

# Procalcitonin Impairs Endothelial Cell Function and Viability

Nana-Maria Wagner, MD,\* Caroline Van Aken, MD,\* Antje Butschkau, PhD,† Laura Bierhansl, MD,\* Patrick Kellner, MD,‡ Viola Schleusener, MSc, PhD,§ Jochen Seggewiss, PhD,|| Brigitte Vollmar, MD,† Gabriele Nöldge-Schomburg, MD,\* and Jan Patrick Roesner, MD\*

**BACKGROUND:** Procalcitonin is used as a diagnostic tool for the identification and risk stratification of septic patients. Procalcitonin plasma concentrations tightly correlate with the severity of the ongoing inflammatory reaction and can rise up to 10,000-fold. Impairment of endothelial cell function plays an important role in the pathogenesis of hypotension and disturbed organ perfusion during sepsis. We investigated the possible effects of procalcitonin itself on endothelial cell function and viability.

**METHODS:** Human endothelial cells were exposed to 0.01 to 100 ng/mL procalcitonin and investigated for endothelial permeability using transwells, migration in a scratch wound assay and new capillary formation on extracellular matrix in vitro. Tumor necrosis factor- $\alpha$  and vascular endothelial growth factor served as positive controls. Procalcitonin's impact on the response of endothelial cells toward ischemia was investigated in vivo in the murine model of unilateral femoral artery ligation. Procalcitonin-exposed endothelial cells were subjected to immunoblot for the investigation of vascular endothelial-cadherin expression and angiogenic signaling pathways. Flow cytometry was used for the detection of inflammatory activation and viability, and genomic analysis was performed. Data are presented as difference in means and 95% confidence intervals; statistical analyses were performed using analysis of variance/Bonferroni, and *P* values are reported as adjusted for multiple comparisons ( $P_{\text{adjust}}$ ).

**RESULTS:** Tumor necrosis factor- $\alpha$  and 0.1 ng/mL procalcitonin induced endothelial barrier disruption after incubation of endothelial monolayers for 6 hours ( $-2.53$  [ $-4.16$  to  $-0.89$ ],  $P = .0008$  and  $-2.09$  [ $-3.73$  to  $-0.45$ ],  $P_{\text{adjust}} = .0064$  compared with vehicle-treated control, respectively). Procalcitonin beginning at concentrations of 0.02 ng/mL reduced endothelial cell migration ( $0.26$  [ $0.06$  to  $0.47$ ],  $P_{\text{adjust}} = .0069$ ) and new capillary formation in vitro ( $0.47$  [ $0.28$  to  $0.66$ ],  $P_{\text{adjust}} < .0001$ ) contrasting the proangiogenic action of vascular endothelial growth factor. Left ventricular injection of procalcitonin in mice on postoperative day 1, 3, and 5 after induction of ischemia impaired new capillary formation and recovery of hindlimb perfusion in vivo (number of capillaries/mm<sup>2</sup> in the ischemic leg of vehicle-treated versus procalcitonin-treated mice,  $852.6$  [ $383.4$ – $1322$ ],  $P_{\text{adjust}} = .0002$ ). Twenty-four-hour incubation with procalcitonin reduced the expression of vascular endothelial-cadherin at 100 ng/mL ( $0.39$  [ $0.06$ – $0.71$ ],  $P_{\text{adjust}} = .0167$ ) and induced endothelial cell death (apoptosis,  $-5.4$  [ $-10.67$  to  $-0.13$ ],  $P_{\text{adjust}} = .0431$ ). No alteration in the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 or extracellular signal-regulated kinase 1/2, and AKT signaling pathways was observed. Genomic analysis revealed regulation of a variety of genes involved in inflammation, angiogenesis, and cell growth.

**CONCLUSIONS:** This study found that procalcitonin itself impaired several aspects of endothelial cell function. Procalcitonin-induced loss of endothelial barrier function may contribute to capillary leakage and therapy-refractory hypotension during sepsis. Anti-angiogenic properties of procalcitonin at low concentrations could also identify procalcitonin as a mediator of vascular disease associated with the metabolic syndrome. Future studies are needed to further test procalcitonin as a potential therapeutic target for preserving vascular dysfunction during acute and chronic inflammatory disorders. (Anesth Analg 2016;XXX:00–00)

Endothelial cells make up the inner lining of all blood vessels. They form a barrier keeping fluid and blood cells restricted to the intravascular space while ensuring oxygen and nutrient delivery to tissues. By controlling

vasomotor tone, endothelial cells participate in the regulation of systemic blood pressure, organ perfusion, and survival. Because of this prominent role in maintaining body homeostasis, endothelial cells are crucial in the pathogenesis of a variety of diseases. In sepsis, for example, endothelial activation, vasodilation, and loss of barrier function contribute to fluid translocation from the intravascular to

From the \*Department of Anesthesiology and Critical Care Medicine and †Institute for Experimental Surgery, University Hospital Rostock, Rostock, Germany; ‡Department of Anesthesiology and Intensive Care, University of Luebeck, University Medical Center Schleswig-Holstein, Luebeck, Germany; §Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany; and ||Institute of Human Genetics, University Hospital Muenster, Muenster, Germany.

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Address correspondence to Nana-Maria Wagner, MD, Department of Anesthesiology, Perioperative and Pain Medicine, Stanford University Medical School, 300 Pasteur Dr, Palo Alto, CA 94035. Address e-mail to [nmwagner@stanford.edu](mailto:nmwagner@stanford.edu).

the extravascular space. The resulting hypotension and tissue edema promote hypoperfusion and organ dysfunction. Preventing impairment of endothelial cell function is thus a promising therapeutic strategy for the treatment of sepsis-related complications. However, no currently available therapy targeting vascular function effectively lowers mortality, suggesting that mechanisms of endothelial cell dysfunction during sepsis are incompletely understood.<sup>1-3</sup>

Procalcitonin is an early sepsis biomarker that stratifies patients by severity of illness and predicts mortality. Procalcitonin is a 116-amino acid precursor protein of calcitonin without known hormonal activity.<sup>4</sup> When pathogens invade the circulation, procalcitonin synthesis is initiated when pathogen-stimulated monocytes adhere to activated endothelium.<sup>5,6</sup> During the course of bacterial sepsis, monocytes further activate parenchymal cells in various organs to produce procalcitonin, increasing procalcitonin plasma concentrations up to 100,000-fold.<sup>5,7,8</sup> The tight correlation of procalcitonin plasma concentration and ongoing sepsis has made procalcitonin an important tool for diagnosis, treatment, estimation of disease severity in, and prognosis of septic patients.<sup>9-12</sup> However, the role of procalcitonin itself in the pathophysiology of sepsis is unknown. In animal models, procalcitonin administration to septic hamsters dramatically increases mortality.<sup>13</sup> Conversely, procalcitonin neutralization during sepsis improves survival in hamsters and pigs.<sup>13,14</sup> These findings suggest that procalcitonin itself may mediate detrimental effects of systemic bacterial infection.<sup>15</sup> We hypothesized that procalcitonin itself, independent of the context of sepsis, affects endothelial cell function and survival.

