Arterial stiffness, endothelial dysfunction and impaired fibrinolysis are pathogenic mechanisms contributing to cardiovascular risk in ANCA-associated vasculitis

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see commentary on page 963

OPEN

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Cardiovascular disease is a complication of systemic inflammatory diseases including anti-neutrophil cytoplasm antibody-associated vasculitis (AAV). The mechanisms of cardiovascular morbidity in AAV are poorly understood, and risk-reduction strategies are lacking. Therefore, in a series of double-blind, randomized case-control forearm plethysmography and crossover systemic interventional studies, we examined arterial stiffness and endothelial function in patients with AAV in long-term disease remission and in matched healthy volunteers (32 each group). The primary outcome for the case-control study was the difference in endothelium-dependent vasodilation between health and AAV, and for the crossover study was the difference in pulse wave velocity (PWV) between treatment with placebo and selective endothelin-A receptor antagonism. Parallel *in vitro* studies of circulating monocytes and platelets explored mechanisms. Compared to healthy volunteers, patients with AAV had 30% reduced endothelium-dependent vasodilation and 50% reduced acute release of endothelial active tissue plasminogen activator (tPA), both significant in the case-control study. Patients with AAV had significantly increased arterial stiffness (PWV: 7.3 *versus* 6.4 m/s). Plasma endothelin-1 was two-fold higher in AAV and independently predicted PWV and tPA release. Compared to placebo, both selective endothelin-A and dual endothelin-A/B receptor blockade reduced PWV and increased tPA release in AAV in the crossover study. Mechanistically, patients with AAV had increased platelet activation, more platelet-monocyte aggregates, and altered monocyte endothelin receptor function, reflecting reduced endothelin-1 clearance. Patients with AAV in long-term remission have elevated cardiovascular risk and endothelin-1 contributes to this. Thus, our data support a role for endothelin-blockers to reduce cardiovascular risk by reducing arterial stiffness and increasing circulating tPA activity.

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KEYWORDS: ANCA vasculitis; cardiovascular risk; endothelin blockade

Cardiovascular disease is a common long-term complication of many systemic inflammatory diseases[1]; however, the mechanisms for this are poorly understood. Anti–neutrophil cytoplasm antibody (ANCA)–associated vasculitis (AAV) is an archetypal systemic inflammatory and autoimmune disease that, left untreated, is almost universally fatal.[2] Current immunosuppressive regimens are highly effective, with ≈90% of patients alive at 1 year following diagnosis.[2] However, long-term survival in AAV remains poor, with atherosclerotic cardiovascular disease being the leading cause of death.[3]

AAV is characterized by autoimmune-mediated injury to the endothelium. In health, endothelial production of nitric oxide (NO) and tissue plasminogen activator (tPA), which promote vasodilation and endogenous fibrinolysis, respectively, is balanced by production of the potent vasoconstrictor and prothrombotic, endothelin (ET)-1.[4] ET-1 exerts its effects via 2 receptor subtypes. Vascular smooth muscle cell ET_A receptors mediate vasoconstriction, whereas endothelial ET_B receptors cause vasodilation and clear ET-1, which, in health, maintains NO/ET-1 balance.[4] These local pathways also contribute to regulation of large artery function, with NO deficiency and ET-1 excess leading to increased arterial stiffness. A few studies have shown endothelial dysfunction[5–7] and arterial stiffness,[8] suggestive of NO deficiency in patients with active AAV, and found them to improve with treatment. However, no one has explored these important, independent cardiovascular risk factors[9,10] or endothelium-dependent fibrinolysis[11] in patients with AAV in long-term disease remission.

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Herein, we have found, for the first time, that optimally managed patients with AAV in long-term disease remission and without overt cardiovascular disease have significant arterial stiffness, endothelial dysfunction, and impaired fibrinolysis compared with healthy volunteers. Our data identify an important and novel contribution of ET-1 to these findings, and, using ET-1 blocking strategies, we demonstrate a reduction in these important cardiovascular risk factors in a randomized, double-blind, placebo-controlled crossover study. Our findings provide insight into the excess cardiovascular risk that defines AAV, and they offer a mechanism-based rationale for a new treatment that might improve long-term patient outcomes.

METHODS

Between September 2016 and November 2019, we enrolled patients with AAV and matched healthy volunteers (n = 32 in each group) into a case-control study, where we assessed arterial stiffness and endothelial vasomotor and fibrinolytic function by forearm plethysmography. Twenty-four patients with AAV from this study then entered a double-blind, randomized, placebo-controlled 3-way crossover study to assess the effects of systemic ET-1 blockade on arterial stiffness, fibrinolysis, and monocyte function *ex vivo* (Supplementary Figure S1). All studies were performed at the University of Edinburgh, according to the principles of the Declaration of Helsinki. They were approved by the regional ethics committee and were performed with written informed consent from each subject.

Please see Supplementary Methods for full methods.

RESULTS

Case-control study: forearm vasomotor function and fibrinolytic capacity in AAV

Thirty-two patients with AAV and 32 age- and sex-matched healthy volunteers were enrolled into this study. Baseline subject characteristics are shown in Table 1 and in Supplementary Table S1. For patients, the median (range) time from AAV diagnosis was 4.2 (1.1–13.7) years, and the median (range) time in remission before study entry was 2.4 (0.6–11.2) years. The median (range) estimated 10-year risk of cardiovascular disease for AAV patients was 4.6% (0.5%–29%), which was no different to that of healthy volunteers (3.8% [0.2%–32%]).[12]

Cardiovascular risk factors in AAV. Twelve (38%) patients with AAV had a diagnosis of hypertension, but overall blood pressure (BP) was no different between patients and healthy volunteers. Total and low-density lipoprotein–associated cholesterol were lower in AAV patients than in healthy volunteers (Table 1). These are all in keeping with optimal management of established cardiovascular risk factors in patients with AAV. However, patients with AAV had increased arterial stiffness (pulse wave velocity [PWV]: 7.3 ± 1.3 vs. 6.4 ± 1.0 m/s [$P = 0.016$]; aortic augmentation index [AIx]: 26% ± 11% vs. 20% ± 10% [$P = 0.031$]).

Vasomotor function. There were no significant differences in resting heart rate, BP, or blood flow in the noninfused forearm between patients and healthy volunteers throughout the duration of the study (Supplementary Table S2). There was a dose-dependent increase in blood flow with each vasodilator in both AAV and in health. However, although endothelium-independent vasodilation was no different between the 2 groups, endothelium-dependent vasodilation was reduced in patients with AAV by a mean (95% confidence interval) difference of –6.1 (–3.2 to –8.9) ml/100 ml of tissue/min during acetylcholine 30 μg/min ($P < 0.001$), and –4.6 (–0.3 to –8.9) ml/100 ml of tissue/min during bradykinin 1000 pmol/min ($P = 0.032$; Figure 1). The overall response to acetylcholine was reduced by 28%, and the response to bradykinin was reduced by 17% ($P < 0.001$ for AAV vs. healthy volunteers for both).

