncommon, they can be significant (e.g., capillary leak syndrome, myocardial infarction, and splenic rupture). Hemorrhage is not a reported side effect; fibrinolysis and NV are not listed on the Filgrastim label.

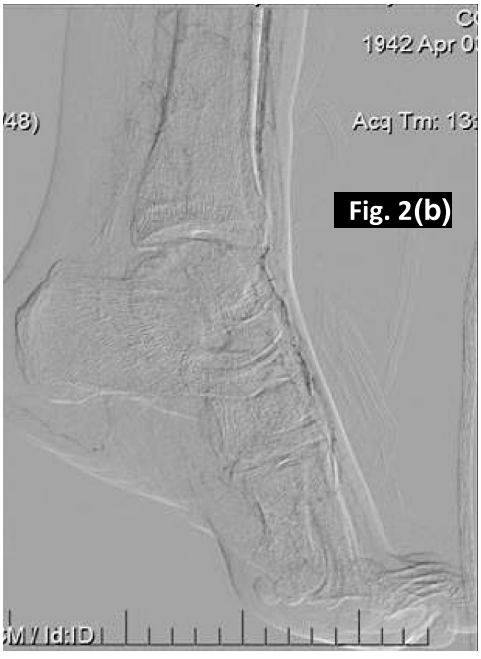
2.6 | PCP

The ArtAssist device (ACI Medical LLC) applies sequential pressure to the calf and foot. PCP was used at home on both legs in the seated position for 3 h daily. Rapid inflation of pneumatic cuffs (0–120 mmHg in <0.3 s) provides an endothelial shear stress stimulus, while at the same time driving in oxygenated nutritive blood flow and facilitating venous return (Table 1). The pressure is held in each of the cuffs for 3 seconds. Rapid deflation follows. Three cycles occur per minute.

2.7 | Phlebotomy

Group 1 patients were treated with PCP alone, and had blood drawn on Day 1 and Day 30. Group 2 patients were treated with Filgrastim and PCP and had three blood samples drawn: on Day 1, on the day





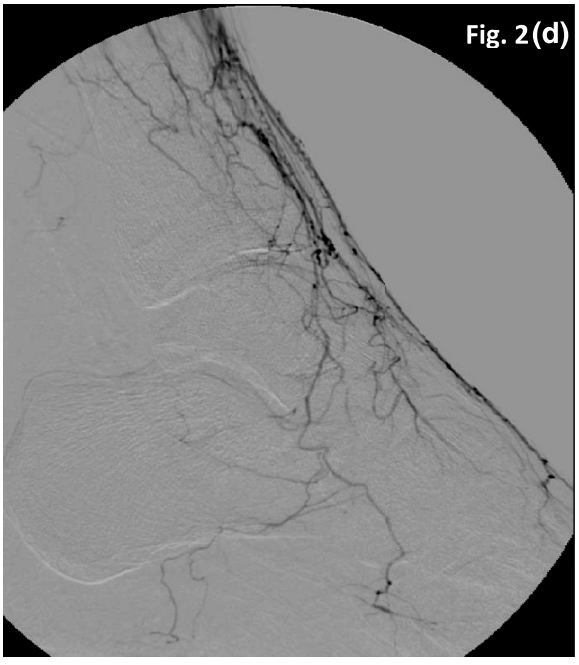


FIGURE 2 75-year-old insulin-dependent diabetic male (Patient 1403) was initially treated with an infrageniculate bypass for dry gangrene. When this failed, he was treated with atherectomy and laser angioplasty of the anterior tibial and dorsalis pedis artery. When this failed, he presented with wet gangrene of toes 1, 2, and 3. Following open amputation, he was treated with G-CSF and PCP in lieu of below knee amputation. (a,b) Pre-treatment distal calf and foot angiogram. (c,d) Operative angiogram with hand injection of contrast through 4F catheter in femoral artery showing recanalization and neovascularization at 9 months after onset of therapy. Patient healed his open toe amputations

after the 5th Filgrastim dose, and on the day after the 10th Filgrastim dose. In both groups, on each day, blood was drawn using a 21-gauge butterfly inserted before and again after 2 h of observed PCP. Blood was collected into serum separator and EDTA plasma separation tubes. Specimens were immediately transported on ice to the independent laboratories at UIC or UC.

2.8 | Serum separation

Whole blood was collected in serum separator tubes. Following clot formation (30–60 min), each tube was centrifuged for 15 min at 1000*g*. Serum aliquots were stored at −80°C in labeled cryovials (to avoid repetitive freeze/thaw) until batch analysis.

TABLE 2 Patient demographics

		Patients who had assays		
	PCP alone	PCP alone	PCP + Filgrastim	
Institution	UC	UC	Weiss	UIC
N	62	19	8	6
Mean age	70 (5)	69 (5)	71 (5)	68 (6)
Male	68%	63%	63%	67%
Hypertension	97%	79%	88%	83%
Hyperlipidemia	74%	53%	75%	67%
Body mass index ≥25 and <34	55%	58%	75%	83%
Congestive heart failure	19%	11%	0%	0%
Myocardial infarction	26%	16%	13%	17%
Stroke	26%	26%	25%	17%
Renal insufficiency	37%	16%	38%	33%
On dialysis	26%	0%	0%	0%
Creatinine	2 (0.9)	1.2 (0.3)	1.3 (0.3)	1.2 (0.4)
Diabetes	66%	53%	63%	50%
Insulin-dependent	44%	32%	25%	17%
Non-insulin dependent	23%	21%	38%	33%
Hemoglobin A1c median	7 (1.4)	7.1 (1.2)	6.9 (1.1)	6.9 (1.2)
Smoking				
Resumed	8%	16%	25%	17%
Former	76%	53%	63%	83%
Medications				
Statin	74%	63%	88%	83%
Aspirin	79%	74%	88%	100%
Angiotensin-receptor blockers	18%	21%	25%	33%
ACE inhibitors	45%	47%	50%	50%
Clopidogrel	44%	37%	38%	33%
Cilostazol	13%	32%	25%	33%
Warfarin	24%	26%	13%	0%
Beta-blockers	68%	53%	75%	50%
Ischemic rest pain				
No ischemic ulcer or gangrene	24%	26%	13%	33%
Ischemic ulcer or gangrene	76%	64%	88%	67%
Ankle brachial index	0.49 ± 0.1	0.4 ± 0.1	0.34 ± 0.1	0.34 ± 0.1

Note: Column 1 is a 62-patient cohort treated with PCP alone at UC and does not include the 14 patients treated with PCP and Filgrastim in this study. Columns 2–4 represent the patients in whom the assays were performed: PCP alone ($N = 19$), PCP and Filgrastim ($N = 14$). Numbers in parentheses are standard deviation from the mean.