## METHODS

### In Vitro Vascular Permeability Assay

Human umbilical vein endothelial cells (HUVECs; Lonza, Germany) were cultured in endothelial cell growth medium (Clonetics EGM-2, Lonza, Germany) and used from passages 2 to 5.  $1 \times 10^5$  endothelial cells were seeded in the upper chamber of transwell migration inserts of 1- $\mu$ m pore size (Greiner Bio-One, Frickenhausen, Germany) and incubated for 72 hours until confluency. Recombinant human procalcitonin (SRP6003; Sigma-Aldrich, Taufkirchen, Germany), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ; R&D Systems, Minneapolis, MN), or vehicle-only was added into the upper transwell chamber together with 50  $\mu$ L of 150-kDa fluorescein isothiocyanate (FITC)-labeled dextran (2% w/v; Sigma, Germany) and 450  $\mu$ L of medium. Samples were taken from the lower chamber every other hour and analyzed for fluorescence intensity employing a Sunrise microplate reader (Tecan Trading AG, Maennedorf, Switzerland). Experiments were performed in triplicate and averaged per independent experiment. The main outcome measure was optical density expressed as times-fold versus control defined as 1.0. Comparisons of treatment groups are shown in a statistical analysis supplemental file (Supplemental Digital Content 1, Supplemental Table, <http://links.lww.com/AA/B504>); correction for multiple comparisons was made by calculating *P* values  $\times$  7.

### In Vitro Endothelial Migration Assay

HUVECs were grown on 12-well cell culture dishes until confluency. Using a pipette tip, endothelial monolayers

were wounded horizontally and vertically as described previously.<sup>16</sup> Cells were stimulated with increasing concentrations of procalcitonin (0.01, 0.02, 0.1, 1, or 100 ng/mL), 50 ng/mL vascular endothelial growth factor (recombinant human VEGF), 10 ng/mL TNF $\alpha$ , or vehicle control. Pictures from scratch crosses were taken immediately and after 8 hours of incubation. Experiments were performed in triplicate and averaged per independent experiment. Cell-free area was quantified using Image-Pro Plus software (version 4.5; Media Cybernetics, Rockville, MD). The main outcome measure was endothelial migration expressed as times-fold versus control defined as 1.0. Correction for multiple comparisons was made by calculating *P* values  $\times$  10.

### In Vitro Angiogenesis Assay

The  $1 \times 10^4$  endothelial cells were seeded in duplicate on growth factor-reduced Matrigel (BD Bioscience, San Jose, CA) in a 96-well format and incubated in the presence of procalcitonin, VEGF, TNF $\alpha$ , or vehicle-only as described previously.<sup>16</sup> Experiments were performed in duplicate and averaged per independent experiment. After 8 hours of incubation, pictures were taken from 4 random fields per well, and tube length was determined using Image-Pro Plus software. The main outcome measure was cumulative network length of capillaries expressed as times-fold versus control defined as 1.0. Correction for multiple comparisons was made by calculating *P* values  $\times$  10.

### Murine Hindlimb Ischemia Model

Animal experiments were approved by the governmental ethical board for animal research in Mecklenburg-Vorpommern (7221.3-1-069/13) and are in accordance with German law on animal protection and the Guide for the Care and Use of Laboratory Animals. Animals were bred under a 12-/12-hour light cycle with ad libitum availability of rodent chow and water and housed at the Institute for Experimental Surgery, Central Animal Care Facility, Rostock University, Rostock, Germany. Eight- to 10-week-old male wild-type (C57BL/6J) mice were subjected to intraperitoneal anesthesia using 6 mg/kg xylazine hydrochloride (CP-Pharma, Burgdorf, Germany) and 100 mg/kg ketamine hydrochloride (Pharmanovo GmbH, Hannover, Germany), and unilateral hindlimb ischemia was then induced as previously described.<sup>16</sup> In brief, the right femoral artery (immediately distal to the branch of the deep femoral artery) and the distal portion of the saphenous artery were permanently ligated using a 7-0 polypropylene suture (Prolene; Ethicon, Norderstedt, Germany). The ligated femoral artery was removed. Wounds were carefully sutured using 6-0 sutures (Prolene). Procalcitonin was injected in the left cardiac ventricle to calculated plasma concentrations of 0.02, 1, and 100 ng/mL assuming approximately 1.5 mL total blood volume on postoperative day (POD) 1, 3, and 5 as described previously.<sup>16</sup>

### Infrared Thermal Perfusion Imaging (thermography)

Before and immediately after surgical ligation of the femoral artery and during follow-up on POD 1, 3, 5, 7, 10, 15, and 21, mice were anesthetized via inhalation of 4 vol%

isoflurane, and infrared imaging was performed with a ThermoCAM B20HS camera (FLIR Systems, Wilsonville, OR) as described previously.<sup>16</sup> Images were analyzed using FLIR QuickReport 1.2 and GraphPad Prism software (version 4.0; GraphPad Prism, La Jolla, CA). The main outcome measure was the difference in temperature ( $\Delta K$ ) between the ischemic and nonischemic leg of each mouse. Both treatment groups (mice injected with NaCl served as vehicle and mice subjected to procalcitonin treatment) were compared at each day of temperature measurement, and  $n = 5$  mice/group were used.