Fibrinolytic capacity. At baseline, mean ± SD tPA activity was lower (0.5 ± 0.3 vs. 0.7 ± 0.2 IU/mL; $P = 0.016$) and the plasma concentration of tPA antigen was higher (11.0 ± 4.6 vs. 6.1 ± 2 ng/ml; $P < 0.001$) in patients with AAV compared with healthy volunteers. The mean ± SD plasma

Table 1 | Baseline characteristics for case-control study

Parameter	AAV	Healthy volunteers
N	32	32
Age, yr	55 ± 13	54 ± 12
Male sex	23 (72)	23 (72)
White race	32 (100)	31 (97)
Smoking	6 (19)	4 (13)
Ex-smoker	26 (81)	28 (87)
Never		
Clinical		
Body mass index, kg/m^2	26.8 ± 3.9	25.0 ± 3.9
Systolic BP, mm Hg	126 ± 16	125 ± 14
Diastolic BP, mm Hg	77 ± 9	77 ± 9
Mean arterial pressure, mm Hg	93 ± 11	93 ± 10
Heart rate, bpm	60 ± 6	59 ± 6
Laboratory		
Hemoglobin, g/L	141 ± 12	138 ± 10
Hematocrit, L/L	0.40 ± 0.04	0.39 ± 0.02
Leukocytes, ×10^6/L	6.7 ± 2.0	5.5 ± 2.0
Platelets, ×10^9/L	260 ± 64	242 ± 43
High-sensitivity C-reactive protein, mg/L	2 ± 2	1 ± 1
Creatinine, mg/dl	0.95 ± 0.19	0.84 ± 0.11
eGFR, ml/min per 1.73 m^2	86 ±17	95 ±13
Glucose, mg/dl	86 ± 7	86 ± 7
Total cholesterol, mg/dl	170 ± 31	197 ± 31
LDL cholesterol, mg/dl	93 ± 31	120 ± 31
HDL cholesterol, mg/dl	58 ± 15	58 ± 15
Triglycerides, mg/dl	106 ± 53	106 ± 71
Lp(a), median (range), mg/dl	7 (1–77)	10 (2–76)
Cardiovascular medications		
Aspirin	5 (16)	N/A
ACE inhibitor or ARB	22 (69)	N/A
Calcium channel blocker	4 (13)	N/A
β-Blocker	4 (13)	N/A
α-Blocker	2 (6)	N/A
Diuretic	1 (3)	N/A
Statin	19 (60)	N/A
Ezetimibe	3 (9)	N/A

AAV, anti–neutrophil cytoplasm antibody–associated vasculitis; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BP, blood pressure; bpm, beats per minute; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); N/A, not applicable.
Data are presented as mean ± SD or n (%), unless otherwise indicated.

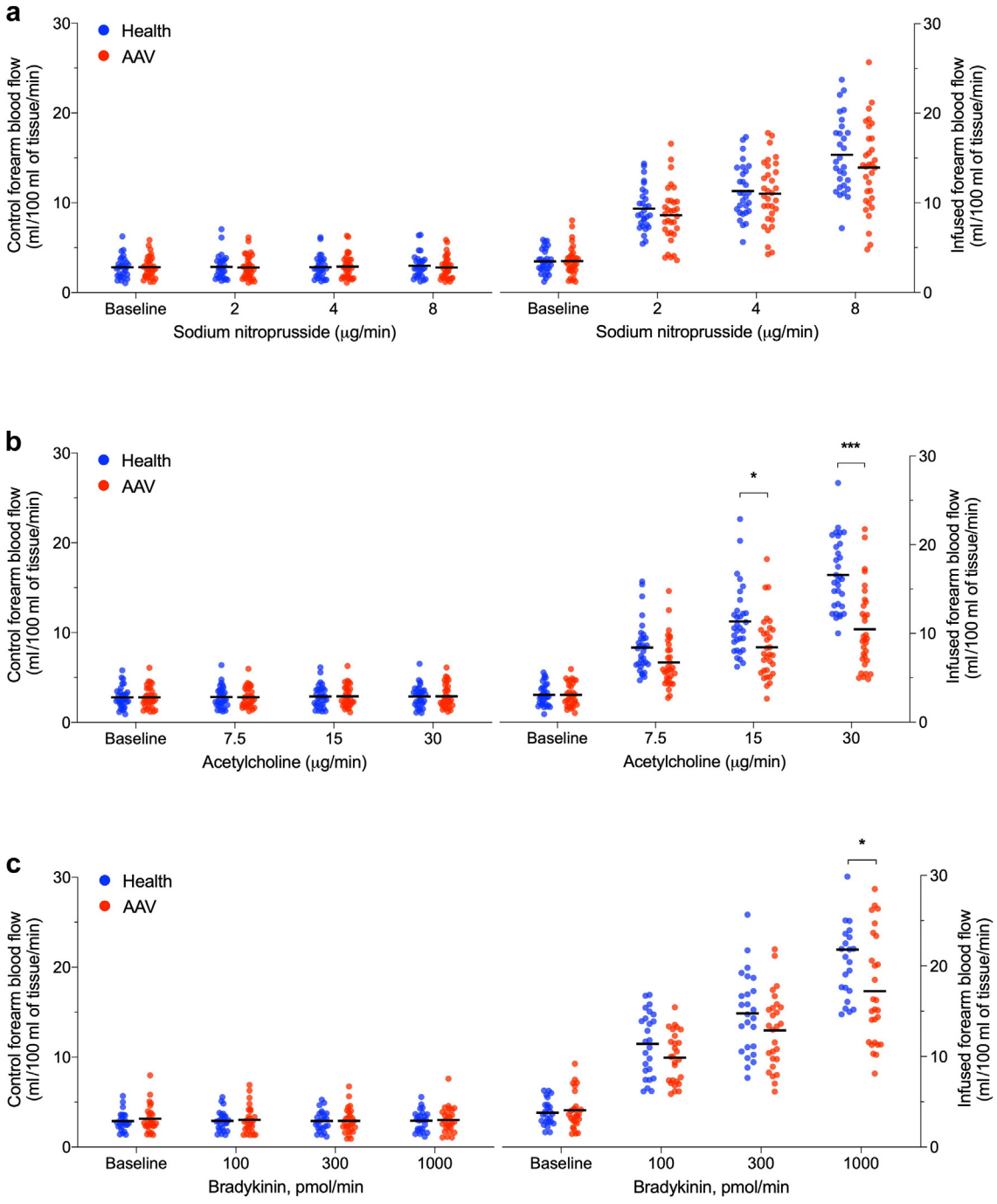


Figure 1 | Vasomotor function in health and anti–neutrophil cytoplasm antibody–associated vasculitis (AAV). Individual forearm blood flow responses during infusion of sodium nitroprusside (**a**), acetylcholine (**b**), and bradykinin (**c**). The horizontal line represents the mean. Analyses by repeated-measures 2-way analysis of variance with Tukey correction for multiple comparisons. (**b**) $^{*}P = 0.012$, $^{***}P < 0.001$. (**c**) $^{*}P = 0.031$.

