



Mechanisms Underlying Vascular Endothelial Growth Factor Receptor Inhibition–Induced Hypertension

The HYPАЗ Trial

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ABSTRACT: Drugs targeting the VEGF (vascular endothelial growth factor) signaling pathway are approved for several malignancies. Unfortunately, VEGF inhibitors lead to hypertension in 30% to 80% patients. Reduced nitric oxide synthase activity, microvascular rarefaction, and increased vascular resistance have been proposed as potential mechanisms. We aimed to assess these mechanisms in patients receiving the VEGF inhibitor, pazopanib, for cancer. Twenty-seven normotensive patients with advanced solid malignancies received pazopanib 800 mg od. Endothelial function was assessed using forearm plethysmography with intraarterial infusions of acetylcholine. Detailed hemodynamic measurements were taken. Density and diameter of the conjunctival and episcleral microvasculature were evaluated using hemoglobin video imaging. Measurements were taken at baseline, 2, and 12 weeks after initiation of pazopanib or earlier if patients became hypertensive. By the end of the trial, systolic blood pressure increased by 12 mm Hg (95% CI, 4–19 mm Hg; $P=0.003$), diastolic by 10 mm Hg (95% CI, 5–15 mm Hg; $P<0.001$), and peripheral vascular resistance by 888 dynes \times s/cm⁵ (95% CI, 616–1168 dynes \times s/cm⁵; $P<0.001$). Forearm blood flow improved: Ratio of acetylcholine response at end of trial/baseline was 2.8 (95% CI, 1.84–4.25; $P<0.001$). Microvascular density in the sclera was reduced by –15.5% (95% CI, –25.7% to –5.3%; $P=0.003$) and diameter by –2.09 μ m (95% CI, –3.95 to –0.19 μ m; $P=0.03$). A post hoc colorimetric assay revealed that pazopanib inhibited acetylcholinesterase activity by –56% (95% CI, –62% to –52%; $P<0.001$). Unexpectedly, pazopanib led to an increase in acetylcholine-mediated forearm blood flow response, likely due to the inhibition of acetylcholinesterase activity. Pazopanib increased peripheral vascular resistance and reduced microvascular density and diameter, suggesting that microvascular rarefaction could be one of the key mechanisms behind VEGF inhibition–induced hypertension.

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A recent large observational study in over 3 million patients has highlighted the increased risk of cardiovascular disease (CVD) in cancer survivors in comparison to the general population.¹ Interestingly,

CVD mortality risk is highest within the first year after cancer diagnosis, suggesting that the high risk of CVD could be explained by aggressive therapy shortly after disease discovery. One of the treatments associated with

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Novelty and Significance

What Is New?

- The HYPАЗ trial (Hypertension Induced by Pazopanib) is the first to examine multiple concurrent potential mechanisms underlying hypertension in humans brought on by pazopanib.
- Pazopanib unexpectedly led to an increase in acetylcholine-mediated forearm blood flow (ie, increased vasodilatation), but this is most likely due to inhibition of acetylcholinesterase activity by the drug, which has not been shown in humans previously.
- Peripheral vascular resistance and minimum forearm resistance were increased and microvascular diameter and density were reduced by the treatment, resulting in an increase in blood pressure.

What Is Relevant?

- Hypertension is a common adverse effect of VEGF (vascular endothelial growth factor) inhibition, and it

appears to be driven by an increase in peripheral vascular resistance.

- Hypertension associated with tyrosine kinase inhibitors, therefore, should be treated with anti-hypertensives with vasodilatory effects, such as calcium-channel antagonists.
- The additional inhibitory effect of pazopanib on acetylcholine esterase could explain other commonly seen side effects, such as bradycardia.

Summary

We have demonstrated that the VEGF inhibitor pazopanib led to structural remodeling of the resistance vasculature, as evidenced by microvascular rarefaction and a significant increase in peripheral vascular resistance, with a subsequent increase in blood pressure.

Nonstandard Abbreviation and Acronyms

AChE	acetylcholinesterase
BP	blood pressure
CVD	cardiovascular disease
eNOS	endothelial nitric oxide synthase
FBF	forearm blood flow
HYPАЗ	Hypertension Induced by Pazopanib
L-NMMA	L-N(γ)-mono-methyl-arginine
MFVR	minimum forearm vascular resistance
PVR	peripheral vascular resistance
TKI	tyrosine kinase inhibitor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

known cardiovascular adverse events is tyrosine kinase inhibitors (TKI) targeting VEGFRs (vascular endothelial growth factor receptors).