### Immunohistochemistry

Before harvest of musculus gastrocnemius tissue on POD 21, 100  $\mu L$  fluorescein griffonia (bandeiraea) simplicifolia lectin I (Vector Laboratories, Burlingame, CA) was applied by left ventricular injection in anesthetized mice for visualization of perfused tissues as described previously.<sup>16</sup> Ten minutes later, mice were anesthetized by inhalation of isoflurane, euthanized by cervical dislocation and perfusion, and capillary density in the gastrocnemius muscle was assessed on 5- $\mu m$ -thick, acetone-fixed frozen sections after staining with antibodies (ABs) against CD31 (1:50 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA) followed by Cy3-labeled secondary ABs (Molecular Probes). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The number of CD31-immunopositive cells per muscle fiber was manually counted on 7 random microscope fields per section ( $\times 200$  magnification). The main outcome measure was the number of CD31/DAPI-double-positive capillaries per  $mm^2$  of musculus gastrocnemius tissue. The number of capillaries in the ischemic leg of the control group treated with NaCl was compared with the number of capillaries in the ischemic legs of mice treated with procalcitonin to concentrations of 0.1, 1, and 100 ng/mL, and  $n = 5$  mice/group were used. Correction for multiple comparisons of capillary density analyses was made by calculating  $P$  values  $\times 4$ .

### Flow Cytometry Analysis

After incubation with procalcitonin, TNF $\alpha$ , or vehicle-only for 24 hours, HUVECs were detached, washed, and resuspended at  $1 \times 10^6$  cells/mL. Volumes of 100- $\mu L$  cell suspension were incubated with reagents of the BD Annexin V: FITC Apoptosis Detection Kit I according to manufacturer's instructions and analyzed using a Becton Dickinson flow cytometer (Bedford, MA). For analysis of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression, antibodies were purchased from R&D Systems. Correction for multiple comparisons was made by calculating  $P$  values  $\times 4$ .

### Western Blot Analysis

After incubation with procalcitonin, TNF $\alpha$ , or vehicle for 24 hours, cells were suspended in lysis buffer, cleared by centrifugation, and equal amounts of protein were loaded and fractionated by electrophoresis on 10% to 12% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes (Immobilon Transfer Membranes; Millipore Corporation, Burlington, MA). Membranes were blocked with 2.5% bovine serum albumin (in tris-buffered

saline (TBS)/0.1% Tween-20) and incubated with primary antibody targeting human vascular endothelial (VE)-cadherin (BD Bioscience) followed by horseradish peroxidase (HRP)-conjugated secondary antimouse IgG (1:2500; Sigma). HRP was then detected with enhanced chemiluminescent substrate (Pierce ECL2; Thermo Scientific, Dreieich, Germany) and autoradiography. Densitometry was performed employing Quantity One software (version 4.6.6.0; Bio-Rad, Dreieich, Germany). For analysis of extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT activation, cells were incubated in growth factor-free medium; stimulated with VEGF, stromal cell-derived factor-1 (SDF-1), or procalcitonin for 10 minutes; lysed; and kept at  $-20^\circ C$  pending analysis using antihuman ERK1/2, phospho-ERK1/2, antihuman AKT, and phospho-AKT antibodies (R&D Systems).

### Affymetrix Gene Chip Analysis

Details of affymetrix gene chip analysis are provided as supplemental methods (Supplemental Digital Content 2, <http://links.lww.com/AA/B552>).

### Statistical Analysis

Statistical analyses were performed using 1-way analysis of variance (ANOVA) followed by pairwise comparison between group levels with  $t$  test followed by correction for multiple testing using the Bonferroni correction method. Adjusted  $P$  values ( $P_{\text{adjust}}$ ) to account for multiple comparisons are reported ( $P_{\text{adjust}} = P \text{ value} \times \# \text{tests}$ ). Treatment effect estimates are presented as difference in means and 95% confidence intervals. Figures show mean  $\pm$  standard deviation. For analyses of thermography data, treatment groups were compared separate for each day using pairwise comparison between group levels with  $t$  test, and  $P$  values were corrected for multiple comparisons. To test for trends over time, 2-way ANOVA was used. All statistical analyses were performed using GraphPad Prism 6 software. Power calculations for the primary endpoint of the in vivo studies (capillary density on POD 21) were performed using software of the University of British Columbia ([www.stat.ubc.ca](http://www.stat.ubc.ca)). On the basis of authors' experience with the in vivo model of hindlimb ischemia, population differences were anticipated as  $n = 5$  procalcitonin-treated mice to exhibit no more than 0.5-fold capillary density (ie, 800 capillaries/ $mm^2$  in mean) compared with  $n = 5$  control-treated mice (ie, 1600 capillaries/ $mm^2$  in mean) and a standard deviation of 400 capillaries/ $mm^2$ , power to exceed 0.8 for an overall  $\alpha$  of .05 corrected for the number of comparisons (3 comparisons). Details of statistical analysis are provided in a statistical analysis supplemental file (Supplemental Digital Content 1, Supplemental Table, <http://links.lww.com/AA/B504>).

## RESULTS

The effects of procalcitonin on endothelial cell function were first investigated using in vitro assays addressing differential aspects of endothelial cell function.

We tested the hypothesis that procalcitonin does not affect endothelial cell barrier function using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on endothelial cell monolayer permeability measuring optical density of

permeated FITC-dextran molecules in procalcitonin-treated versus vehicle-treated samples using pairwise comparison between treatment groups with *t* test followed by correction for multiple testing using the Bonferroni correction method. In the presence of control vehicle-only, no fluorescent signal was detected on the other side of the monolayer, indicating intact endothelial barrier function. In the presence of 0.1 ng/mL procalcitonin, increases in fluorescent signal were seen on the other side of the monolayer over the 6-hour time course compared with control (difference in mean,  $-2.09$ ; 95% confidence interval,  $-3.73$  to  $-0.45$ ; adjusted *P* value [ $P_{\text{adjust}}$ ] = .0064). This permeabilizing effect of procalcitonin on the monolayer was comparable with that of TNF $\alpha$  (TNF $\alpha$  vs control,  $-2.53$  [ $-4.16$  to  $-0.89$ ];  $P_{\text{adjust}}$  = .0008; Figure 1).