concentration of plasminogen activator inhibitor-1 (PAI-1), the endogenous inhibitor of tPA, was higher at baseline in AAV (4.7 ± 3.5 vs. 2.7 ± 3.5 ng/ml; $P = 0.015$; Supplementary Table S2). Bradykinin caused a dose-dependent increase in mean (95% CI) net release of active tPA and tPA antigen in both groups, but these effects were markedly reduced in patients with AAV compared with healthy volunteers (–32 [–27 to –38] IU/100 ml of tissue/min and –95 [–73 to –116] ng/100 ml of tissue/min, respectively, during bradykinin 1000 pmol/min; $P < 0.001$ for both vs. healthy volunteers; Figure 2). Overall, net release of active tPA and tPA antigen in response to bradykinin was 50% lower in patients with AAV compared with healthy volunteers. The plasma concentration of PAI-1 was unaffected by bradykinin

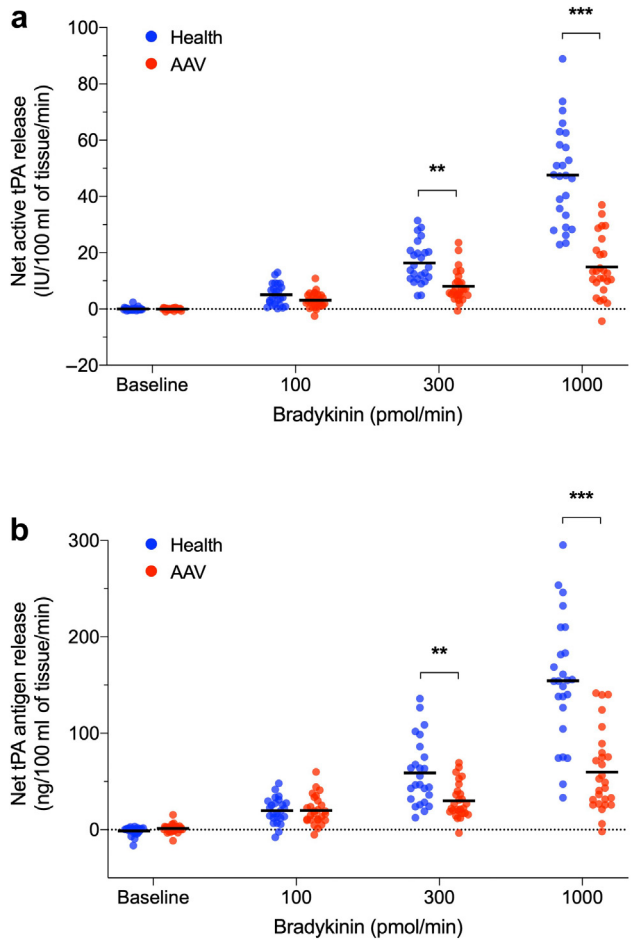


Figure 2 | Fibrinolytic capacity in health and anti–neutrophil cytoplasm antibody–associated vasculitis (AAV). Individual estimated net tissue plasminogen activator (tPA) activity (**a**) and tPA antigen (**b**) release during infusion of bradykinin. The horizontal line represents the mean. Analyses by repeated-measures 2-way analysis of variance with Tukey correction for multiple comparisons. (**a**) $^{**}P = 0.009$, $^{***}P < 0.001$. (**b**) $^{**}P = 0.004$, $^{***}P < 0.001$.

infusion in both groups. Exploratory subgroup analyses showed worse fibrinolytic capacity in patients with proteinase-3 compared with myeloperoxidase AAV (Supplementary Figure S2). However, there were no differences in arterial stiffness, endothelium-dependent vasodilation, or fibrinolytic capacity, depending on whether patients were receiving inhibitors of the renin-angiotensin-aldosterone system or statins, or not (Supplementary Figures S3 and S4).

ET-1 as a determinant of increased cardiovascular risk. Plasma ET-1 concentration was 2-fold higher in patients with AAV compared with healthy volunteers (median [range]: 1.8 [0.6–6.8] vs. 0.9 [0.5–1.5] pg/ml; $P < 0.001$). In all subjects, a higher concentration of plasma ET-1 correlated with increased arterial stiffness, reduced endothelium-dependent vasodilation, and lower basal and stimulated release of active tPA and tPA antigen (Figure 3). After adjusting for age, sex, body mass index, BP, heart rate, and kidney function, a higher plasma ET-1 concentration remained significantly associated with increased arterial stiffness, reduced endothelium-dependent vasodilation, and a lower net release of active tPA and tPA antigen in multiple linear regression models of all subjects (Supplementary Table S3 and Supplementary Figure S5).

The association of plasma ET-1 with PWV and tPA release persisted in adjusted analyses restricted to AAV patients alone. Interestingly, AAV patients receiving current maintenance immunosuppression had greater circulating ET-1 concentrations and worse endogenous fibrinolytic capacity compared with those patients receiving no maintenance treatment (Supplementary Figure S6). Patients receiving no current immunosuppression had been in disease remission for twice as long as those still receiving immunosuppression (5.6 ± 2.3 vs. 2.8 ± 2.8 years; $P = 0.005$). However, there was no relationship between duration of remission and our primary outcomes (PWV: $r = 0.21$, $p = 0.27$; infused forearm blood flow during acetylcholine 30 μg/min: $r = –0.22$, $P = 0.27$; net active tPA release: $r = 0.23$, $P = 0.24$; plasma ET-1: $r = 0.24$, $P = 0.21$; Supplementary Figure S7).

Randomized crossover study: effects of selective and dual ET receptor antagonism on arterial stiffness and fibrinolysis in AAV

Twenty-four patients with AAV from the case-control study entered a fully randomized, double-blind, placebo-controlled, 3-way crossover study. Baseline characteristics were no different to those from the plethysmography study (Supplementary Table S4). All enrolled subjects completed all 3 study visits (placebo, selective ET_A receptor antagonism, and dual $ET_{A/B}$ receptor antagonism) with no adverse events following administration of drugs. At baseline, all parameters studied were no different between the 3 treatment phases (Table 2).

During placebo, PWV and AIx increased by 0.5 ± 0.4 m/s and 3% ±3%, respectively, over the time course of the study, in keeping with waning effects of antihypertensive medications. In contrast, both selective ET_A and dual $ET_{A/B}$ receptor antagonism reduced PWV and AIx to a similar extent. At maximum, and compared with placebo, PWV decreased by ≈10% and AIx decreased by ≈20% during ET antagonism (Figure 4a and b).

Although placebo had no effect, both selective ET_A and dual $ET_{A/B}$ receptor blockade increased the plasma tPA activity and tPA antigen by ≈100% and ≈25%, respectively. Interestingly, these effects were still developing at the end of the study when systemic vascular effects had worn off (Figure 4c and d). Circulating PAI-1 activity and antigen decreased over the course of the study and did not differ between the 3 phases (Supplementary Figure S8).

In addition to their effects on arterial stiffness and fibrinolytic capacity, both selective and dual ET receptor antagonism reduced BP modestly, and to a similar extent, but neither treatment affected heart rate, cardiac output, or stroke index (Supplementary Figure S9). Over the course of the study, we observed an increase in plasma ET-1 with placebo ($P < 0.05$ vs. baseline) but greater, and similar, increases with both ET_A and $ET_{A/B}$ receptor antagonism (Figure 4e).