Pazopanib is an orally bioavailable, small-molecule multi-targeted TKI of VEGF receptors 1, 2, and 3, platelet-derived growth factor receptor α and β , and tyrosine-protein kinase (c-KIT), also known as CD117.² It is an established treatment for several solid tumors and has been licensed for use in metastatic renal cell carcinoma and soft tissue sarcoma.³ It works by inhibiting angiogenesis, thereby leading to tumor hypoxia and shrinkage. Unfortunately, treatment with VEGF inhibitors is complicated by cardiovascular side effects, most commonly hypertension.⁴ Hypertension is a class effect of VEGF inhibitors, and the incidence of hypertension varies depending on the agent used, occurring in $\approx 40\%$

of patients taking pazopanib.⁵ An increase in blood pressure (BP) is seen in most patients on these therapies, and 15% of patients develop severe hypertension. Although the incidence of hypertension is well documented with VEGF inhibitors, the underlying pathophysiology remains unclear.

Two main mechanisms for hypertension have been suggested: endothelial dysfunction and microvascular rarefaction.⁶ In health, binding of VEGF to its receptors leads to activation of multiple pathways including the eNOS (endothelial nitric oxide synthase) pathway, resulting in increased NO production and vasodilatation. Thus, when VEGF signaling pathway is inhibited, the NO pathway is suppressed and the endothelin-1 pathway stimulated, promoting vasoconstriction and consequent hypertension.⁷ An alternative mechanism is microvascular rarefaction. In health, VEGF maintains the integrity of the capillary and arteriole network. Experimental data suggest that when this pathway is inhibited, both microvascular density and diameter are reduced.⁷ The subsequent diminution of the microvascular surface area leads to an increase in peripheral vascular resistance and resultant increase in BP.⁸ However, these pathways have mainly been studied in animals, and the existing data in humans are conflicting with various vascular parameters measured independently, or biomarkers being assessed in separate small studies, using a variety of drugs (both TKIs and monoclonal antibodies) to inhibit VEGF.

Therefore, we designed a mechanistic clinical trial (HYPАЗ, an open label investigation into hypertension induced by pazopanib therapy) where our primary objective was to determine whether reduced NO bioavailability was the causative mechanism underlying a rise in BP induced by the TKI, pazopanib. Secondary and exploratory end points

included determining the effect of pazopanib on capillary rarefaction, peripheral vascular resistance, cardiac output, and arterial stiffness to draw a comprehensive picture of the possible mechanisms behind VEGF-induced hypertension.

METHODS

Patients

Patients with advanced solid malignancies for whom pazopanib was considered a reasonable option by their oncologist were recruited. Patients were eligible if they were aged ≥ 18 years old, had an Eastern Cooperative Oncology Group performance status⁹ of 0 to 1, measurable disease by Response Evaluation Criteria In Solid Tumours 1.1¹⁰ (at least one lesion measuring 20 mm), and had adequate baseline organ function. Patients were excluded if they were hypertensive (BP $>150/90$ mmHg), had any history of acute cardiovascular events within 6 months, or were considered to be at an increased risk from treatment with antiangiogenic therapy. A total of 31 eligible patients were recruited into the study from June 2011 to July 2014. Approval for this clinical trial was obtained from the Medicines Healthcare Regulatory Agency and the Research Ethics Committee (10/H0304/72). Written informed consent was received from each participant before performing any study-related procedures. The studies were performed in accordance with institutional guidelines and the International Council for Harmonisation Good Clinical Practice. The trial was registered and was sponsored jointly by Cambridge University Hospitals National Health Service Foundation Trust and the University of Cambridge. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Protocol

This was an open-label, nonrandomized, exploratory, targeted safety phase IIB study to investigate the mechanisms of hypertension caused by pazopanib therapy. The primary end point was change in forearm blood flow (FBF) ratio in response to intra-arterial acetylcholine infusion (endothelium-dependent dilatation) using the technique of venous occlusion plethysmography. Secondary end points included FBF ratio responses to intra-arterial sodium nitroprusside infusion (endothelium-independent dilatation) and L-N(γ)-mono-methyl-arginine (L-NMMA) infusion (basal NO production), as well as changes in BP, augmentation index, aortic pulse wave velocity, cardiac output, peripheral vascular resistance (PVR), minimum forearm vascular resistance (MFVR), and conjunctival and episcleral microvascular density and diameter.

The study design is depicted and detailed in the [Data Supplement](#) (Figure S1 in the [Data Supplement](#)). Patients made up to 8 visits; baseline research assessments were taken at visit 2, repeated 2 weeks later on visit 3, and the last research measurements were taken 12 weeks after the initiation of treatment (visit 8) or earlier if the patient became hypertensive, thus called visit hypertensive.