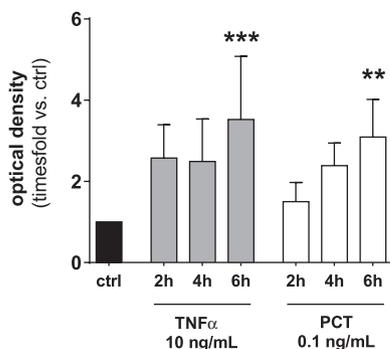
We then tested the hypothesis that procalcitonin does not impair the ability of endothelial cells to repair wounded endothelial monolayers using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on wound closure size within the endothelial cell monolayers using pairwise comparison between treatment groups with *t* test followed by correction for multiple testing using the Bonferroni correction method. At 0.01 ng/mL, no effect of procalcitonin on endothelial cell migration was observed (0.03 [ $-0.18$  to 0.24];  $P_{\text{adjust}}$  = .9999). At 0.02 ng/mL, endothelial cell migration was reduced control (0.26 [0.05–0.47];  $P_{\text{adjust}}$  = .0069). Higher concentrations (0.1–100 ng/mL) decreased endothelial cell migration to a degree comparable with 0.02 ng/mL. The anti-migratory effects of procalcitonin were comparable with that caused by 10 ng/mL TNF $\alpha$  (TNF $\alpha$  vs control; 0.23 [0.02–0.44];  $P_{\text{adjust}}$  = .0251). In contrast, endothelial cell migration was enhanced in the presence of 50 ng/mL of VEGF, a proangiogenic cytokine that stimulates endothelial cell migration VEGF ( $-0.28$  [ $-0.49$  to  $-0.06$ ];  $P_{\text{adjust}}$  = .0041; Figure 2, A and B).

Formation of new capillaries is a pivotal function of endothelial cells. Incubation of endothelial cells on Matrigel induces tube formation that mirrors their basal angiogenic activity.<sup>17</sup> We tested the hypothesis that procalcitonin does not affect the ability of endothelial cells to form capillary networks on Matrigel using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on the cumulative capillary

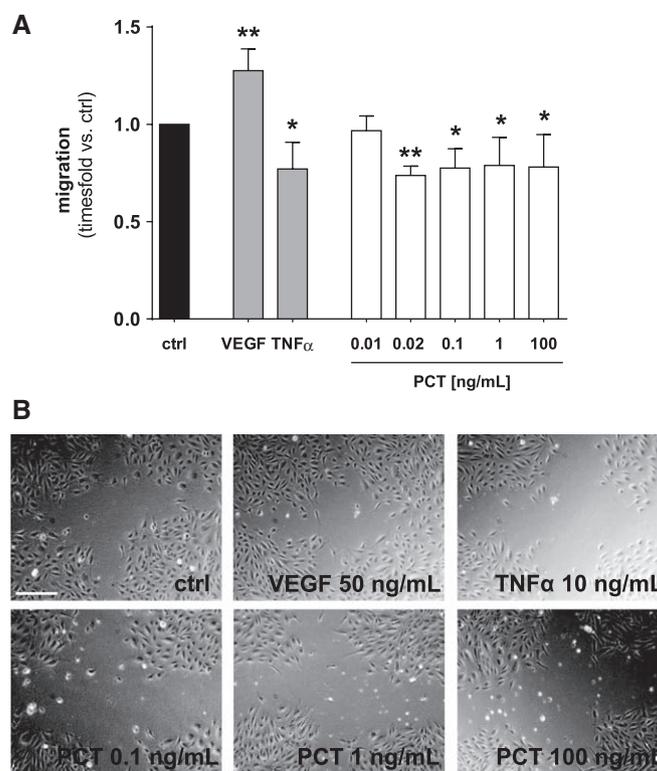
network length in vitro using pairwise comparison between treatment groups with *t* test followed by correction for multiple testing using the Bonferroni correction method. Procalcitonin attenuated basal angiogenic activity at concentrations of 0.02 (0.02 ng/mL vs control; 0.47 [0.27–0.67];  $P_{\text{adjust}}$  < .0001), 0.1 (0.1 ng/mL vs control; 0.33 [0.14–0.53];  $P_{\text{adjust}}$  = .0001), 1 (1 ng/mL vs control; 0.41 [0.22–0.60];  $P_{\text{adjust}}$  < .0001), and 100 ng/mL (100 ng/mL vs control; 0.40 [0.21–0.59];  $P_{\text{adjust}}$  < .0001) but not at 0.01 ng/mL procalcitonin (0.03 [ $-0.17$  to 0.22];  $P_{\text{adjust}}$  > .9999; Figure 3, A and B).

Hypoxia is one of the strongest inducers of new vessel formation in vivo.<sup>18</sup> In the murine model of hindlimb ischemia, hypoxia is induced in musculus gastrocnemius muscle tissue by permanent ligation of the proximal femoral artery.<sup>19</sup> The endothelial cell response to hypoxia can then be readily assessed by immunohistochemistry and measurements of perfusion.<sup>16</sup> In  $n = 5$  mice, a procalcitonin dose calculated to produce a 1 ng/mL plasma concentration was administered via left ventricular injection on POD 1, 3, and 5 after unilateral hindlimb ischemia induction. Five mice were given the vehicle as controls. We tested the hypothesis that procalcitonin does not affect recovery of perfusion in a murine model of hindlimb ischemia at any time point after induction of ischemia using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on difference in temperature of the operated versus nonoperated hindlimb using pairwise comparison between treatment groups at each day of measurement with *t* test followed by correction for multiple testing using the Bonferroni correction method. During the course of 21 PODs, hindlimb thermography revealed increased temperature differences between operated and nonoperated hindlimbs in mice treated with procalcitonin compared with vehicle on POD 3, indicating delayed recovery of perfusion (2.04 [0.34–3.74];  $P_{\text{adjust}}$  = .0035 on POD 3; Figure 4, A and B).<sup>16</sup> Further analysis revealed no group-by-time interaction ( $P = .3257$ ) but a significant treatment effect over time ( $P = .0002$ ). We then tested the hypothesis that procalcitonin does not reduce hypoxia-induced neovascularization using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on capillary density in musculus gastrocnemius tissue using pairwise comparison between treatment groups on POD 21 with *t* test followed by correction for multiple testing using the Bonferroni correction method. Hindlimb immunohistochemistry on POD 21 revealed reduced numbers of capillaries (852.6 [354.3–1351];  $P_{\text{adjust}}$  = .0002 CD31/DAPI-double-positive cells in operated hindlimb of procalcitonin-treated mice vs the operated hindlimb of vehicle-treated mice) in procalcitonin-treated mice and reduced lectin perfusion (Figure 4, C and D). The use of lower (0.02 ng/mL) and higher (100 ng/mL) concentrations of procalcitonin revealed similar impairment of capillary growth and perfusion in response to ischemia (ischemic leg of 0.02 and 100 ng/mL procalcitonin-treated mice vs control mice treated with NaCl; 765.8 [296.6–1235];  $P_{\text{adjust}}$  = .008 and 944.8 [475.6–1414];  $P_{\text{adjust}}$   $\leq$  .0001, respectively; Supplemental Digital Content 3, Supplemental Figure, <http://links.lww.com/AA/B505>).