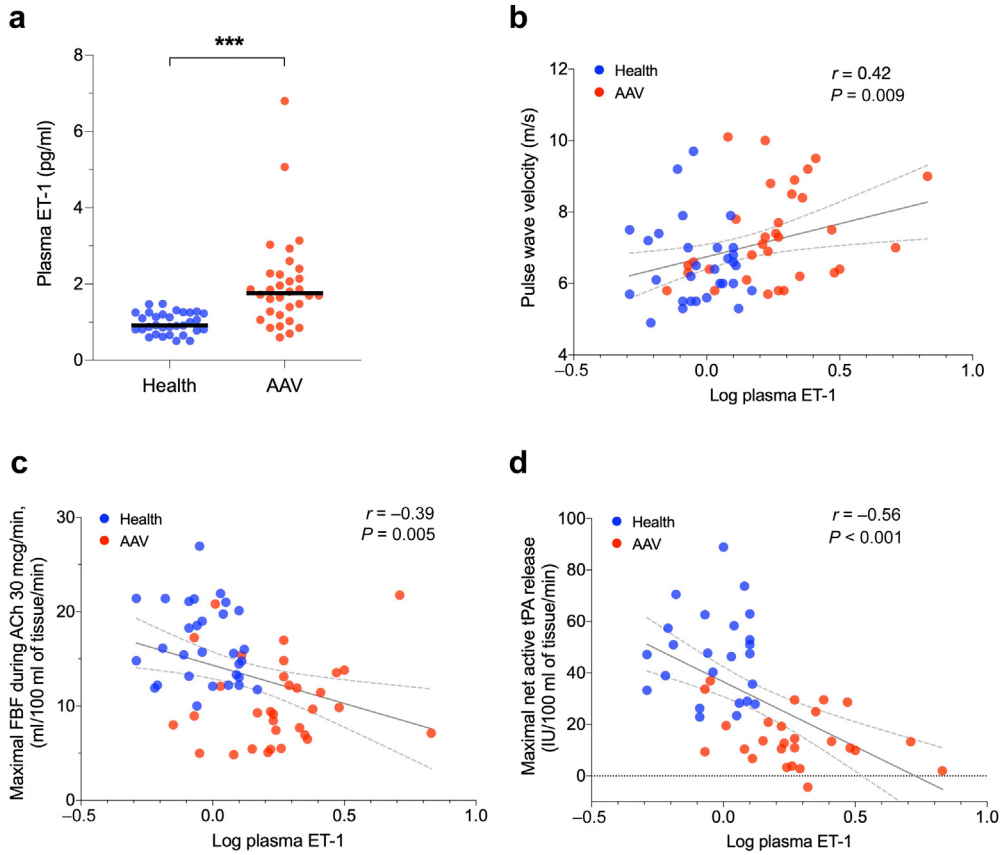


Figure 3 | Plasma ET-1 and cardiovascular risk factors. (**a**) Plasma endothelin-1 (ET-1) in patients with anti–neutrophil cytoplasm antibody–associated vasculitis (AAV) and healthy volunteers. The horizontal line represents the median. Analysis by unpaired *t*-test. ***$P < 0.001$. Scatterplots of individual pulse wave velocity and log plasma ET-1 for all subjects (**b**), individual maximal forearm blood flow (FBF) during acetylcholine (ACh) and log plasma ET-1 for all subjects (**c**), and maximal net tissue plasminogen activator (tPA) activity release during bradykinin and log plasma ET-1 for all subjects (**d**). *r* values are Pearson coefficients. Dashed lines are 95% confidence interval bands.

In vitro study: monocyte clearance of ET-1 in AAV

Our clinical data show that plasma ET-1 is increased in patients with AAV compared with healthy volunteers, and that systemic antagonism of either the ET_A receptor alone or in combination with ET_B increases plasma ET-1 further. We have recently shown that monocytes provide an important clearance mechanism for ET-1 through an ET_B-dependent mechanism.[13] Monocytes are cells of the innate immune system that are central to disease pathogenesis and a key therapeutic target in AAV.[14] Therefore, we hypothesized that monocyte ET_B-dependent clearance of ET-1 is impaired in AAV and, to compensate, that monocyte ET_A take on a role in clearing ET-1 from their surroundings. To examine this, we isolated peripheral blood monocytes from a subset of patients with AAV and healthy volunteers who took part in the case-control study and examined their responses *in vitro* (Figure 5a).

Healthy monocytes were exposed to ET-1 in their surrounding media. Over a 24-hour period, the concentration of ET-1 in the media decreased by ≈60%. This was unaffected by selectively blocking the monocyte ET_A receptor but completely abrogated by ET_B receptor blockade. These data are consistent with our previous reports that monocytes clear ET-1 via the ET_B receptor. Monocytes taken from patients with AAV in long-term disease remission showed impaired ET-1 clearance ($P < 0.001$ vs.

Table 2 | Baseline characteristics for randomized 3-way crossover study

Parameter	Placebo	BQ123	BQ123 + 788
Hemodynamics			
PWV, m/s	7.4 ± 1.5	7.4 ± 1.7	7.5 ± 1.8
AIx, %	24 ± 10	24 ± 11	23 ± 10
Systolic BP, mm Hg	122 ± 10	123 ± 12	123 ± 12
Diastolic BP, mm Hg	74 ± 7	74 ± 9	74 ± 9
Heart rate, bpm	60 ± 8	60 ± 9	60 ± 8
Cardiac index, L/min per m^2	3.2 ± 0.4	3.1 ± 0.4	3.1 ± 0.4
Stroke index, ml/m^2	53 ± 9	53 ± 8	53 ± 9
Biochemistry			
tPA activity, IU/ml	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.3
tPA antigen, ng/ml	8.2 ± 3.8	7.3 ± 3.6	7.9 ± 3.6
PAI-1 activity, IU/ml	9 ± 12	10 ± 10	9 ± 12
PAI-1 antigen, ng/ml	41 ± 57	41 ± 59	38 ± 60
Plasma ET-1, pg/ml	1.3 ± 0.5	1.2 ± 0.5	1.2 ± 0.5

AIx, aorta augmentation index; BP, blood pressure; bpm, beats per minute; ET-1, endothelin-1; PAI-1, plasminogen activator inhibitor-1; PWV, pulse wave velocity; tPA, tissue plasminogen activator.
Values are mean ± SD. Analysis by analysis of variance with Tukey correction for multiple comparisons. No differences between groups for any variable.