Abbreviation: PCP, programmed compression pump.

2.9 | Plasma separation

It was used for preparation of samples for ELISA, and for cytometry. Whole blood was collected in EDTA-treated tubes. Red cells and platelets were removed after centrifugation at 2000*g* for 20 min at 4° C. Plasma aliquots were stored at −80°C.

2.10 | Enzyme-linked immunosorbent assay

Protein concentrations were measured in duplicate in plasma or in serum using Human ELISA Kits according to each kit's instructions (Table 3). **Plasmin** and fibrin degradation products **(FDP)** were measured in plasma at UIC and in serum at UC to assess fibrinolysis. Proteins associated with NV included **HGF** (Kaga et al., 2012), **VEGF-A** (Nagy et al., 2002), **MMP-9** (Wang & Khalil, 2018), **Angipoietin**-1 (Koh, 2013), **PDGF-AA**, **PDGF-BB**, **PDGF-AB** (Hellberg et al., 2010), **TNF-α** (Leibovich et al., 1987), **MCP**-1 (Shyy et al., 1994) and **TGF-β** (Goumans et al., 2009), **PLGF** (De Falco, 2012), **IL-6** (Villar-Fincheira et al., 2021), and **IGF-1** (Lin et al., 2017). All patient-specific samples were analyzed together using a single ELISA kit.

2.11 | Cytometry

Mature endothelial **CD31+** (PECAM-1), progenitor **CD34+**, and endothelial progenitor **CD309+** (**VEGFR2+**) cell populations were measured (Table 3). White blood cell (WBC) and differential counts were obtained on plasma in the hospital hematology laboratories.

2.12 | Serum nitrite

Serum nitrite, a breakdown product of nitric oxide (NO), reflects nitric oxide synthase (NOS) activity. It was measured before and after 2 h of supervised PCP with a quantitative fluorometric assay based on the reaction of nitrite with 2,3-diaminonaphthalene under acidic conditions to form fluorescent 1-(*H*)-naphthotriazole (Table 3).

2.13 | Statistics

Each patient had two internal controls. The first internal control was obtained on Day 1, prior to PCP or Filgrastim. Changes resulting from daily PCP use for 30 days (Group 1) or caused by Filgrastim (Group 2) were referenced to data from Day 1. The second control was obtained on arrival (at $T = 0$) to each 2 h observed PCP session, and was used to ascertain the effect of PCP (at $T = 2$ h) on Day 1 for all patients, on Day 30 for the PCP alone group, and on the day after the 5th and the 10th doses of Filgrastim in the Filgrastim + PCP group. Percent changes are reported relative to each of these two controls (change from control divided by the control). The paired Student *t*-test was used to estimate a *p* value directly from the ELISA data (not from the percent changes). An unpaired *t*-test was used when comparing the aggregated ELISA data in all patients obtained before any Filgrastim exposure to all data obtained after Filgrastim was given. *p* Values <0.05 are considered significant. The significant findings in the UC study led to the confirmatory laboratory study at UIC in six CLTI patients. The UIC sample size was calculated from the UC data to provide 80% power to detect changes in protein levels of 0.66 standard deviations assuming a two-sided significance level of 0.05.

TABLE 3 Assays and equipment

ELISA Readers: at UIC: Synergy HT, at UC: EL800, both from BioTek (Winooski, VT)
ELISA kits:
Fibrinolysis assays: From MyBioSource Company (San Diego, CA):
Plasmin: MBS026652
Fibrin degradation products (**FDP**)**:** MBS032783
NV assays: From R&D systems Inc. (Minneapolis, MN):
Vascular endothelial growth factor A (**VEGF-A**): Catalog No.DVF00
Human placenta growth factor (**PLGF**): DPG00
Platelet-derived growth factor: **PDGF-AA:** DAA00 B, **PDGF-BB:** DBB00**, PDGF-AB**: DHA00 C
Angiopoietin 1: DANG10
Hepatocyte growth factor (**HGF**): DHG00
Matrix Metalloproteinase-9 (**MMP-9**): DMP900
Human tumor necrosis factor alpha (**TNF-α**)**:** DTA00 C
Transforming growth factor beta 1 (**TGFβ**-1): DB100 B
Interleukin-6 (**IL-6**): D6050
Insulin-like growth Factor-1 (**IGF-1**):DG100
Monocyte chemotactic protein 1 (**MCP-1**)**:** DCP00
Cytometer/FACS
BD LSRII cytometer (Becton Dickinson, Franklin Lakes, NJ) at UC
BD FACS Canto II (No.V96300101) at UIC
Reagents for cytometry:
Human CD31 FITC WM59 Becton Dickinson (BD)
Ms IgG1 Kappa ItCl FITC MOPC-21 from BD
FCM lysing solution from BD
FITC anti-human CD34+ ab from ABCAM (Cambridge, MA)
Anti-VEGFR2-Biotin from ABCAM
FITC Mouse IgG1 k Isotype control ab from ABCAM
Mouse IgG1 (biotin)-Isotype control ab from ABCAM
DYLIGHT549 labeled Strptavidin from KPL (Milford, MA)
Serum nitrite
NanoDrop 3300 Microvolume UV-Vis Spectrophotometer from Thermo Scientific (Waltham, MA)
Reagent: 2,3- diaminonaphthalene (DAN; Aldrich, Milwaukee, WI)

3 | RESULTS

Abbreviations are listed in Table 3. Brackets "[ ]" = "concentration of"; for example,: [FDP] = concentration of FDP.