We tested the hypothesis that procalcitonin does not affect apoptosis/necrosis of endothelial cells using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on the



**Figure 1.** Procalcitonin (PCT) induced permeability of endothelial cell monolayers indicated by increasing concentrations of fluorescence-labeled macromolecules in the lower chamber of transwell inserts over the time course of 6 hours. The effects were comparable with those of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ). Quantitative summary of  $n = 5$  independent experiments. Mean and standard deviation, \*\*\*TNF $\alpha$  versus control,  $P = .0008$ ; \*\*6-hour procalcitonin control,  $P = .0064$ .



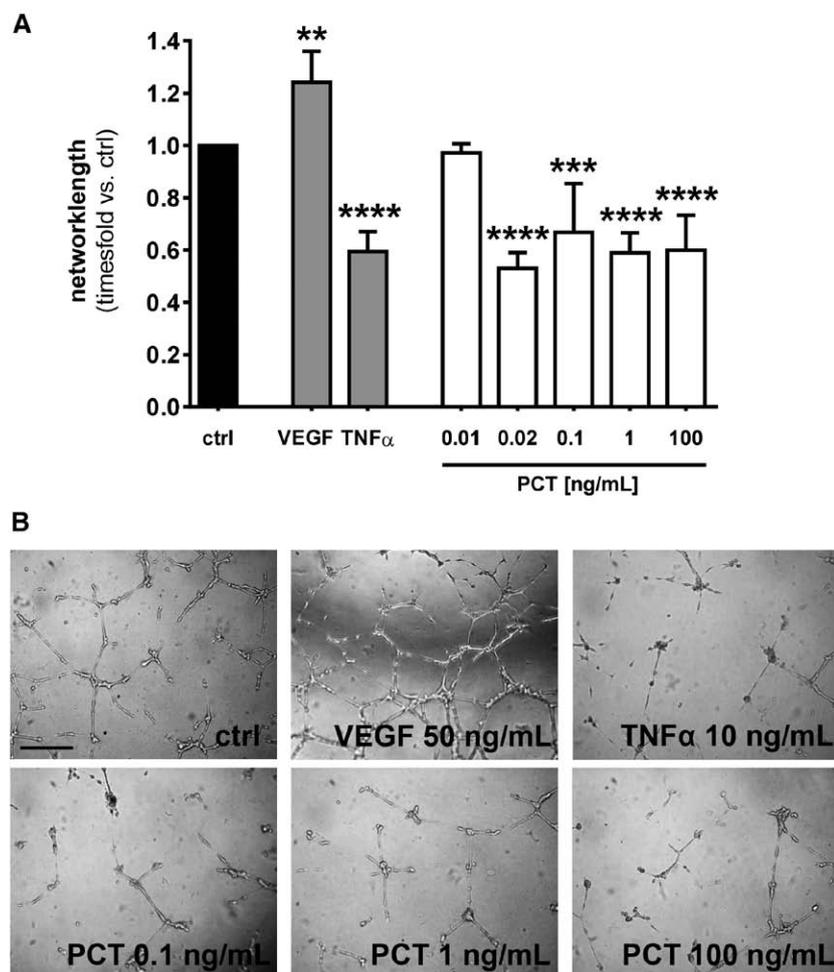
**Figure 2.** A, Procalcitonin (PCT) impaired endothelial migration in a scratch-wound assay in vitro. Vascular endothelial growth factor (VEGF) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) served as positive and negative controls, respectively. Quantitative summary of  $n = 5$  independent experiments after incubation for 8 hours. Mean and standard deviation, \*\*VEGF versus control,  $P = .0041$ ; \*TNF $\alpha$  versus control,  $P = .0251$ ; \*\*0.02 ng/mL procalcitonin versus control,  $P = .0069$ ; \*0.1 ng/mL procalcitonin versus control,  $P = .0299$ ; \*1 ng/mL procalcitonin versus control,  $P = .0484$ ; \*100 ng/mL procalcitonin versus control,  $P = .0366$ . B, Representative pictures of endothelial cell migration. Scale bar indicates 200  $\mu\text{m}$ .

number of apoptotic and necrotic cells using pairwise comparison between treatment groups with  $t$  test followed by correction for multiple testing using the Bonferroni correction method. Compared with vehicle-treated cells, 100 ng/mL procalcitonin induced endothelial cell apoptosis and necrosis ( $-5.4 [-10.67 \text{ to } -0.13]$ ;  $P_{\text{adjust}} = .0431$  and  $-4.0 [-6.52 \text{ to } 1.49]$ ;  $P_{\text{adjust}} = .0012$ , respectively; Figure 5, A and B). Both 0.1 and 1 ng/mL procalcitonin had no impact on necrosis. We also tested the hypothesis that procalcitonin does not downregulate the adherens junction protein VE-cadherin involved in both endothelial barrier function and angiogenesis using an  $\alpha$  of .05.<sup>20,21</sup> We assessed the treatment effect of procalcitonin on VE-cadherin protein content using pairwise comparison between treatment groups with  $t$  test followed by correction for multiple testing using the Bonferroni correction method; 100 ng/mL procalcitonin induced downregulation of VE-cadherin ( $0.38 [0.06-0.71]$ ;  $P_{\text{adjust}} = .0167$  compared with control; Figure 5, C and D). This effect was comparable with the effect of TNF $\alpha$  ( $0.54 [0.22-0.87]$ ;  $P = .0010$  vs vehicle). We next tested the hypothesis that procalcitonin does not modulate the expression of leukocyte adhesion molecules such as ICAM-1 and VCAM-1 (Supplemental Digital Content 4, Supplemental Figure, <http://links.lww.com/AA/B506>) using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on fluorescence intensity derived from fluorescence-labeled antibodies targeting ICAM-1 and VCAM-1 using pairwise comparison between treatment groups with  $t$  test followed by correction for multiple testing using the Bonferroni correction method. In contrast to TNF $\alpha$ ,

procalcitonin had no effect on the expression either ICAM-1 or VCAM-1. We also tested the hypothesis that procalcitonin does not modulate the activation of VEGF-induced or SDF-1-induced proangiogenic signaling pathways such as ERK1/2 or AKT/protein kinase B (Supplemental Digital Content 5, Supplemental Figure, <http://links.lww.com/AA/B507>) using an  $\alpha$  of .05. We assessed the treatment effect of procalcitonin on ERK1/2 and AKT phosphorylation using pairwise comparison between treatment groups with  $t$  test followed by correction for multiple testing using the Bonferroni correction method. Procalcitonin did not affect ERK1/2 or AKT activation. Genomic analysis revealed the regulation of a variety of genes related to inflammation, angiogenesis, and cell growth (Tables 1 and 2).