Hemodynamic Measurements

All studies were conducted in a quiet, temperature-controlled room by trained members of the study team. Further details about the methods can be found in the [Data Supplement](#).

Venous Occlusion Plethysmography

FBF was measured using venous occlusion plethysmography (Hokanson, Inc, Bellevue) with calibrated mercury-in-silastic strain gauges, as previously described.¹¹ Endothelium-dependent dilatation was assessed by incremental infusions of acetylcholine (Novartis Pharmaceuticals, Basel, Switzerland) at doses of 7.5 and 15 $\mu\text{g}/\text{min}$, endothelium-independent dilatation by sodium nitroprusside (Nitroprussiat Fides, Madrid, Spain) at doses of 3 and 10 $\mu\text{g}/\text{min}$, and basal NO production/the effect of nonselective NOS inhibition was assessed by an infusion of L-NMMA (Bachem Distribution Services GmbH, Weil am Rhein, Germany) at doses of 2 and 4 $\mu\text{mol}/\text{min}$.

BP and Arterial Stiffness

Measurements were made in seated and supine position following at least 5 minutes of rest. Brachial BP was recorded using a validated oscillometric technique (HEM-705CP; Omron Corp, Japan). Arterial stiffness (augmentation index and aortic pulse wave velocity) was measured using the SphygmoCor system, as described previously.¹²

Minimum Forearm Vascular Resistance

Venous occlusion plethysmography was set up as described for FBF but without arterial cannulation. After at least 10 minutes of supine rest, the baseline FBF was assessed. This was followed by inflation of a brachial cuff to suprasystolic pressure for 13 minutes in the nondominant arm. Immediately after deflation of the cuff, FBF was measured for 3 minutes. The average of the 3 highest FBF rates (flow_{max}) during the recording was used for the subsequent calculation of MFVR by dividing mean arterial pressure by flow_{max} . Vascular resistance is given in resistance unit (R unit); 1 $\text{mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100\text{ mL}^{-1}$ forearm tissue.^{13,14}

Cardiac Output

Cardiac output was assessed using a noninvasive, inert gas rebreathing technique.¹⁵

Hemoglobin Video Imaging of Conjunctiva and Episclera

Hemoglobin video imaging of the conjunctiva and episclera is a technique by which blood flow through the entire ocular surface microcirculation can be imaged,¹⁶ using a slit-lamp (Zeiss SL130) modified for hemoglobin imaging, as described previously.¹⁷ We aimed to image these circulations during visits 2, 3, and 8. However, by visit 8, many patients found the technique too demanding, and only 4 complete sets of good-quality video sequences could be obtained. Our formal analysis was therefore restricted to visits 2 and 3.

Video Analysis

Microvascular density (percentage coverage) and diameter distribution were obtained by applying the pretrained U-Net convolutional neural network^{18–20} on processed images using multiple open-source toolboxes.^{21–23}

Acetylcholinesterase Inhibition Assay

Whole blood samples were collected from 10 healthy volunteers and spiked with a range of concentrations (0.1–183 $\mu\text{mol/L}$) of pazopanib (GW-786034, BioVision) and AChE (acetylcholinesterase) activity was measured colorimetrically using a commercially available assay kit (no. MAK119, SigmaAldrich/Merck).

Safety Assessments

Safety data were collected continuously from patients until 28 days after the discontinuation of pazopanib. Adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0.

Statistical Analysis

A hierarchical mixed-effects model was used to analyze the effect of pazopanib on FBF with fixed effects for dose, visit, and their interaction and unstructured random dose effects at the patient and patient-visit level. The effect of pazopanib on the other hemodynamic measurements (BP, augmentation index, aortic pulse wave velocity, cardiac output, PVR, MFVR) was analyzed using a simple ANCOVA model, with fixed effects for visit, adjusting for baseline value. All parameters are reported with estimates and 95% CIs or means and SDs. A detailed description of the statistical analysis used in the trial can be found in the [Data Supplement](#).

RESULTS

Ninety-five patients were given study information, of whom 53 consented to screening. Thirty-one patients were initially considered eligible, but safety concerns were identified at the baseline visit in 4 patients (tumor adjacent to major vessels in 3 and new bleeding in 1) and a clinical decision was made not to commence pazopanib. Therefore, 27 patients, with a mean age of 60 years, were initiated on treatment with pazopanib at the standard dose of 800 mg daily. See Table 1 for baseline characteristics and demographics at the screening visit. Patients had a wide range of malignant diseases. Patients were normotensive with an average BP of $122/76 \pm 15/8$ mmHg at the screening visit. The flow of the patients through the study can be seen in Figure S2.