3.1 | Filgrastim and PCP

3.1.1 | Fibrinolysis

ELISA was used to measure the [plasmin] and [FDP] in plasma (Figures 3a and 4a), and in serum (Figures 3b and 4b). Compared to Day 1 (prior to Filgrastim), [plasmin] increased 1600% ± 2603% ($p < 0.002$) and [FDP] increased 554% ± 475% ($p = 0.006$) when each was measured a day after the fifth dose of Filgrastim. After the 10th dose of Filgrastim, [plasmin] increased 2231% ± 2913% ($p < 0.0004$) and [FDP] increased 555% ± 442% ($p = 0.004$) compared to Day 1. These data were PCP independent (Tables A1 and A2).

Patients P008, 1403, and 1406 each had elevated plasmin and FDP on Day 1. Each had recent thrombotic complications, suggesting endogenous activation of the fibrinolytic system. Figures 3 and 4 show the minimal change in these three patients relative to their high baselines on the day after the 5th and 10th Filgrastim doses. The

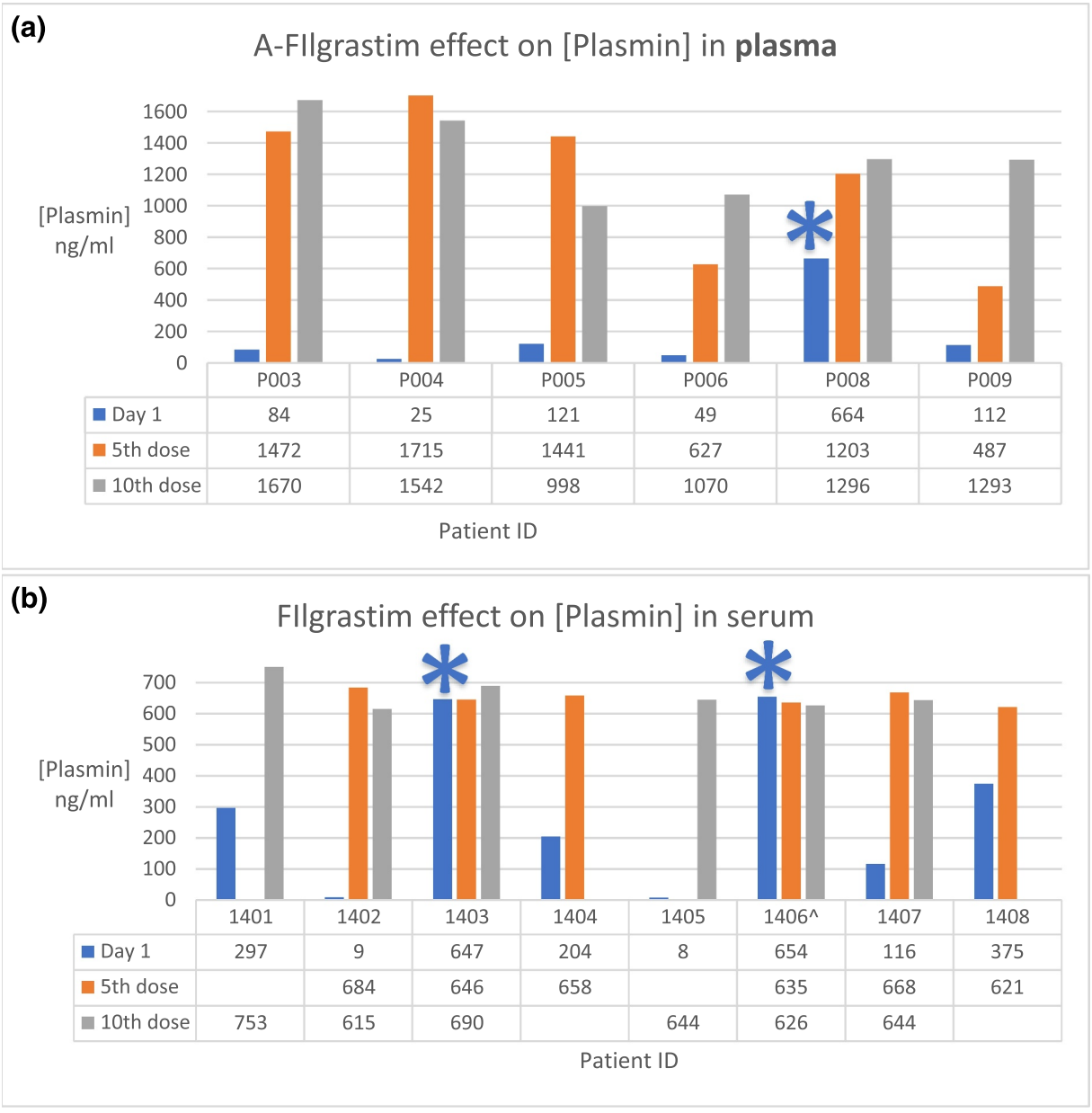


FIGURE 3 Filgrastim effect on the concentration of plasmin ("[Plasmin]") for each patient on the day after the 5th and 10th doses, relative to Day 1 (at $T = 0$). The [Plasmin] was higher in plasma (a) than in serum (b). Patients P008, 1403, and 1406 had recent post intervention thromboses; their elevated [plasmin] and [FDP] on Day 1 (blue*) likely reflect endogenous fibrinolysis following thrombosis. ^Patient 1406 only had 7 doses of filgrastim: [plasmin] after the 7th dose is substituted for the 10th dose [Colour figure can be viewed at wileyonlinelibrary.com]