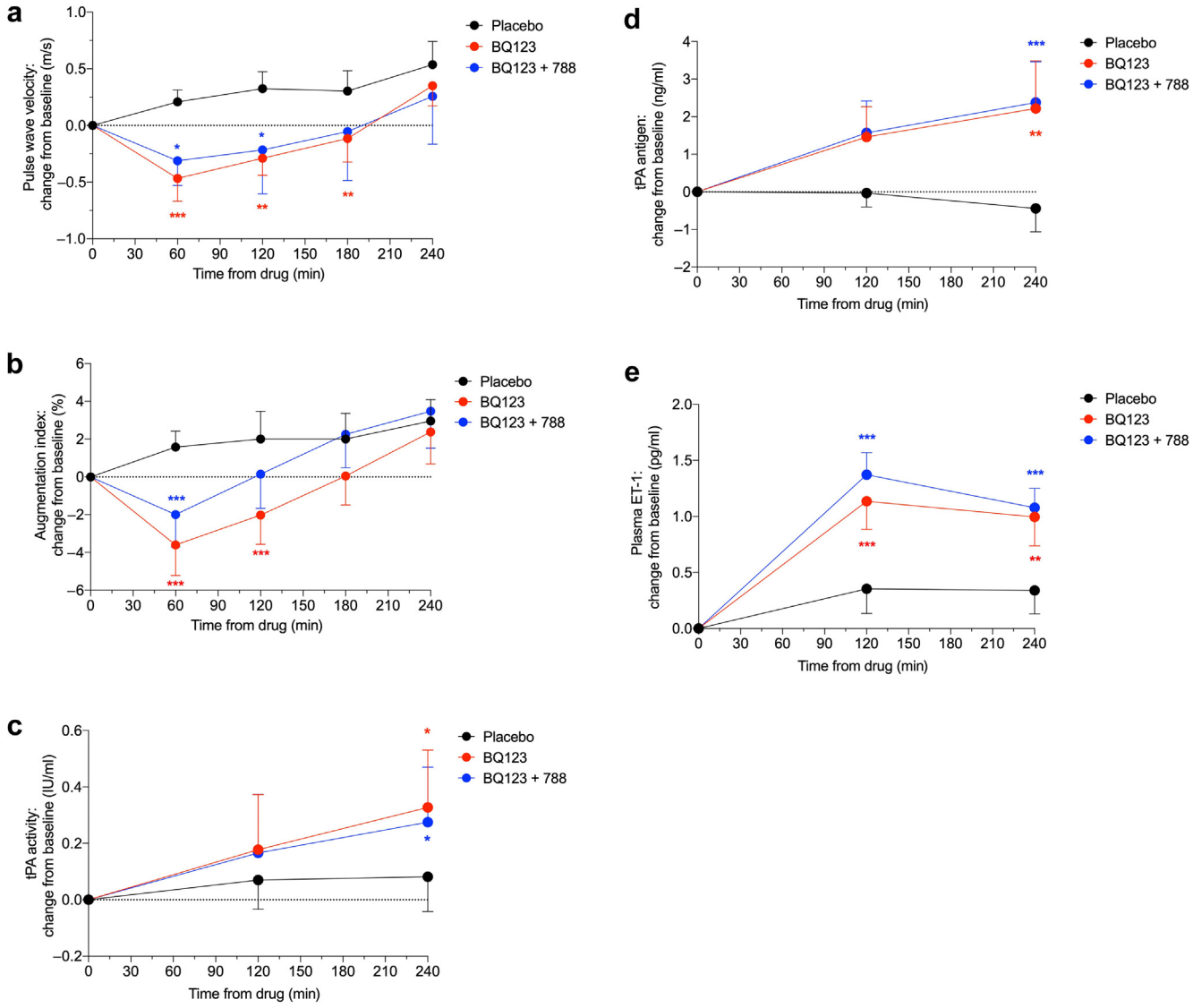
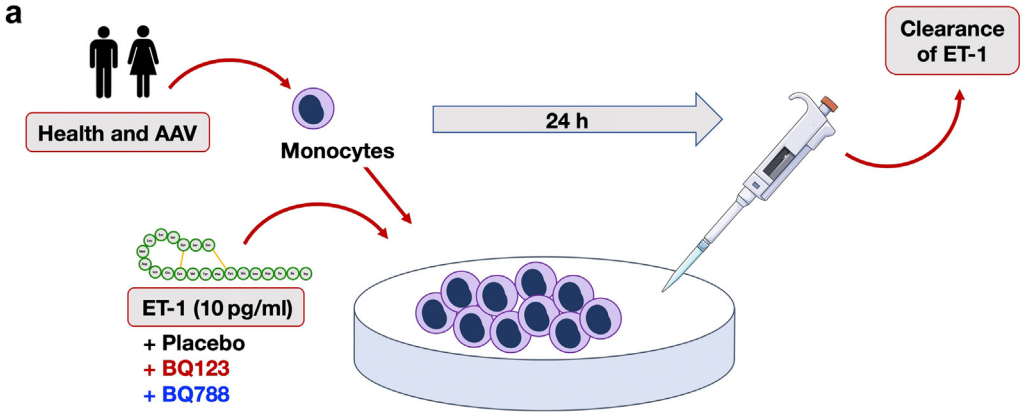


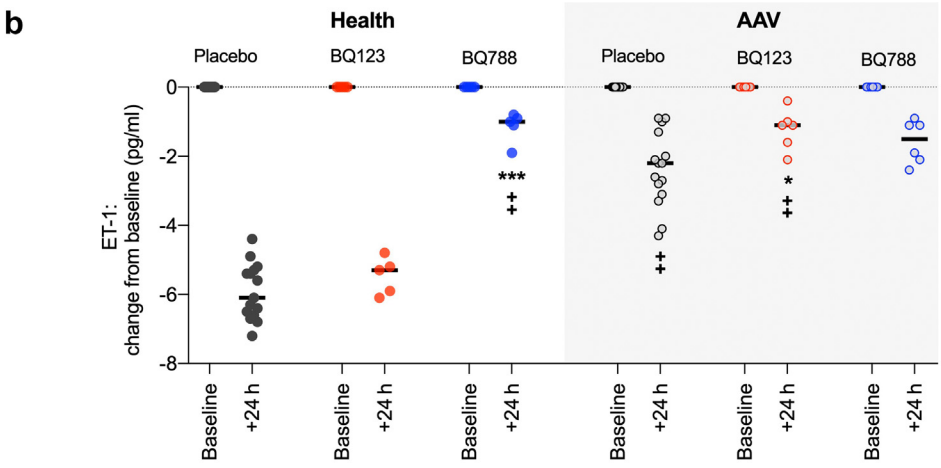
Figure 4 | Effects of endothelin receptor blockade on arterial stiffness and fibrinolysis in anti–neutrophil cytoplasm antibody–associated vasculitis. (**a**) Change in pulse wave velocity from baseline. BQ123: ***$P < 0.001$, **$P = 0.002$ at 120 minutes, **$P = 0.005$ at 180 minutes versus placebo. BQ123 + 788: *$P = 0.013$ at 60 minutes, *$P = 0.041$ at 120 minutes versus placebo. (**b**) Change in augmentation index from baseline. BQ123: ***$P < 0.001$ at 60 minutes, ***$P < 0.001$ at 120 minutes versus placebo. BQ123 + 788: ***$P < 0.001$ versus placebo. (**c**) Change in circulating tissue plasminogen activator (tPA) activity from baseline. BQ123: *$P = 0.021$ versus placebo. BQ123 + 788: *$P = 0.042$ versus placebo. (**d**) Change in circulating tPA antigen from baseline. BQ123: **$P = 0.005$ versus placebo. BQ123 + 788: ***$P < 0.001$ versus placebo. (**e**) Change in plasma endothelin-1 (ET-1) from baseline. BQ123: ***$P < 0.001$, **$P = 0.003$ versus placebo. BQ123 + 788: ***$P < 0.001$ versus placebo. Data are mean ± 95% confidence intervals. Analyses by repeated-measures 2-way analysis of variance with Tukey correction for multiple comparisons.

healthy volunteers); this clearance was reduced further by blocking *either* the ET_A or ET_B receptor (Figure 5b). Interestingly, monocytes isolated from patients with newly diagnosed active AAV (and before receiving any immunosuppressive treatment) also showed impaired clearance of ET-1 ($P < 0.001$ vs. healthy volunteers) but to a lesser extent. In these patients, monocyte clearance of ET-1 was unaffected by ET_A receptor blockade but reduced by blocking ET_B, effects similar to those seen in monocytes from healthy volunteers (Supplementary Figure S10). Preincubation with a range of immunosuppressive drugs had no effect on clearance of ET-1 by monocytes isolated from healthy volunteers (Supplementary Figure S11).