HEMODYNAMIC MEASUREMENTS

BP, Cardiac Output and Arterial Stiffness

Changes in hemodynamic parameters following pazopanib are listed in Table 2. After 2 weeks of treatment (visit 3), systolic BP increased by 12 mmHg (95% CI, 6–18 mmHg; $P < 0.001$) and diastolic BP by 8 mmHg (95% CI, 5–12 mmHg; $P < 0.001$) and remained at the similar level at visit 8/HYP. Similarly, after 2 weeks of treatment, we saw 24-hour ambulatory systolic BP increase by 6 mmHg (95% CI, –16 to 25 mmHg; $P = 0.02$) and diastolic BP by 6 mmHg (–6 to 18 mmHg; $P < 0.001$), and BP continued

Table 1. Subject Demographics and Characteristics

Variable	Mean \pm SD; n \pm SD
n	27
Type of malignant disease	Metastatic renal cell carcinoma, soft tissue sarcoma, melanoma, cervical and ovarian cancer, thymic carcinoma, gastrointestinal and hepatobiliary system malignancies
Age, y (range)	60 \pm 13 (25–82)
Sex	Women=17, men=10
Ethnicity, n	26 White people, 1 Chinese people
Smoking status, n	5 current smokers, 7 past smokers, 15 non-smokers
Height, cm	168 \pm 9
Weight, kg	70 \pm 17
Total cholesterol, mmol/L	4.8 \pm 1.0
Glucose, mmol/L	5.6 \pm 0.9
Blood pressure, mm Hg	122/76 \pm 15/8
ECOG status, n	0=7, 1=20
Target lesion size, mm	102 \pm 51

ECOG denotes The ECOG performance status. ECOG indicates Eastern Cooperative Oncology Group.

to stay elevated at visit 8/HYP. A large increase in PVR of +504 dynes \cdot s/cm 5 (95% CI, 288–728 dynes \cdot s/cm 5 ; $P < 0.001$) was observed at visit 3 and +888 dynes \cdot s/cm 5 (95% CI, 616–1168 dynes \cdot s/cm 5 ; $P < 0.001$) at visit 8/HYP. Aortic pulse wave velocity increased by 1.3 (95% CI, 0.3–2.2 m/s; $P = 0.01$) at visit 8/HYP; importantly this change remained significant after adjusting for the change in mean arterial pressure. MFVR increased after the initiation of pazopanib from $3.27 \text{ mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$ (95% CI, 2.82–3.72 $\text{mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$) to $4.38 \text{ mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$ (95% CI, 3.88–4.88 $\text{mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$); $P = 0.01$ at visit 3 and remained high at visit 8/HYP, $4.93 \text{ mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$ (95% CI, 3.57–6.29 $\text{mmHg}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot 100 \text{ mL}^{-1}$). The increase in MFVR was more pronounced in those patients who developed clinical hypertension with pazopanib compared with those who did not ($P = 0.023$).

Forearm Blood Flow

The primary end point of the trial was the change in FBF ratio in response to intraarterial acetylcholine infusion. We saw a significant interaction between dose and visit in acetylcholine-mediated (endothelium-dependent) dilatation following pazopanib treatment ($P = 0.0006$). This was apparent at visit 3 and at visit 8/HYP. See Figure 1 for the representation of the FBF results (Figure 1A: acetylcholine). There was no change in endothelium-independent dilatation ($P = 0.75$), as assessed by the change in FBF ratio during sodium nitroprusside infusion nor in basal NO production, as assessed by change in FBF during L-NMMA infusion ($P = 0.9$; Figure 1B: sodium nitroprusside and Figure 1C: L-NMMA).