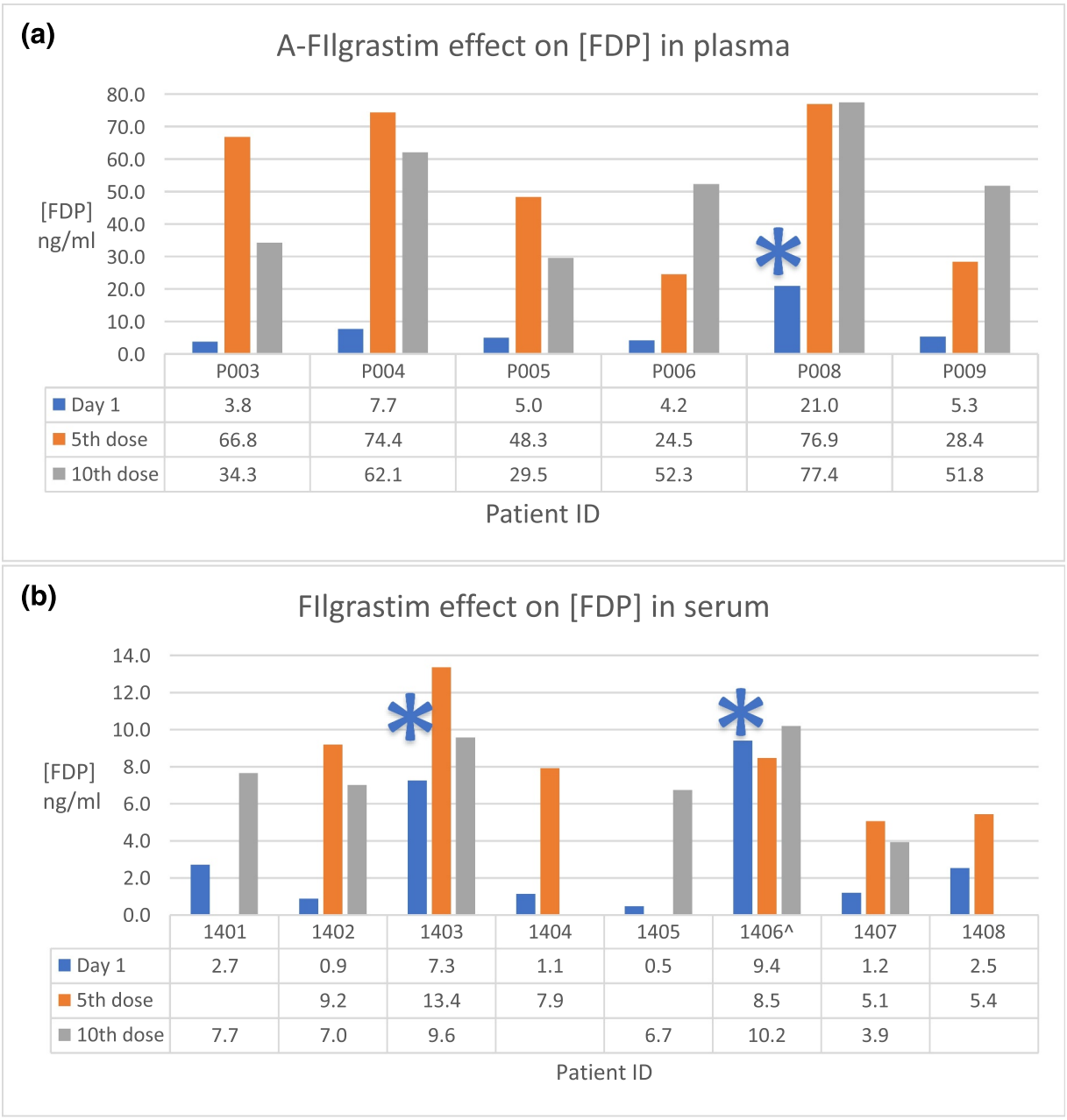


FIGURE 4 Filgrastim effect on the concentration of FDP ("[FDP]") for each patient on the day after the 5th and 10th doses, relative to Day 1. The [FDP] was higher in plasma (a) than in serum (b). Patients P008, 1403, and 1406 had recent post-intervention thromboses; their elevated [plasmin] and [FDP] on Day 1 (blue*) likely reflect endogenous fibrinolysis following thrombosis. ^Patient 1406 only had 7 doses of filgrastim: [FDP] after the 7th dose is substituted for the 10th dose [Colour figure can be viewed at wileyonlinelibrary.com]

significant increases in the other patients approximately match in magnitude the Day 1 data in these three patients, supporting the notion that Filgrastim promotes fibrinolysis at a physiologic level over the course of treatment. Acute hemorrhage did not occur during the 29 days of Filgrastim exposure. The [Plasmin] and [FDP] were higher in the plasma samples compared to the serum samples, likely because of clot formation in the serum separator tubes; however, the percent changes to Day 1 for each patient were similarly significant (Tables A1 and A2).

3.1.2 | Neovascularization

Analysis paired to controls

One day following the 5th and 10th Filgrastim doses, there was a significant percent increase in the serum concentrations of NV associated proteins relative to Day 1 in CLTI patients at both institutions (Table 4). ELISA concentrations are tabulated for each patient in Tables A3–A15 before and after 2 h of PCP. These findings were PCP independent, except for [**MCP-1**]. [**MCP-1**] decreased

TABLE 4 Effect of filgrastim (paired to control data)