Flow cytometry study of monocyte-platelet aggregates in AAV

Monocytes interact with the endothelium and with platelets to promote endothelial dysfunction and thrombosis, respectively.[15,16] Activation of the monocyte ET_B receptor leads to the generation of NO, which limits these adverse cell-cell interactions. Thus far, we have shown that patients with AAV in long-term disease remission have impaired clearance





	Health			AAV		
	Placebo	BQ123	BQ788	Placebo	BQ123	BQ788
Change in [ET-1] at 24 h (pg/ml)	−5.9 ± 0.8	−5.5 ± 0.5	−1.1 ± 0.4	−2.4 ± 1.1	−1.2 ± 0.6	−1.6 ± 0.6
P vs. placebo		0.93	**<0.001**		**0.023**	0.34
P vs. BQ123			**<0.001**			0.99
P vs. health				**<0.001**	**<0.001**	0.98

Figure 5 | Monocyte clearance of endothelin-1 (ET-1) in health and anti–neutrophil cytoplasm antibody–associated vasculitis (AAV). Peripheral blood monocytes were isolated from venous blood from healthy volunteers and patients with AAV. Monocytes were then incubated with human ET-1 (10 pg/ml) following treatment with placebo, BQ123, or BQ788. (**a**) After 24 hours, the concentration of ET-1 in the culture medium was quantified to provide a measure of 24-hour monocyte clearance of ET-1. (**b**) Graph and table of 24-hour clearance of 10 pg/ml ET-1 in presence of placebo (black solid circles), BQ123 (red solid circles), and BQ123/788 (blue solid circles) by monocytes from healthy volunteers (left panel) and from patients with AAV (right panel; placebo: open circles; BQ123: red open circles; BQ788: blue open circles). The horizontal line represents the median. Health: BQ788: ***$P < 0.001$ versus placebo and BQ123. AAV: placebo: $^{\ddagger}P < 0.001$ versus health; BQ123: $^{\ddagger}P < 0.001$ versus health, *$P = 0.02$ versus placebo. Values in table are mean ± SD. Analysis by 2-way analysis of variance with Tukey correction for multiple comparisons.

of circulating ET-1 and reduced acetylcholine-dependent vasodilation and bradykinin-stimulated tPA release. These reflect impairment of monocyte ET_B receptor and endothelial function, respectively. So finally, we examined monocytes and their interactions with platelets using fluorescently labeled cell sorting.

The number of circulating classic ($CD14^{++}CD16^{-}$) monocytes was lower in patients with AAV compared with healthy volunteers (Figure 6a), whereas nonclassic and intermediate monocytes did not differ (Supplementary Table S5). Interestingly, a lower classical monocyte count was associated with a higher plasma ET-1 and lower

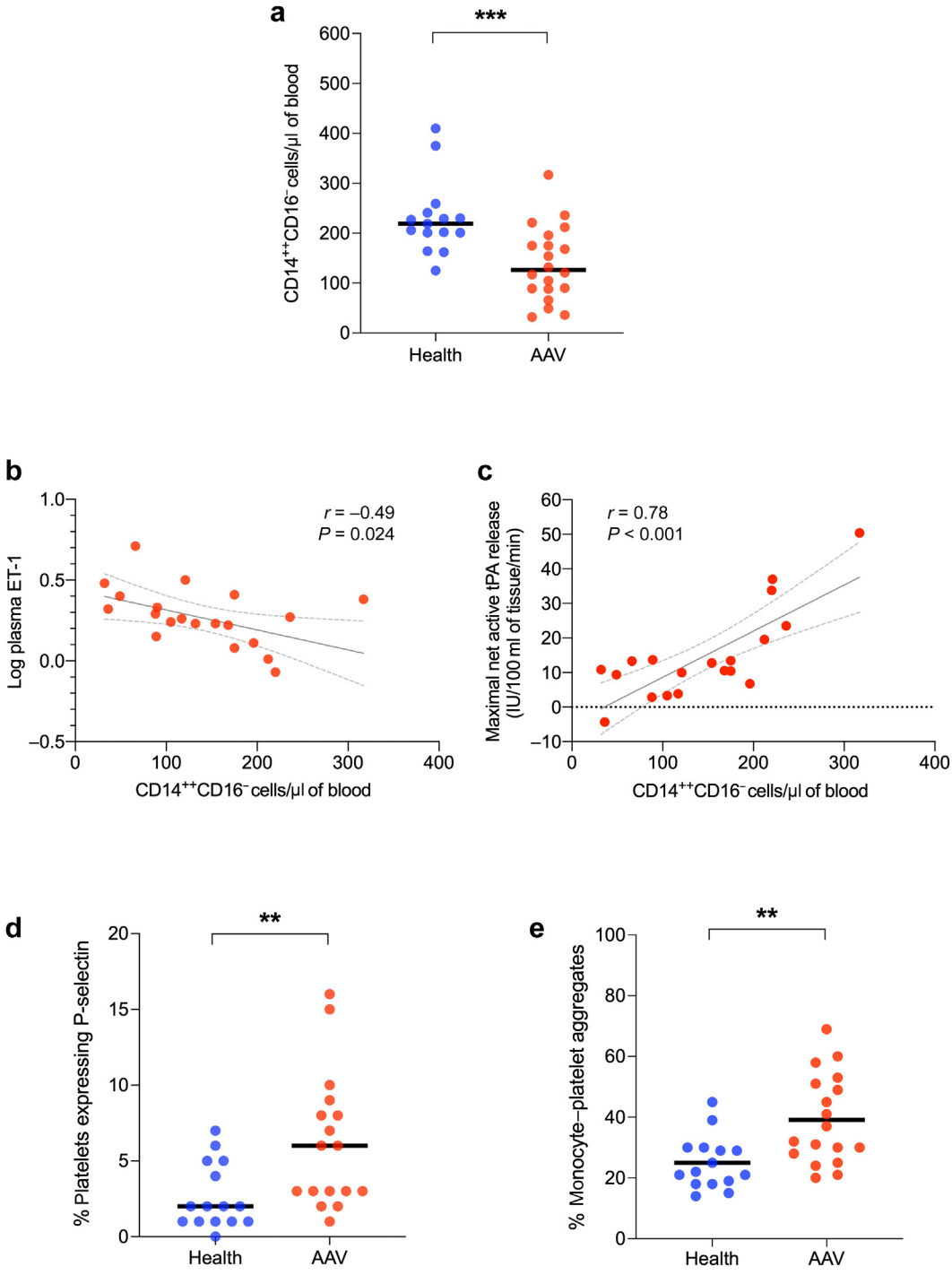


Figure 6 | Monocytes and platelet activation in anti–neutrophil cytoplasm antibody–associated vasculitis (AAV) patients. (**a**) Circulating classic monocytes in healthy volunteers and patients with AAV. The horizontal line represents the median. Analysis by Mann-Whitney test. ***$P < 0.001$. Scatterplots of circulating classic monocytes and log plasma endothelin-1 (ET-1) (**b**) and maximal net release of tissue plasminogen activator (tPA) (**c**) from patients with AAV. *r* values are Pearson coefficients. Dashed lines are 95% confidence interval bands. (**d**) Platelet P-selectin expression in healthy volunteers and patients with AAV. The horizontal line represents the median. Analysis by Mann-Whitney test. **$P = 0.004$. (**e**) Monocyte-platelet aggregates in healthy volunteers and AAV. The horizontal line represents the median. Analysis by Mann-Whitney test. **$P = 0.002$.