## DISCUSSION

Endothelial cells are a highly active and heterogeneous cell population that constantly responds to a variety of physical, chemical, cellular, and hormonal stimuli. This functional plasticity involves programmed cell death, migratory processes, angiogenic responses towards oxygen deprivation, and maintenance of an intact vascular barrier function. In diseases affecting vascular function, endothelial cells thus continuously modulate vascular behavior.<sup>22</sup> In this study, we show that procalcitonin adversely affects several aspects of endothelial cell function. Beginning at a concentration as low as 0.02 ng/mL and up to 100 ng/mL, procalcitonin impaired endothelial migration and new vessel formation both in vitro and in vivo; and 0.1 ng/mL procalcitonin



**Figure 3.** A, Procalcitonin (PCT) inhibited capillary formation on Matrigel basement membrane in vitro. Vascular endothelial growth factor (VEGF) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) served as positive and negative controls, respectively. Quantitative summary of  $n = 5$  independent experiments. Mean and standard deviation, \*\*VEGF versus control,  $P = .0069$ ; \*\*\*\*TNF $\alpha$  versus control,  $P < .0001$ ; \*\*\*\*0.02 ng/mL procalcitonin versus control,  $P < .0001$ ; \*\*\*0.1 ng/mL procalcitonin versus control,  $P = .0001$ ; \*\*\*\*1 ng/mL procalcitonin versus control,  $P < .001$ ; \*\*\*\*100 ng/mL procalcitonin versus control,  $P < .0001$ . B, Representative pictures of capillary formation after 8 hours of incubation. Scale bar indicates 200  $\mu\text{m}$ .

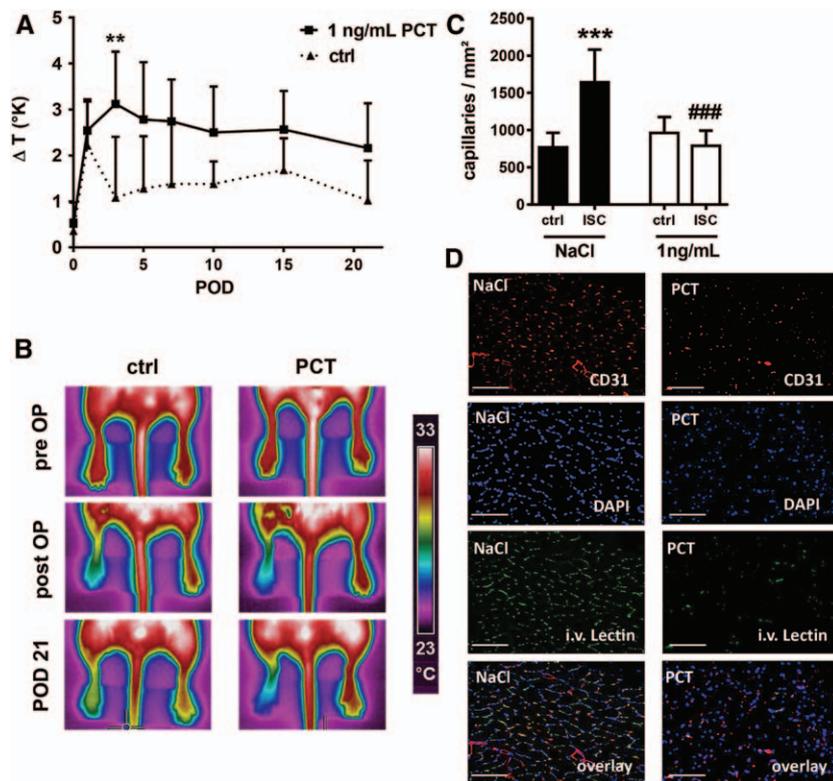
initiated loss of endothelial barrier function and higher concentrations (100 ng/mL) downregulated VE-cadherin expression and induced endothelial cell death. Our data suggest that procalcitonin itself is a potent modulator of endothelial integrity and that blocking procalcitonin effects may be a potential therapeutic target for preserving vascular function.

During sepsis, proinflammatory mediators such as TNF $\alpha$  induce ubiquitous endothelial cell activation and a procoagulatory phenotype. This generalized response contributes to the dysregulated and undirected inflammation and coagulation that occurs in the systemic inflammatory response syndrome and multiple organ dysfunction syndrome.<sup>1</sup> However, clinical trial-based animal models targeting either TNF $\alpha$ -induced endothelial cell activation or the preponderance of endothelium-dependent coagulation initiation (eg, by administration of activated protein C) have not shown benefit.<sup>23-25</sup> Previous in vitro studies report that procalcitonin augments TNF $\alpha$ -induced and lipopolysaccharide-induced production of nitric oxide in vascular smooth muscle cells, potentially contributing to the vasoplegia associated with the septic condition.<sup>26</sup> However, potential mechanisms induced directly by procalcitonin have not been investigated.