		% Change due to Filgrastim (Relative to Day 1 T=0)							
		Day after 5th Dose				Day after 10th Dose			
		N	Mean	SD	P Value	N	Mean	SD	P Value
Plasmin ng/ml	ALL	12	1600%	2603%	**0.002**	12	2231%	2913%	**0.0004**
	Plasma	6	1859%	2496%	**0.01**	6	1994%	2151%	**0.001**
	Serum	6	1341%	2919%	**0.04**	6	2469%	3729%	**0.03**
FDP ng/ml	ALL	12	554%	475%	**0.006**	12	555%	442%	**0.0040**
	Plasma	6	766%	506%	**0.003**	6	718%	308%	**0.0004**
	Serum	6	341%	364%	**0.02**	6	391%	520%	**0.01**
HGF pg/ml	Serum	12	316%	222%	**0.0001**	12	419%	301%	**0.0001**
MMP-9 ng/ml		12	805%	1211%	**0.00001**	12	984%	1241%	**0.000003**
VEGF-A pg/ml		11	191%	367%	**0.03**	11	666%	1217%	**0.01**
Angiopoietin-1 pg/ml		11	779%	2186%	0.1	11	878%	2203%	**0.02**
PDGF-AA pg/ml		6	544%	1032%	0.2	6	824%	1521%	**0.05**
PDGF-BB pg/ml		6	291%	462%	0.3	6	268%	297%	**0.03**
PDGF-AB pg/ml		6	293%	457%	0.3	5	437%	542%	0.08
TNF-α pg/ml		6	72%	144%	0.3	6	173%	166%	**0.01**
TGF-β pg/ml		6	9%	13%	0.1	5	83%	105%	**0.05**
IGF-1 ng/ml		12	40%	112%	0.3	11	15%	31%	0.3
PLGF pg/ml		6	293%	457%	0.3	6	437%	542%	0.08
IL-6 pg/ml		6	-35%	35%	0.2	6	43%	106%	0.5
MCP-1 pg/ml		6	-1%	34%	0.5	5	-15%	25%	0.3
Nitrite nM/ml		8	72%	37%	**0.0002**	6	268%	159%	**0.003**

Note: The relative percent change in concentration was calculated using data from Day 1 (before Filgrastim and PCP) as baseline for each patient. Paired *t*-tests using the concentrations were used to derive the *p* values. [Plasmin] and [FDP] were measured in plasma at UIC and in serum at UC. The other proteins were measured in serum. *p* Values ≤0.05 are in bold. Despite the magnitude of increase, some did not reach significance due to the wide standard deviation (SD) in this small population.

Abbreviations: HGF, Hepatocyte Growth Factor; IGF, Insulin-like Growth Factor; IL-6, interleukin 6; MCP-1, Monocyte Chemoattractant Protein; MMP-9, Matrix Metalloproteinase-9; PLGF, Human Placenta Growth Factor; TGF, Transforming Growth Factor; VEGF-A, Vascular Endothelial Growth Factor A.

during Filgrastim exposure −22% ± 29% (p = 0.04) after the 5th dose, and −27% + 23% (p = 0.07) after the 10th dose compared to Day 1. These were partially offset by 2 h of PCP, which increased [**MCP-1**] 41% ± 44% on Day 1, 18% ± 27% after the 5th Filgrastim dose, and 12% ± 28% after the 10th Filgrastim dose.

Unpaired group analysis

The Filgrastim impact on the concentrations of NV proteins is evident when data are aggregated into two groups. Group A was derived from the ELISA data obtained in the absence of any prior Filgrastim exposure (includes all Day 1 data on all patients, and Day 30 Data in the PCP alone group). This was justifiable due to the lack of significant impact of PCP *alone* on most of the NV protein concentrations (Table 6). Group B was derived by combining all ELISA protein concentrations obtained after the 5th and 10th Filgrastim doses. Table 5 lists the highly significant impact of Filgrastim: all the NV protein concentrations were increased in Group B versus Group A, except for TGF-β and MCP-1. The decreases measured in the latter two were also statistically significant.

3.1.3 | Progenitor cell mobilization

The circulating progenitor cell populations (CD34+ and VEGFR2+) were significantly increased 1 day after the 5th and 10th Filgrastim doses compared to Day 1 (Table 7) in CLTI patients treated with Filgrastim and PCP. Individual patient cell fraction data are reported in Table A18. After the 5th Filgrastim dose, CD34+ and VEGFR2+ fractions increased 49% ± 46% (p = 0.004) and 52% ± 42% (p = 0.02), respectively. After the 10th Filgrastim dose, CD34+ and VEGFR2+ fractions increased 83% ± 57% (p = 0.0004) and 93% ± 598% (p = 0.02), respectively, versus Day 1. Small further increases in the number of these cells were consistently measured in the circulation after 2 h of PCP on each day (Table A18).

TABLE 5 Filgrastim effect in CLTI (unpaired Group analysis)

	Group A: NO FILGRASTIM EXPOSURE			Group B: FILGRASTIM				
	N	Mean	SD	N	Mean	SD	% Change	P Value
Plasmin ng/ml	136	157	196	96	894	362	**468%**	**<0.001**
FDP ng/ml	184	2	2	46	8	2	**308%**	**<0.001**
HGFpg/ml	192	1999	1783	90	6526	3337	**227%**	**<0.001**
VEGF-A pg/ml	186	130	183	85	509	293	**291%**	**<0.001**
MMP=9 ng/ml	208	415	521	90	2156	759	**419%**	**<0.001**
Angiopoietin pg/ml	116	15257	18159	88	41999	14882	**175%**	**<0.001**
PDGF=AA pg/ml	184	1032	1480	44	4244	1519	**311%**	**<0.001**
PDGF=BB pg/ml	184	1046	714	44	3832	2302	**266%**	**<0.001**
PDGF-AB pg/ml	100	85	82	42	255	108	**200%**	**<0.001**
IGF-1 ng/ml	136	74	33	88	98	30	**33%**	**<0.001**
TGF-βpg/ml	184	2018	1224	42	1481	483	**-27%**	**0.006**
TNF-α pg/ml	184	3	2	44	6	2	**99%**	**<0.001**
PLGF pg/ml	184	20	9	44	41	19	**109%**	**<0.001**
IL-6 pg/ml	184	20	41	44	52	84	**158%**	**0.0003**
MCP-1 pg/ml	164	312	153	42	258	97	**-17%**	**0.03**

Note: This is a summary comparison of ELISA concentrations aggregated into 2 groups. Group A: no Filgrastim exposure (includes all Day 1 data on all patients, and Day 30 data in the PCP alone group). Group B: all data obtained one day after both the 5th and 10th Filgrastim doses. *p* Values are derived from unpaired *t* test of ELISA data. *N* is the number of ELISA results.