fibrinolytic capacity (Figure 6b and c). Although the number of circulating platelets did not differ between the 2 groups, patients demonstrated a greater degree of P-selectin expression, in keeping with increased platelet activation (Figure 6d). Finally, patients with AAV had a greater number of circulating monocyte-platelet aggregates compared with

healthy volunteers—another robust measure of platelet activation[17] and cardiovascular risk[18] (Figure 6e)—and this associated with higher ET-1 concentrations ($r = 0.45$, $P < 0.05$).

DISCUSSION

Cardiovascular disease is the leading cause of death in patients with AAV in the longer term. However, the mechanisms underlying this increased cardiovascular risk are poorly understood. Herein, we have shown that, despite optimal management of conventional risk factors, patients with AAV in long-term disease remission have increased arterial stiffness, impaired endothelial vasomotor and fibrinolytic function, and increased platelet activation. We have also shown that these pathogenic mechanisms for acute cardiovascular events are strongly associated with the ET system and can be ameliorated by ET blockade. Our data suggest that clinical trials targeting the ET system are warranted to reduce the burden of cardiovascular disease in patients with AAV.

We specifically enrolled a low-risk group of patients with AAV. The estimated 10-year risk of cardiovascular disease of our patient cohort was within the lowest-risk tertile defined by the American College of Cardiology Atherosclerotic Cardiovascular Disease Risk Estimator.[12] Despite this, we found that PWV and AIx, gold standard measures of arterial stiffness, were ≈15% and ≈20% higher, respectively, in patients with AAV compared with healthy volunteers. These were not explained by differences in BP or heart rate, and so they likely represent the direct effects of the disease on arterial structure. The magnitude of these differences is associated with a 10% to 15% increased risk of future cardiovascular events and cardiovascular mortality, even following adjustment for age and baseline risk factors, like hypertension and chronic kidney disease.[19] More important, reducing PWV improves outcomes.[20] Altered arterial structure is linked to altered endothelial function. In keeping with this, patients with AAV had ≈25% reduced response to acetylcholine, an endothelium- and NO-dependent vasodilator. This degree of endothelial dysfunction is characteristic of patients with coronary artery disease,[21] where a focus on reducing cardiovascular risk is established. Our independent associations between higher ET-1 concentrations, greater arterial stiffness, and poorer endothelium-dependent vasodilation are consistent with the known vascular effects of ET-1.[22,23] These are novel findings in AAV and suggest that an upregulated ET system is central to the development and progression of cardiovascular risk in this at-risk patient group. Previous small studies have shown increased arterial stiffness and endothelial dysfunction in active AAV, which improved following treatment.[5–8] Our data from patients in long-term disease remission show marked systemic vascular dysfunction, suggesting that such improvements may be transient or limited.[24]

The risk of arterial and venous thromboses is increased in AAV, during both active disease and in remission.[25,26] A few studies have explored the role of impaired fibrinolysis as one explanation for this risk using surrogate measures, such as concentrations of clotting factors and fibrin degradation products.[27,28] Our data are the first to directly assess the functional capacity of the endothelium in this respect. We show an ≈50% reduction in stimulated tPA release in patients with AAV in disease remission, which is similar or worse than in patients with previous myocardial infarction[21] and advanced chronic kidney disease.[29] More important, impaired endothelial tPA release is associated with risk of future cardiovascular events.[11] Our early studies in healthy volunteers suggested no relationship between ET-1 and tPA release.[30,31] The presence of compensatory endothelial NO generation pathways that contribute to tPA release in health[32] may explain these contrasting results. Our findings in AAV likely reflect the combined effects of ET-1–mediated inhibition of endothelial NO generation[33–35] and enhanced monocyte adhesion due to impaired ET_B function, both of which contribute to regulation of endogenous fibrinolysis.[32,36] In addition, at baseline, tPA activity was lower in AAV than in health, and this might be explained, in part, by the higher plasma concentration of PAI-1, the endogenous inhibitor of tPA, in AAV. PAI-1 is a damage response protein produced by the endothelium, the liver, and activated platelets,[37] all of which may be affected by AAV in the long-term.

We found no difference in arterial stiffness, endothelial function, and tPA release in AAV patients receiving renin-angiotensin-aldosterone system inhibitors or statins compared with those who were not. Given these medications improve vascular function in other high-risk groups,[38–41] these data highlight that current cardiovascular risk reduction strategies may be ineffective in AAV and that novel treatments are urgently needed. Similarly, arterial stiffness and endothelial function were no different between those currently receiving immunosuppression and those on no regular immunosuppression, although we did observe poorer fibrinolytic capacity and higher plasma ET-1 concentrations in the former. The need for immunosuppression might suggest active subclinical inflammation, which has been linked to increased ET-1 generation.[42,43] In contrast, acute local and systemic inflammation results in an *increase* in endothelial tPA release, potentially as a protective mechanism against thrombosis.[44,45] Whether this holds true in chronic inflammation is not known. Although we cannot exclude the contribution of active, low-level inflammation to our findings, we took all reasonable steps to minimize this as a source of confounding.

ET-1 is a powerful endogenous vasoconstrictor and contributes to the development and progression of autoimmune and cardiovascular disease.[46] We found that patients with AAV had a higher plasma ET-1 concentration compared with matched healthy volunteers and that a higher plasma ET-1 independently predicted increased arterial stiffness and reduced endothelial function. Thus, we hypothesized that antagonism of the ET system might improve these independent cardiovascular risk factors. Currently, there are 3 ET receptor antagonists licensed for the orphan indications of pulmonary arterial hypertension and scleroderma digital

ulceration. These drugs have variable ET_A:ET_B receptor-blocking ability, and only the dual $ET_{A/B}$ antagonist, bosentan, is available off patent. To provide proof of concept, we used the well-established and widely available probe compounds, BQ123 and BQ788, to provide pharmacologically effective ET_A and ET_B receptor antagonism, respectively.[47,48] We show, for the first time, that both selective ET_A and dual $ET_{A/B}$ receptor blocking approaches reduce PWV and increase plasma tPA in patients with AAV. These effects, if sustained with regular therapy, could reduce the risk of acute cardiovascular events in these patients. Notably, PWV decreased by ≈10% during ET blockade, and a reduction of this magnitude in groups of patients with varying baseline cardiovascular risk is associated with ≈10% and ≈40% relative reductions in mortality and cardiovascular events, respectively.[20,49] Although we observed a small reduction in BP (≈5 mm Hg), this is unlikely to have contributed significantly to these effects as the reduction in PWV was greater than might be expected and persisted after BP had returned to baseline.[50] Similarly, the increase in tPA continued after BP had returned to the pretreatment baseline.