Table 2. Baseline Hemodynamics and the Change at Week 2 and Week 12

Variable	Baseline (visit 2) mean \pm sd	Week 2 (visit 3) change (95% CI)	Week 12/HYP (visit 8/HYP) change (95% CI)
Systolic BP, mmHg	117 \pm 13	+12 (6 to 18); $P<0.001$	+12 (4 to 19); $P<0.001$
Diastolic BP, mmHg	73 \pm 9	+8 (5 to 12); $P<0.001$	+10 (5 to 15); $P<0.001$
MAP, mmHg	85 \pm 10	+9 (4 to 13); $P<0.001$	+10 (5 to 16); $P<0.001$
Heart rate, bpm	89 \pm 14	-11 (-16 to -7); $P<0.001$	-9 (-15 to -3); $P<0.001$
Cardiac output, L/min	4.88 \pm 1.42	-0.95 (-1.25 to -0.65); $P<0.001$	-1.35 (-1.73 to -0.98); $P<0.001$
Cardiac Index, L/min/m ³	2.70 \pm 0.72	-0.51 (-0.76 to -0.27); $P<0.001$	-0.86 (-1.18 to -0.53); $P<0.001$
PVR, dynes \times s/cm ⁵ *	1552 \pm 576	+504 (288 to 728); $P<0.001$	+888 (616 to 1168); $P<0.001$
AIX, %†	16 \pm 10	+7 (4 to 11); $P<0.001$	+6 (1 to 11); $P<0.001$
Aortic PWV, m/s‡	8.0 \pm 2.1	+0.4 (-0.3 to 1.2); $P=0.23$	+1.3 (0.3 to 2.2); $P<0.01$
24-h ambulatory SBP, mmHg	118 \pm 12	+6 (-16 to 25); $P=0.02$	+6 (-12 to 24); $P=0.08$
24-h ambulatory DBP, mmHg	75 \pm 8	+6 (-6 to 18); $P<0.001$	+5 (-5 to 19); $P=0.01$

The significance was determined using a simple ANCOVA model, with fixed effects for visit, adjusting for baseline value. AIX indicates augmentation index; BP, blood pressure; DBP, diastolic BP; HYP, hypertensive; MAP, mean arterial pressure; PVR, peripheral vascular resistance; PWV, pulse wave velocity; and SBP, systolic BP.

*Derived from $PVR=(MAP/CO)\times 80$.

†Adjusted for heart rate.

‡Adjusted for MAP and heart rate.

Hemoglobin Video Imaging of Conjunctiva and Episclera

Representative images from the conjunctival hemoglobin video imaging can be seen in Figure 2A). Following 2 weeks of treatment (at visit 3), there was a significant change in both the density -6.49% (95% CI, -10.31% to -2.66% ; $P=0.003$) and diameter $-2.09\ \mu\text{m}$ (95% CI, -3.95 to $-0.19\ \mu\text{m}$; $P=0.03$) of vessels in the ocular surface microcirculations (Figure 2B).

Pazopanib Plasma Concentrations

Trough plasma pazopanib concentrations ranged from 4.3 to 210 μM . Geometric mean pazopanib concentration was $75\pm 2.0\ \mu\text{M}$ at visit 3 and $75\pm 1.7\ \mu\text{M}$ at visit 8/HYP.

AChE Activity

In whole blood samples from healthy subjects, pazopanib led to a dose-dependent decrease in AChE activity (Figure 3). At the highest tested dose of pazopanib

(183 $\mu\text{mol/L}$), there was a 55.9% (95% CI, -61.8% to -50.1% ; $P<0.001$) inhibition of AChE activity. At the pazopanib concentration (57.9 μM) that most closely represented the observed mean trough concentration in the trial, there was 40% (95% CI, -43.3% to -36.7% ; $P<0.001$) inhibition of AChE activity.

Adverse Events

Adverse events were collected for all subjects for the duration of the study and are listed in Table S1 (related adverse events) and S2 (serious adverse events). Further details can be found in the [Data Supplement](#).

Related adverse events were in line with the adverse events reported for other, similar populations of patients treated with pazopanib.

DISCUSSION

Hypertension is a common and serious side effect associated with VEGF inhibitors. The HYPAZ trial

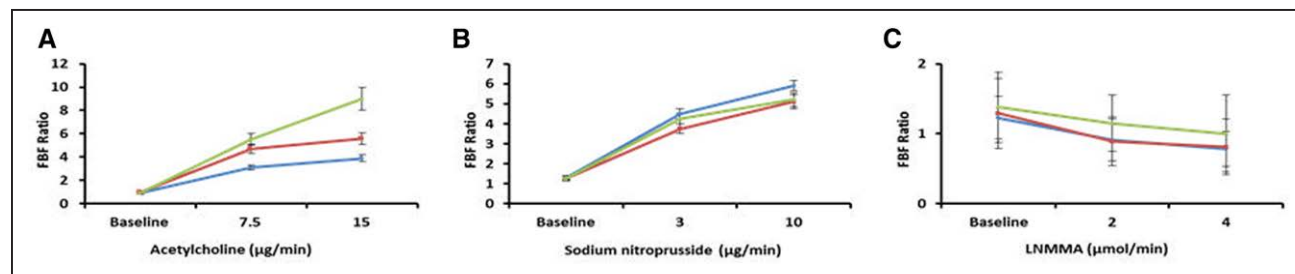


Figure 1. Change in the forearm blood flow ratio (FBF; infused/control arm).