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; HGF, Hepatocyte Growth Factor; IGF, Insulin-like Growth Factor; IL-6, interleukin 6; MCP-1, Monocyte Chemoattractant Protein; MMP-9, Matrix Metalloproteinase-9; PLGF, Human Placenta Growth Factor; TGF, Transforming Growth Factor; VEGF-A, Vascular Endothelial Growth Factor A.

3.1.4 | Hematology

One day following Filgrastim administration, the hematologic evaluation identified a 438% ± 200% increase in the leukocyte count ($P = 0.0001$), a 664% ± 251% increase in the absolute neutrophil count ($p = 0.0003$), a 104% ± 63% increase in the absolute lymphocyte count ($p = 0.02$), and a 134% + 161% increase in the monocyte count ($p = 0.07$). An average of 4.3 ± 3.2 bands appeared per high power field (none on Day 1).

3.1.5 | Serum nitrite

Relative to Day 1 (prior to Filgrastim), serum [nitrite] (nM/ml) increased 72% ± 37% ($p < 0.001$) and 268 ± 159% ($p = 0.003$) 1 day after the 5th and 10th Filgrastim doses, respectively. These increases were measured prior to onset of PCP ($T = 0$) on those days (Table 4). Two hours of PCP further increased serum [nitrite] 30% ± 27% and 9% ± 5% on those days ($p = 0.01$; Table A20).

3.2 | PCP alone

CLTI patients were treated for 3 h daily with PCP alone. Blood was drawn before and after 2 h of observed PCP use on Day 1 and Day 30. Data from arrival on Day 1 were pooled for baseline [proteins], cells, and [nitrite] ($N = 28$). On Day 1, 2 h of PCP led to increases in serum [IL-6] (21% ± 35%, ($p = 0.04$), [nitrite] (28% ± 28%, $p < 0.0001$), and [MCP-1] (19% ± 32%, $p = 0.006$; Tables A16 and A19). On Day 30, 2 h of PCP led to an increase in TNF-α (37% ± 71%, $p = 0.04$) and [nitrite] (7% ± 6%, $p < 0.0001$). Thirty days of 3 h daily PCP use did not significantly impact concentrations of any of the proteins but did lead to a 169% ± 158% ($p < 0.001$) increase in [nitrite] (Table 6 and Tables A21–A35). Cytometry data are listed in Table A17.

4 | DISCUSSION

4.1 | Filgrastim and fibrinolysis

In 1989, Filgrastim was reported to stimulate activity of plasminogen activator in both extracellular and intracellular milieus of endothelial cells obtained from bovine arteries (Kojima et al., 1989). This effect was dependent on the concentration of Filgrastim added to the culture medium and on the treatment time. Analyses by fibrin and reverse fibrin autography revealed that activity of plasminogen activator increased more than its inhibitor in endothelial cells treated with Filgrastim. In 2006, in vitro experiments suggested Filgrastim-associated fibrinolysis was directly attributable to the increase in

TABLE 6 Effect of PCP alone

	Effect of PCP alone											
	% Change due to 2 hours of PCP (Relative to T=0)								Effect of 30 days PCP Relative to Day 1 (T=0)			
	Day 1				Day 30							
	N	Mean	SD	P Value	N	Mean	SD	P Value	N	Mean	SD	P Value
Plasmin ng/ml	9	41%	143%	0.2	9	43%	143%	0.3	9	-7%	63%	0.4
FDP ng/ml	19	-1%	47%	0.6	17	-16%	22%	0.07	17	35%	158%	0.9
HGF pg/ml	19	-1%	16%	0.4	17	-1%	16%	0.5	17	7%	40%	0.9
MMP=9 ng/ml	19	37%	106%	0.6	17	12%	63%	0.3	17	65%	195%	0.7
VEGF-A pg/ml	19	0%	35%	0.8	17	83%	306%	1.0	17	18%	60%	0.3
Angiopoietin-1 pg/ml	19	7%	76%	0.9	17	24%	53%	0.2	17	1%	26%	0.9
PDGF=AA pg/ml	19	-11%	31%	0.1	17	4%	53%	0.9	17	23%	74%	0.5
PDGF=BB pg/ml	19	13%	103%	0.8	17	43%	132%	0.3	17	10%	43%	0.6
PDGF-AB pg/ml	10	45%	165%	0.5	9	32%	72%	0.1	8	2%	23%	1.0
TNF-α pg/ml	19	22%	52%	0.3	17	37%	71%	**0.04**	17	6%	55%	1.0
TGF-β pg/ml	19	4%	56%	0.5	17	12%	56%	0.5	17	12%	61%	0.7
IGF-1 ng/ml	10	1%	4%	0.5	10	-1%	4%	0.5	10	4%	29%	0.8
PLGF pg/ml	19	-1%	15%	1.0	17	6%	14%	0.2	17	3%	22%	0.6
IL-6 pg/ml	19	21%	35%	**0.04**	17	2%	21%	0.5	17	37%	122%	0.8
MCP-1 pg/ml	28	19%	32%	**0.006**	17	-1%	14%	0.3	17	9%	32%	0.2
Nitrite nM/ml	28	28%	28%	**<0.001**	17	7%	6%	**<0.001**	17	169%	158%	**<0.001**

Note: The relative percent change in concentration was calculated using data from Day 1, $T = 0$ (before onset of PCP) as baseline for each patient. Paired *t*-tests using the concentrations were used to derive the *p* values. P values ≤0.05 are in bold.

Abbreviations: HGF, Hepatocyte Growth Factor; IGF, Insulin-like Growth Factor; IL-6, interleukin 6; MCP-1, Monocyte Chemoattractant Protein; MMP-9, Matrix Metalloproteinase-9; PLGF, Human Placenta Growth Factor; TGF, Transforming Growth Factor; VEGF-A, Vascular Endothelial Growth Factor A.