In this study, we investigated the role of procalcitonin on different aspects of endothelial cell function independent of

other stimuli endothelial cells are exposed to during septic conditions. We found that procalcitonin itself caused loss of endothelial barrier function at 0.1 ng/mL, a concentration far below any plasma concentration considered as clinically relevant for the diagnosis of sepsis.<sup>27</sup> This result suggests that procalcitonin may already be affecting endothelial cell function before clinical signs of vascular dysfunction occur. We also observed significant reductions in VE-cadherin expression at procalcitonin concentrations of 100 ng/mL after 24 hours of incubation. Because VE-cadherin is 1 of the most important determinants of vascular endothelium barrier function, subtle changes in VE-cadherin expression remaining undetected by immunoblot or mechanisms such as VE-cadherin dephosphorylation may have accounted for the increased permeability of the endothelial monolayer at lower concentrations after 6 hours of incubation.<sup>20</sup> Our data identify neutralization of the procalcitonin effect as a potential therapeutic tool to mitigate endothelial barrier disruption and therapy-refractory hypotension. However, further studies are needed to clarify procalcitonin's role in the pathophysiology of human sepsis and to specifically characterize differential responses toward procalcitonin of endothelial cells at different locations in the vascular tree.

One of the characteristics that makes procalcitonin useful as a diagnostic tool in bacterial sepsis is the tight correlation between procalcitonin plasma concentrations and the



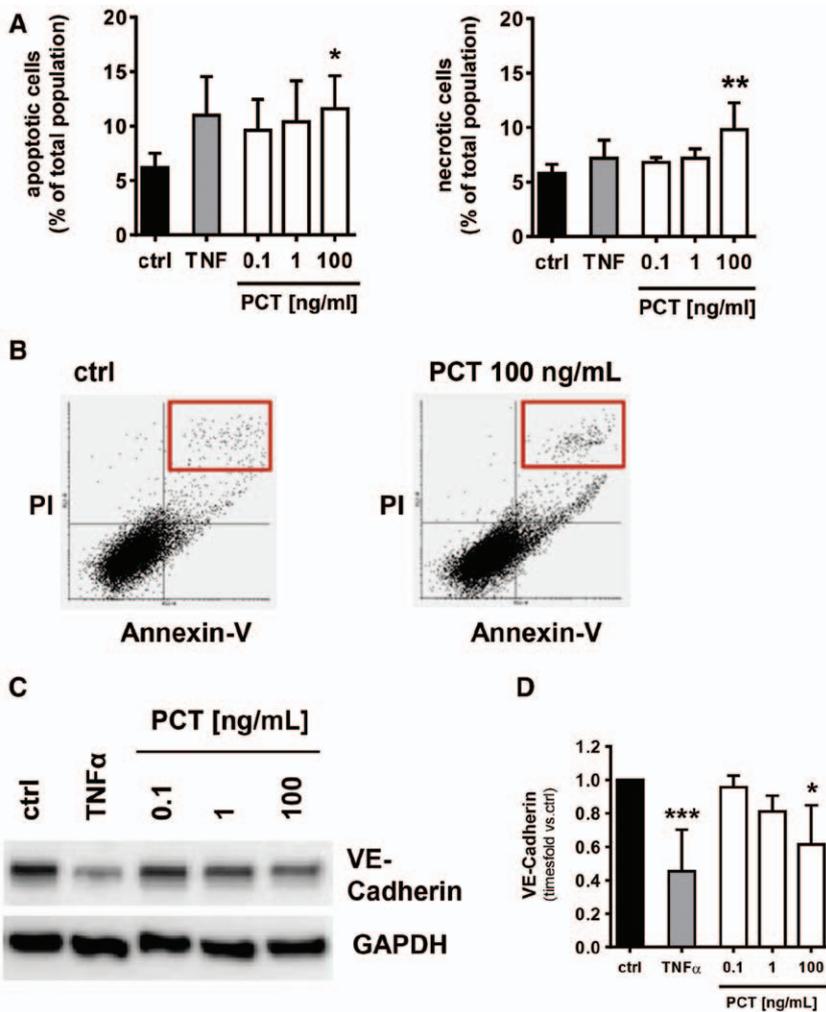
**Figure 4.** Procalcitonin impaired endothelial cell response to hypoxia in vivo. **A**, Thermography of both hindlimbs (ischemic and nonischemic) revealed comparable temperature differences ( $\Delta K$ ) on postoperative day (POD) 1 after the induction of ischemia verifying reduced perfusion of musculus gastrocnemius muscle tissue in both NaCl (ctrl)-treated and procalcitonin (PCT)-treated mice. These differences quickly recovered in NaCl-treated but not procalcitonin-treated mice toward POD 3. Mean and standard deviation, \*\*procalcitonin versus control on POD 3,  $P = .0035$ ;  $n = 5$  mice/group. **B**, Representative thermography pictures before femoral artery ligation (preoperation [OP]), on POD 1 (post-OP) and on POD 21. Indicators of temperature are indicated on the right. **C**, Quantitative results of capillary density assessment (number of CD31-/DAPI-double positive cells/ $\text{mm}^2$  of musculus gastrocnemius tissue on POD 21) after vehicle treatment of procalcitonin (to 1 ng/mL calculated plasma concentrations). ISC, ischemic hindlimb; ctrl, nonoperated hindlimb. Mean and standard deviation, \*\*\*ctrl versus ISC of vehicle-treated mice,  $P = .0009$ ; ###ischemic leg of vehicle-treated versus procalcitonin-treated mice,  $P = .0002$ ;  $n = 5$  mice/group. **D**, Representative pictures of histological analyzes of musculus gastrocnemius muscle tissue on POD 21 after induction of ischemia in mice treated with procalcitonin to final concentrations of 0.02 ng/mL. Endothelial cells were identified by expression of CD31 (red) and nuclear staining with DAPI (blue). Perfusion and capillary function were verified by left ventricular injection of fluorescent lectin (green). Picture overlay shows merge of CD31/DAPI/lectin staining. Scale bars indicate 100  $\mu\text{m}$ .