The higher plasma ET-1 concentration in patients with AAV may be due to increased generation or reduced clearance of the peptide, the latter being largely ET_B receptor dependent.[46] Given the generalized endothelial dysfunction we observed in AAV, it is likely that endothelial ET_B function will be disrupted, which would impair ET-1 clearance. Our findings also suggest a role for monocytes in this regard. These innate immune cells are critical to the pathogenesis of AAV and are also a treatment target.[14] Recently, we have shown a role for monocytes in the cardiovascular system, where they provide a novel clearance mechanism for ET-1 via surface ET_B receptors with little contribution from ET_A.[13] This pathway is important in limiting the development and complications of hypertension. In the current study, we show that monocyte ET_B clearance of ET-1 is impaired in AAV and that, as a compensatory mechanism, monocyte ET_A receptors take on this role. This paradigm is supported by our crossover study, where both selective ET_A and dual $ET_{A/B}$ antagonism increased plasma ET-1, typically recognized as a biomarker of ET_B blockade alone.[51] To our knowledge, this is the first report to show the ET_A receptor playing a role in ET-1 clearance *in vitro* and in clinical studies.

Interestingly, monocytes from patients with active AAV before receiving immunosuppressive treatment did not show the same degree of impairment in clearance of ET-1 or a role for ET_A receptors in this. Thus, impaired monocyte ET-1 clearance in patients with AAV in long-term remission might be partly treatment related and suggests the potential for immunosuppression to contribute to cardiovascular risk. This is supported by our finding of higher plasma ET-1 concentrations and poorer fibrinolytic capacity in those patients receiving maintenance immunosuppression compared with those on no treatment. More disease relapses, and so more cumulative immunosuppression, in patients with proteinase-3–AAV may explain their reduced fibrinolytic capacity compared with those with myeloperoxidase-AAV. Although preincubation with immunosuppression did not impair clearance of ET-1 by healthy monocytes *in vitro*, we suggest that immunosuppression might still contribute to this in patients over the longer-term. In addition, monocyte[52] and platelet[53] ET_B receptor activation generates NO as a strategy to inhibit these cells interacting and triggering vascular injury and thrombosis. The increased number of monocyte-platelet aggregates we observed in AAV compared with health, and their association with higher ET-1 concentrations, provides another line of evidence supporting impaired monocyte ET_B function in these patients. Together, these findings suggest a chronic, systemic impairment in NO signaling across the endothelium, monocytes, and platelets in AAV. From a clinical perspective, this may be restored using an ET-blocking approach.

Our study using local, systemic, and *in vitro* approaches has several strengths. We used a randomized, double-blind, placebo-controlled design throughout and enrolled well-matched controls. The demographics and disease characteristics of the patients studied herein are typical of patients presenting to the clinic and similar to those seen in other landmark studies in the field, making our findings generalizable.[54,55] We focused on AAV patients in stable remission to exclude the confounding effects of acute inflammation on arterial stiffness and endothelial function.[5,8] We excluded those with diabetes, previous cardiovascular disease, current smoking, and chronic kidney disease to minimize confounding. We also excluded patients who had untreated or treated hypertension and dyslipidemia *before* the diagnosis of AAV to further minimize confounding. Thus, AAV patients with hypertension and dyslipidemia included in our study may be considered to have developed these risk factors as a consequence of AAV and/or its treatment.[13,25,56] Moreover, two-thirds of patients were receiving a renin-angiotensin-aldosterone system blocker and a statin, both standard-of-care cardioprotective medications that also improve endothelial function.[38–41] Consequently, BP and circulating lipids were well controlled and no different to healthy volunteers. These strict inclusion criteria allow us to infer with confidence that the observed differences reflect the dramatic legacy of AAV and its treatment on the cardiovascular system. We suspect that these differences would be even greater in a broader cohort of AAV patients. Our findings may also be of relevance to other systemic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematous, both of which are associated with an upregulated ET system and increased cardiovascular risk.[57,58]

A limitation of our study is the narrow ethnicity of our subjects, but this reflects our local population. In addition, we did not assess AAV patients for anti-tPA or plasminogen antibodies, which have been shown to impair fibrinolysis *in vitro*.[59] However, the low prevalence of these antibodies in AAV patients in remission[59] suggests that such antibodies are unlikely to account for the differences observed. Finally, whether long-term ET blockade would translate into a reduced incidence of cardiovascular events in AAV is unclear, but this has been shown in other patient groups.[60]

AAV is a systemic inflammatory disease characterized by widespread endothelial injury with severe manifestations, such as rapidly progressive glomerulonephritis and pulmonary hemorrhage. The treatment of these life-threatening disease features, and modulation of the immune response more broadly, has been the major focus of clinical studies in AAV to date. Current immunosuppressive regimens have transformed AAV into a chronic, relapsing, and remitting disease, with cardiovascular disease emerging as its most common complication. There is a clear unmet need for targeted interventions that address this risk. Our data from observational and interventional clinical studies, and *in vitro* experimental work, provide deep phenotyping of the long-term cardiovascular risk in patients with AAV. They provide a rationale for future studies to assess the potential of ET-blocking strategies, using currently available drugs, to reduce this risk.

DISCLOSURE

ND has acted as a consultant for Travere Therapeutics. All the other authors declared no competing interests.

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Clinical Trial Registration: https://clinicaltrials.gov. Unique Identifier: NCT02062346

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Table S1. Characteristics of anti–neutrophil cytoplasm antibody–associated vasculitis (AAV) patients in case-control study.

Table S2. Baseline blood flow and fibrinolytic parameters.

Table S3. Multiple linear regression models.

Table S4. Baseline characteristics of anti–neutrophil cytoplasm antibody–associated vasculitis (AAV) patients in crossover study.

Table S5. Monocytes in health and anti–neutrophil cytoplasm antibody–associated vasculitis (AAV).

Figure S1. CONSORT flow diagram.

Figure S2. Fibrinolytic capacity in anti–neutrophil cytoplasm antibody (ANCA)–associated vasculitis (AAV) patients by ANCA type.

Figure S3. Effects of renin-angiotensin-aldosterone system (RAAS) inhibitor (RAASi) use.

Figure S4. Effects of statin use.

Figure S5. Plasma endothelin-1 (ET-1) univariate associations.

Figure S6. Endothelin-1, fibrinolysis, and immunosuppression.

Figure S7. Effects of immunosuppression.

Figure S8. Plasminogen activator inhibitor-1 during endothelin (ET) antagonism.

Figure S9. Systemic hemodynamics during endothelin (ET) antagonism.

Figure S10. Monocyte clearance of endothelin-1 (ET-1).

Figure S11. Immunosuppressive drugs and clearance of endothelin-1 (ET-1).

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