TABLE 7 Filgrastim effect on circulatory ells in CLTI patients treated with Filgrastim and PCP

	INFLUENCE OF FILGRASTIM After 5th and 10th doses relative to DAY 1							
	5th Dose				10th dose			
	N	Mean	SD	P Value	N	Mean	SD	P Value
CD31+	4	11%	12%	**0.03**	4	17%	15%	**0.03**
CD34+	4	49%	46%	**0.004**	5	83%	57%	**0.004**
VEGFR2+	4	52%	42%	**0.02**	5	93%	59%	**0.02**
WBC	8	438%	200%	**<0.001**				
Neutophil	6	664%	251%	**<0.001**				
Lymphocyte	6	104%	63%	**0.02**				
Monocyte	6	134%	161%	0.07				

Note: Cytometry confirmed the significant percentage increase in CD34+ progenitor and VEGFR2+ endothelial progenitor cells one day after each Filgrastim dose. The DIfferential blood cell count also shows the percentage increase in white blood cell count (WBC) and neutrophils the day after the 5th Filgrastim dose.

circulating neutrophils (Stief, 2006). Neutrophils also release enzymes having both pro- and anti-angiogenic effects (Tazzyman et al., 2009).

Despite these in vitro observations, clinical assessment of the fibrinolytic potential of Filgrastim has not occurred. Our investigation was prompted by segmental tibial artery recanalization observed on angiogram in CLTI patients treated with Filgrastim and PCP. 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 would facilitate management of ischemia in CLTI and in other tissue beds.

Filgrastim has a half-life of 3.5 h, yet plasmin and FDP were elevated a day after each administration. Moreover, the elevation was to the same order of magnitude as in the three patients who

likely entered this evaluation in a fibrinolytic state due to a recent thrombotic event. Gradual physiologic fibrinolysis has the advantage of being able to reach thrombus wherever the blood travels, no matter how small the vessel, for prolonged periods of time. Follow-up angiograms show a distinctly faster contrast transit time through what were ischemic tissues. No hemorrhagic complications occurred during the month of Filgrastim use. While PCP seemed to have no influence on the [plasmin] or [FDP], the cyclic deformation it causes to the vessel walls may promote fissuring within the thrombus, exposing more surface area to the plasmin in blood entering from the adjacent patent lumen or the vaso-vasorum. The next step is to measure the plasma concentrations of plasmin-alpha 2 antiplasmin (PAP; Chandler et al., 2000) and of tissue plasminogen activator-plasmin activator inhibitor complexes (tPA-PAI1).

4.2 | G-CSF and neovascularization

In 1989, G-CSF and GM-CSF were reported to induce endothelial cells to express an activation/differentiation program (including proliferation and migration) related to angiogenesis (Bussolino et al., 1989, 1993). Others reported the release of NV proteins in serum after G-CSF administration (Fujii et al., 2004; Willis et al., 2008). Many NV clinical trials have been performed with G-CSF, and less so with GM-CSF. Results in the coronary (Abdel-Latif et al., 2008) and lower extremity (Arai et al., 2006; Capoccia et al., 2006; Gloekler et al., 2013; McDermott et al., 2017; Minamino et al., 2005; Subramaniyam et al., 2009; van Royen et al., 2005) circulations have been mixed and have not changed standard of care. The duration of G-CSF administration in these trials may have been too short to effect significant fibrinolysis or NV. The extended dosimetry proposed herein has the potential to improve outcomes. Our therapy is presently 1 month long, not 5–10 days long. Our dosing interval is 72 h, not 24 h. The longer interval is sufficient to lower the intensity of the G-CSF effect, making treatment potentially safer, while at the same time prolonging the interval during which lysis and NV are promoted. Moreover, in the case of CLTI, the absence of PCP in previous CLTI trials leaves the first five obstacles to NV (Table 1) unaddressed.

Filgrastim significantly increases most of the NV proteins based on the group analysis (Table 6). The paired patient data analysis (Table 4) mirrored many of those results but did not precisely match due to the magnitude of the standard deviations in this small population. Two proteins did decrease in response to Filgrastim, TGF-β, and MCP-1 in the larger group analysis. The impact of Filgrastim on [TGF-β] will need to be re-studied in plasma to reduce possible contribution from TGF-β released during in-tube clot formation in serum assays. Plasma ELISA is recommended for future assessment of VEGF-A and PDGF for the same reason.

As expected, Filgrastim significantly increased the circulating number of leukocytes, neutrophils, monocytes, CD34 + progenitor cells, and VEGFR2+ endothelial progenitor cells.

4.3 | PCP alone

Endothelial shear stress (required for arteriogenesis) activates the endothelium initiating arteriogenesis. Rapid PCP compression induces high shear stress, which has been reported to promote NOS activity (Chen et al., 1985; Kelm et al., 1999). We measured NOS activity indirectly by measuring serum [nitrite]. However, serum [nitrite] does not differentiate between NO produced by inflammatory cells (iNOS) versus NO produced from the endothelium (eNOS). We also measured an increase in MCP-1, a protein that acts as a homing signal for circulatory monocytes and progenitor cells. Activated endothelium express adhesion molecules (e.g., PECAM-1) which capture these cells and drag them into the sub-endothelium where they begin the process of vascular remodeling. In the absence of correlation of [MCP-1] in the serum to [MCP-1] in the endothelial glycocalyx, its decrease in the serum during Filgrastim use requires further investigation. Elevations of serum [MCP-1] and serum [nitrite] were observed after 2 h of PCP. This supports, but does not prove, our hypothesis that endothelial activation occurs from the endothelial shear stress generated by the rapid rise time of the ArtAssist device (from 0 to 120 mmHg in 0.3 s).