FBF responses to acetylcholine (A), sodium nitroprusside (B), and L-N(γ)-mono-methyl-arginine (L-NMMA; C) at baseline (blue line), at 2 wk after initiation of pazopanib (red line) and at 12 wk (green line) or when subject became hypertensive (visit hypertensive). Data are expressed as a geometric mean FBF ratio (infused to control arm) \pm SEM. * $P<0.001$ (a hierarchical mixed-effects model).

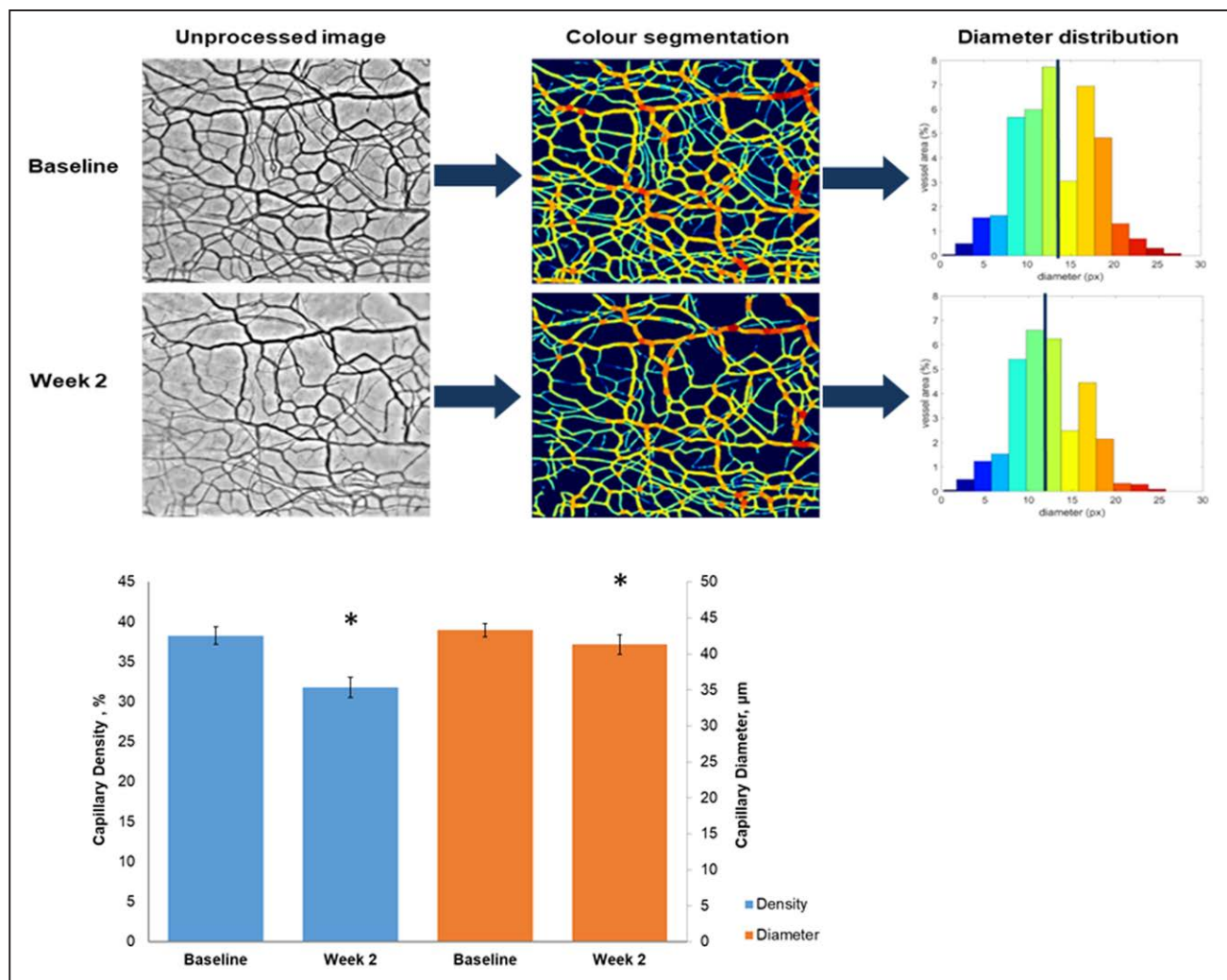


Figure 2. Hemoglobin imaging.