severity of ongoing inflammation.<sup>28</sup> Although the cellular interaction of activated monocytes with the endothelium accounts for an initial rise, procalcitonin plasma concentrations then increase rapidly because of monocyte-induced procalcitonin secretion.<sup>5</sup> Although increased procalcitonin synthesis has been detected in lungs, kidney, liver, and many other organs, the cellular interaction of monocytes that trigger procalcitonin synthesis in parenchymal cells has only been verified for adipose tissue.<sup>5,29,30</sup> Visceral adipose tissue is thus a potent source of procalcitonin during sepsis. In healthy humans, increased weight circumference and high body mass index (BMI) in humans correlate with increased plasma procalcitonin levels.<sup>31</sup> Obesity is a major risk factor for vascular disease such as atherosclerosis, hypertension, and thrombosis, but the underlying mechanisms remain not completely understood.<sup>32</sup> In this study, we found that procalcitonin plasma concentrations similar to those in individuals with increased BMI (ie, 0.02 ng/mL in males with a mean BMI of 26.8 kg/m<sup>2</sup> and females, 27.8 kg/m<sup>2</sup>)<sup>31</sup> impair endothelial migration and vessel growth in vitro and in vivo. Procalcitonin may thus play a role in vascular disease related to obesity and the metabolic syndrome. For example,

our findings of procalcitonin-induced disturbance of capillary formation and ischemia-induced neovascularization suggest an underlying mechanism of myocardial pathology associated with adiposity. However, a major limitation of the results derived from the in vivo model is that no sample size calculation had been performed a priori. Although alterations of VE-cadherin expression may crucially affect endothelial migratory and angiogenic properties,<sup>21</sup> we could not identify a mechanism clearly identifying the molecular cause of procalcitonin's effect on endothelial cell function at lower concentrations. Because evidence points toward procalcitonin as a potent inducer of endothelial cell apoptosis, one mechanism may simply be the death of cells.

## CONCLUSIONS

The present study identifies procalcitonin as an inducer of endothelial cell functional impairment at both concentrations found in the metabolic syndrome and under septic conditions. Elevated procalcitonin levels could thus identify patients at risk for the development of vascular complications related to acute or chronic inflammatory conditions. Blocking these vascular effects of procalcitonin



**Figure 5.** Mechanisms mediating effects of procalcitonin (PCT) on endothelial cell function. A, Quantitative summary of n = 5 independent experiments identifying Annexin-V-positive (apoptotic, left panel) and propidium-iodide-positive (PI, necrotic, right panel) cells by flow cytometry analysis after 24 hours of incubation with 10 ng/mL tumor necrosis- $\alpha$  (TNF $\alpha$ ) or procalcitonin. Mean and standard deviation (SD); \*100 ng/mL procalcitonin versus control (apoptosis),  $P = .0431$ ; \*\*100 ng/mL procalcitonin versus control (necrosis),  $P = .0012$ . B, Representative dot plots of control- and 100 ng/mL procalcitonin-treated cells. C, Representative immunoblot analysis for the expression of VE-cadherin protein in endothelial cell lysates after incubation of endothelial cells with TNF $\alpha$  or procalcitonin for 24 hours. GAPDH indicates equal loading. D, Quantitative summary of n = 4 immunoblot analyzes. Mean and SD; \*100 ng/mL versus control,  $P = .0167$ ; \*\*\*TNF $\alpha$  versus control,  $P = .0010$ .

**Table 1. PCT 0.02 ng/mL Versus Control-Treated Endothelial Cells**

Gene Symbol	Gene Title	Median Fold Change	Involvement in Cellular Processes
CCL8	Chemokine (C-C motif) ligand 8	-1.90	Inflammation
INFA13	Interferon, alpha 13	-1.90	Inflammation
MIR200c	MicroRNA 200c	-1.68	Angiogenesis
ANKRD30A	Ankyrin repeat domain 30A	-1.61	Cell growth/maintenance
TPI1P2	Triosephosphate isomerase 1 pseudogene 2	-1.58	Cell growth/maintenance
TBLY	Transducin (beta)-like, Y-linked	-1.57	Inflammation
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	-1.54	Vascular disease
XPNPEP3	X-prolyl aminopeptidase (aminopeptidase P) 3, putative	1.53	Cell growth/maintenance
PNRC2	Proline-rich nuclear receptor coactivator 2	1.54	Cell growth/maintenance
POLK	Polymerase (DNA directed) kappa	1.57	Cell growth/maintenance
CCL7	Chemokine (C-C motif) ligand 7	1.59	Inflammation
GGT3P	Gamma-glutamyltransferase 3 pseudogene	1.82	Cell growth/maintenance

**Table 2. PCT 1 ng/mL Versus Control-Treated Endothelial Cells**

Gene Symbol	Gene Title	Median Fold Change	Involvement in Cellular Processes
MIR1224	MicroRNA 1224	-1.89	Inflammation
MIR3781	MicroRNA3781	-1.60	Angiogenesis
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	-1.55	Vascular disease
KMO	Kynurenine 3-monoxygenase (kynurenine 3-hydroxylase)	-1.55	Inflammation
ANKRD30A	Ankyrin repeat domain 30A	-1.52	Cell growth/maintenance
GSTTP2	Glutathione S-transferase theta pseudogene 2	-1.51	Cell growth/maintenance
ITFG1	Integrin alpha FG-GAP repeat containing 1	1.58	Angiogenesis

may be a therapeutic option for the treatment of vascular disease related to excess body weight and the preservation of vascular function in the context of sepsis. Future studies are needed to further elucidate the underlying molecular mechanisms of procalcitonin's possible role in modulating endothelial cell function.

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#### DISCLOSURES

**Name:** Nana-Maria Wagner, MD.

**Contribution:** This author helped design the study, conduct parts of the study, collect and analyze the data, and prepare the manuscript.

**Name:** Caroline Van Aken, MD.

**Contribution:** This author helped conduct parts of the study, collect and analyze the data, and revise the manuscript.

**Name:** Antje Butschkau, PhD.

**Contribution:** This author helped conduct parts of the study, collect and analyze the data, and revise the manuscript.

**Name:** Laura Bierhansl, MD.

**Contribution:** This author helped conduct parts of the study, collect and analyze the data, and revise the manuscript.

**Name:** Patrick Kellner, MD.

**Contribution:** This author helped analyze the data and revise the manuscript.

**Name:** Viola Schleusener, MSc, PhD.

**Contribution:** This author helped contribute to computation of the statistical revision and revise the manuscript.

**Name:** Jochen Seggewiss, PhD.

**Contribution:** This author helped conduct the genomic analyzes and revise the manuscript.

**Name:** Brigitte Vollmar, MD.

**Contribution:** This author helped design the study, analyze the data, and revise the manuscript.

**Name:** Gabriele Nöldge-Schomburg, MD.

**Contribution:** This author helped design the study and revise the manuscript.

**Name:** Jan Patrick Roesner, MD.

**Contribution:** This author helped design the study, analyze the data, and prepare the manuscript.

**This manuscript was handled by:** Edward Joseph Mascha, PhD, and Avery Tung, MD, FCCM.

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