PCP was also used to manage the unhealthy ischemic tissue environment, helping to "pump" in oxygenated nutritive blood flow and important cellular elements, while pumping out toxic metabolic products and protein "distress" signals needed to recruit salutary cells (monocytes and progenitor cells). In the absence of oxygenated nutritive blood flow, oxidative phosphorylation becomes impaired, glycolysis is initiated, lactate is produced, and if there is insufficient local buffering, the tissue pH drops. The latter can denature proteins, alter receptor activity, and impair enzyme function. As ischemia progresses the tissue temperature drops, which affects the pKa of water and worsens protein dysfunction. Another benefit of PCP is the finding that the number of circulating CD31+, CD34+, and VEGFR2+ cells increase slightly during PCP use; this may improve delivery of the salutary progenitor cells to the ischemic tissue.

PCP is used as a stand-alone limb salvage tool, particularly in patients who are not candidates for invasive limb revascularization, or who have had re-occlusion following previous such attempts (Kavros et al., 2008; Sultan et al., 2011). Serial hemodynamic testing in 62 patients (Table 2) at UC (2010–2014) showed a non-statistically significant 9% rise in ABI ($p = 0.5$) after a prescribed 3 h of daily PCP for a mean of 5 months. Adding 1 month of Filgrastim during the first month of PCP (rationale in Table 1), with PCP continuation until CLTI resolves, led to a consistent 50% increase in ABI by 6 months.

4.4 | Clinical impact

The goal of this treatment is to improve blood flow. Achieving limb salvage also requires assiduous wound care, infection control, nutrition, avoidance of trauma, and patient compliance. Ischemic rest pain, coldness, and numbness typically begin to improve in the second

week. 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. As with all vascular interventions, the earlier the intervention the better. The longest "no-option" patient that achieved limb salvage was treated in 2008 (Figure 1) and remains amputation free as of 2021. Long-term durability is an anticipated benefit of NV, without the risks of recurrent intervention failures. This novel therapy does not preclude invasive revascularization if improvement is not achieved. Moreover, improvement in arterial runoff using a G-CSF and PCP would improve the patency of a subsequent intervention. Lastly, this systemic therapy improved the circulation in the contralateral limb when PCP was used in both legs.

Stem cell mobilization with G-CSF is not effective in 15%–20% of patients, particularly in diabetics (Semad et al., 2005). Diabetics are prone to accelerated atherosclerosis leading to advanced CLTI. The concentration of proteins associated with fibrinolysis and NV in patients with G-CSF resistance is yet to be delineated. The present study was too small to accurately observe differential influence of diabetes (Greenbaum & Link, 2011). Plerixafor (Mozobil, Genzyme) binds to CXCR4 and blocks the binding of its cognate ligand CXCL12 (Stroma-derived factor 1 alpha; Fricker, 2013). The combination of G-CSF with plerixafor shows promise in overcoming ineffective hematopoietic stem cell mobilization and may be a solution to this problem if it arises in CLTI.

Thromboangiitis obliterans results in a severe vasculitis. When tobacco use stops, symptoms often improve. We have successfully used PCP and Filgrastim when symptoms persist after the vasculitis has ended. Whether the sequelae of microvascular thrombosis following resolution of other forms of vasculitis (e.g., after COVID) in other tissue beds can be treated with Filgrastim would need further investigation.

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 (Cruciani et al., 2013). The role of Filgrastim in assisting in the management of infection in CLTI wounds will need further investigation at the dosimetry we used.

Based on this report, 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. While PCP is not feasible in central ischemic beds, it likely would not be necessary due to proximity to the force of the pumping heart.

4.5 | Limitations

This investigation was not a clinical efficacy trial. These patients had significant anatomic alterations from prior failed invasive revascularization procedures and were at the terminal end of the disease spectrum. The thrust of this work is the biological evidence supporting further study. Due to the advanced nature of the ischemia, and the a priori lack of knowledge of the discoveries we now report, this investigation encompasses three non-concurrent single-arm evaluations, while managing the clinical conditions. Any contribution to the protein concentrations from the local tissue response to subcutaneous injection will require future placebo comparison.

4.6 | Conclusion

Ten doses of Filgrastim (approximately 10 mcg/kg SQ every 72 h) consistently elevated serum concentrations of proteins associated with fibrinolysis (plasmin and FDP) and NV at two independent institutions. Acute hemorrhagic complications were not observed. Filgrastim decreases serum levels of MCP-1. PCP increases [MCP-1] and serum [nitrite], both features of endothelial activation required to initiate arteriogenesis. This project supports a new hypothesis: NV and safe physiologic fibrinolysis may be possible to orchestrate together.

ACKNOWLEDGMENTS

Cathy Pabst coordinated the care of the patients at Weiss Memorial Hospital, where Zaid Sayed processed the clinical data. Ed Arkans, CEO of ACI Medical, LLC, provided the ArtAssist® devices and many of the assay kits used at University of Chicago and University of Illinois at Chicago. Yuanfan Hong meticulously processed the ELISA assays at UIC. [Correction added on 15 March 2021, after first online publication: Acknowledgments section has been updated]

CONFLICT OF INTEREST

Authors report no specific financial conflict of interest. ACI Medical, LLC Inc (Ed Arkans CEO) provided funding for some of the UC assays and provided the ArtAssist devices for patients. Darwin Eton: Science and clinical advisor at Vasogenesis Inc (Brookline, MA). [Correction added on 21 March 2021, after first online publication: Conflict of Interest have been updated to reflect full company name and correct spelling of CEO in the second sentence and correct company name in the third sentence.]

AUTHOR CONTRIBUTIONS

Darwin Eton originated the study; methodology, assays, and data summary were performed at UC by Guolin Zhou under the supervision of Tong-Chuan He, and at UIC by Yuanfan Hong and Rachana Patil under the guidance of Amelia Bartholomew. Clinical data review was done by Zaid Syed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the DRYAD repository at https://doi.org/10.5061/dryad.b2rbnzsgw.

ORCID

Darwin Eton https://orcid.org/0000-0001-5937-1971

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How to cite this article: Eton, D., Zhou, G., He, T.-C., Bartholomew, A., & Patil, R. (2022). Filgrastim, fibrinolysis, and neovascularization. *Journal of Tissue Engineering and Regenerative Medicine*, *16*(5), 496–510. https://doi.org/10.1002/term.3284