A, Representative images from the conjunctival and episcleral hemoglobin video imaging (1 pixel equals 10 μm^2). One-minute videos were recorded using a modified slit-lamp. The videos were aligned and overlaid to yield a single image, which was segmented and stratified according to the vessel diameter using a pretrained U-Net convolutional neural network. The stratification is represented by the different colors in the images shown. **B**, Microvascular density (blue) and diameter (orange) at baseline and at 2 wk after initiation of treatment. Data are expressed as a mean \pm SEM. * $P < 0.05$.

aimed to gain a better understanding of the underlying mechanisms in otherwise normotensive patients with cancer.

As expected, trough concentrations of pazopanib reached steady state after 2 weeks of dosing. Both systolic and diastolic BP increased after 2 weeks of treatment and this increase was sustained throughout the study. Nearly all patients experienced a rise in BP from their baseline values, and 4 out of 13 patients who finished the trial reached the predetermined static threshold definition of hypertension (defined as SBP of >160 mm Hg or DBP of >100 mm Hg, as per national treatment guidelines at the time of the study protocol submission) and were then initiated on anti-hypertensive therapy. Our data are in keeping with the published literature which demonstrates that $\approx 90\%$ of patients taking VEGF inhibitors experience a rise in BP, with

15% of patients developing a severe form of clinically diagnosed hypertension.⁵

Our study saw an increase in acetylcholine-mediated dilatation following pazopanib. These unexpected results contradict previous data from animal and human studies that have repeatedly demonstrated a key role of the NO pathway in VEGF inhibitor-induced hypertension. Facemire et al²⁴ demonstrated in a murine model, that VEGFR2 blockade leads to reduced eNOS expression, thus clearly showing the deleterious effect of VEGF blockade on eNOS. Subsequently, in human studies, plasma nitrate, a biomarker of NO bioavailability, is reduced following treatments inhibiting VEGF.²⁵ Using Doppler flowmetry and iontophoresis of pilocarpine, Mourad et al²⁶ demonstrated reduced vasodilation following bevacizumab in patients with metastatic colorectal cancer. Finally, using the same technique as used in

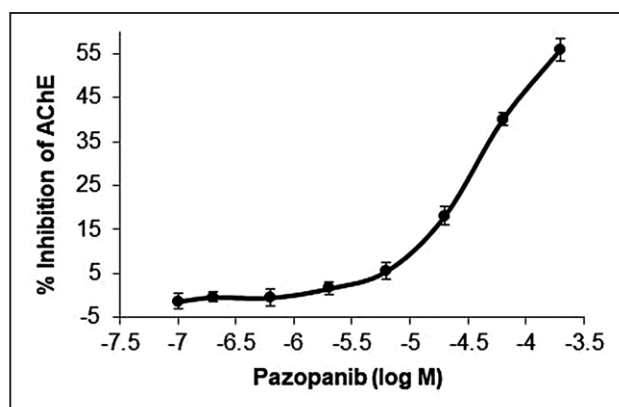


Figure 3. Percentage inhibition of AChE (acetylcholinesterase) activity.

AChE activity was assessed in whole blood samples taken from 10 healthy volunteers and spiked in a range of concentration of pazopanib. ANOVA $P < 0.001$. Data are expressed as a mean \pm SEM.

the present study, Thijs et al²⁷ demonstrated that acute bevacizumab infusion reduced the vasodilatory effect to acetylcholine in healthy volunteers.

The unpredicted results of our FBF study led us to consider, post hoc, that pazopanib may act as an inhibitor of AChE, leading to the observed improvement in acetylcholine-mediated dilatation, without improving endothelial function per se. AChE is the enzyme responsible for the hydrolysis of acetylcholine, and hence its inhibition would increase the half-life of acetylcholine, leading to increased local concentrations of acetylcholine in the forearm. This would potentially confound our efforts to assess endothelial function with this particular challenge agent. In subsequent ex vivo study in healthy volunteers, we demonstrated that in the presence of 210 μ M pazopanib, there was a 55% inhibition of AChE activity. Importantly, a 40% inhibition was seen at the pazopanib concentration (57.9 μ M), which most closely represented the observed trough concentration in the trial, therefore suggesting that the inhibition seen in ex vivo studies is likely to be clinically relevant. We think this is the first study to demonstrate that pazopanib has an inhibitory effect on AChE in humans. Following the completion of our study, a computational analysis reported that pazopanib shares structural and functional properties with donepezil, a marketed AChE inhibitor used for the treatment of dementia.²⁸ This unexpected pharmacological profile of pazopanib would also explain the discrepancy between the findings of Thijs et al²⁷ and our data because bevacizumab is a monoclonal antibody against VEGF with a different molecular structure to pazopanib, thus would not lead to AChE inhibition. Our data relating to AChE inhibition could explain the observed vagal adverse events including bradycardia and reduced stroke volume, resulting in decreased cardiac output and GI toxicity.

Ocular surface hemoglobin video imaging was performed, and a pretrained machine learning code was applied to interpret the data. Following only 2 weeks of

treatment, a significant reduction in both microvascular density and diameter was observed, demonstrating clearly that pazopanib leads to microvascular rarefaction. Our study is the first to investigate the effect of VEGF inhibition on microvascular rarefaction using a technique in which microcirculations can be revisited, allowing a comparison of exactly the same arterioles, capillaries, and venules over time. Our results confirm previous findings in humans, albeit using very different methodology, to look at microvascular effects of VEGF inhibition.^{26,29}

Our data demonstrate that treatment with pazopanib led to a large increase in peripheral vascular resistance. Our findings support the results of a previous study demonstrating that treatment with sunitinib increased PVR.³⁰ Together with the fact that cardiac output was reduced, as a result of the fall in heart rate and stroke volume, our results highlight the importance of increased PVR as the causative hemodynamic mechanism behind the BP increment seen in this trial. Additionally, our study demonstrated an increase in minimum forearm vascular resistance following VEGF inhibition. Although, there are no reference values for MFVR, a study by Svendsen et al¹⁴ gives an indication of normative values by demonstrating that MFVR was 3.7 R units in normotensive and, 3.9 R units in hypertensive subjects. This indicates that the MFVR in our study was normal at baseline, with 3.27 R units, and grossly elevated at the end of the trial at 4.93 R units. Together with the changes seen in the ocular microvasculature, the elevated MFVR suggests a structural remodeling of resistance vessels.

Our study demonstrated significant increases in arterial stiffness as measured by augmentation index and aortic pulse wave velocity following pazopanib. Importantly, these changes remained significant after adjusting for confounding factors, such as mean arterial pressure and heart rate. The increase observed in augmentation index could be a result of vasoconstriction at the sites of pressure wave reflection, as indicated by the increase in PVR and microvascular rarefaction. This would lead to an increase in the magnitude of the reflected pressure wave and subsequent increase in systolic BP. The observed pressure-independent increase in aortic pulse wave velocity suggests that there may be direct structural changes in aortic wall properties, perhaps via changes in the composition of aortic extracellular matrix or fragmentation of elastic fibers. Our data are supportive of the results of Alivon et al³¹ who demonstrated that treatment with TKIs led to increased large artery stiffness, but in contrast to our study, they showed no change in augmentation index.

The deleterious changes we observed in both the microvasculature and large arteries could contribute towards the increased risk of CVD in cancer patients. This was highlighted recently by a large study of cancer survivors in whom CVD mortality was highest within the first year of cancer diagnosis.¹ Together, our

results and the observations in the cancer survivors, highlight the importance of the burgeoning area of cardio-oncology in attempt to minimize CVD complications in oncology patients. A detailed understanding of the underlying cardiovascular mechanisms of anti-cancer therapies could lead to better stratification and treatment of the hemodynamic burdens imposed on this population to minimize CVD complications and maximize survival rates.

Limitations

The complex study design and the underlying malignant disease made recruitment and retention of patients difficult, resulting in the study being relatively small and having a high dropout rate due to early disease progression in patients (n=14). Therefore, we were not able to compare the hemodynamic changes in hypertensive versus nonhypertensive patients, as originally planned. The small number of patients may have led to some of the secondary outcomes being underpowered, such as the L-NMMA response. No placebo group was enrolled as it was unethical to deny treatment from cancer patients who had already exhausted other treatment options. The unexpected AChE inhibition by pazopanib underlines the difficulty of working with drugs with a wide range of pharmacological activities. Thus, as it stands, the current study is unable to draw definitive conclusions about the role of endothelial dysfunction in VEGF-induced hypertension. Ideally, an alternative endothelium-dependent challenge agent, not hydrolyzed by AChE, such as bradykinin, should be used to determine if there is a direct endothelium-dependent effect of pazopanib on NO bioavailability.

Perspectives

We have demonstrated that the VEGF inhibitor pazopanib led to structural remodeling of the resistance vasculature, as evidenced by microvascular rarefaction and a significant increase in PVR, with a subsequent increase in BP. Our findings highlight the importance of altered microvascular structure and increased peripheral vascular resistance as the key mediators of hypertension seen with pazopanib and potentially other VEGF inhibitors. Our data support the current expert opinion for the use of calcium-channel blockers and potassium-sparing diuretics³² in the treatment of VEGF inhibition-induced hypertension due to their ability to reduce PVR. However further studies are needed, particularly as drugs that reduce resistance by relaxing precapillary resistance vessels may not be specific when rarefaction is the primary cause of increased PVR.

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