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Potential Vascular Mechanisms of Ramipril Induced Increases in Walking Ability in Patients with Intermittent Claudication.

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ABSTRACT (249 words)

Rationale – We recently reported that ramipril more than doubled walking times in peripheral artery disease patients with intermittent claudication.

Objective - To conduct exploratory analyses of the effects of ramipril therapy on circulating biomarkers of angiogenesis/arteriogenesis, thrombosis, inflammation and leukocyte adhesion in patients with intermittent claudication.

Methods and Results - 165 patients with intermittent claudication (mean (SD), 65.3 (6.7) years), were administered ramipril 10mg/d (n=82) or matching placebo (n=83) for 24 weeks, in a randomized, double-blind study. Plasma biomarkers of angiogenesis/arteriogenesis (vascular endothelial growth factor, VEGF-A; fibroblast growth factor, FGF-2), thrombosis (D-dimer; von Willebrand Factor, vWF; thrombin-antithrombin III, TAT), inflammation (high sensitivity C-reactive protein, hsCRP; osteopontin, OPN), and leukocyte adhesion (soluble vascular cell adhesion molecule-1, sVCAM-1; soluble intracellular adhesion molecule-1, sICAM-1) were measured at baseline and 24 weeks. Relative to placebo, ramipril was associated with increases in VEGF-A by 38% (95% CI, 34 to 42%) and FGF-2 by 64% (44 to 85%; P<0.001 for both), and reductions in D-dimer by 24% (-30 to -18%), vWF by 22% (-35 to -9%), TAT by 16% (-19 to -13%), hsCRP by 13% (-14 to -9%), OPN by 12% (-14 to -10%), sVCAM-1 by 14% (-18 to -10%), and sICAM-1 by 15% (-17 to -13 %; all P<0.001). With the exception of vWF all the above changes correlated significantly with the change in maximum walking time (P= 0.02-0.001) in the group treated with ramipril.

Conclusions – Ramipril is associated with an increase in biomarkers of angiogenesis/arteriogenesis and reduction in markers of thrombosis, inflammation and leukocyte adhesion. This study informs strategies to improve mobility in patients with intermittent claudication.

ClinicalTrials.gov Identifier, NCT00681226

Key Words: biomarkers; peripheral artery disease; inflammation; angiogenesis; thrombosis

Non-standard Abbreviations and Acronyms:

ABI; ankle brachial index

ACE; angiotensin converting enzyme

Ang II; angiotensin II

ARBs; angiotensin receptor blockers

BK; bradykinin

ELISA; enzyme-linked immunosorbent assay

FGF-2; fibroblast growth factor

hsCRP; high sensitivity C-reactive protein

NO; nitric oxide

OPN; osteopontin

PAD; peripheral artery disease

sICAM; soluble intracellular adhesion molecule-1

sVCAM-1; soluble vascular cell adhesion molecule-1

TAT; thrombin-antithrombin III

VEGF-A; vascular endothelial growth factor

VEGFR₁; vascular endothelial growth factor receptor 1

VEGFR₂; vascular endothelial growth factor receptor 2

sVEGFR₁; soluble vascular endothelial growth factor receptor 1

vWF; von Willebrand factor

We have recently demonstrated that treatment with ramipril significantly improves walking ability and the physical functioning aspects of quality of life in peripheral artery disease (PAD) patients who have intermittent claudication.¹ Ramipril induced a 123% (range, 103% to 142%) increase in maximum walking time when administered over 24 weeks.

Understanding the mechanisms contributing to this improvement in functional capacity may identify important targets for improving the medical management of intermittent claudication.

Angiotensin converting enzyme (ACE) inhibitor therapy may mediate improved functional capacity through multiple mechanisms including increased peripheral blood flow and adaptations in skeletal muscle structure and function.¹ Elevated blood flow may be mediated via vasodilatation through reduction in angiotensin II (AngII), sympathetic inhibition, improvement in endothelial function through preservation of bradykinin (BK) and formation of new collaterals (arteriogenesis) or muscle capillaries (angiogenesis).² There is substantial evidence to support that both the renin-angiotensin and bradykinin pathways play a crucial role in the regulation of arteriogenesis and angiogenesis through modulation of a number of angiogenic factors including vascular endothelial growth factor (VEGF-A) and fibroblast growth factor (FGF-2).³

Activation of the renin-angiotensin system, through increase in the production of AngII is also closely related to local vascular inflammation.⁴ AngII functions as a potent inflammatory stimulus and up-regulates the expression of many redox-sensitive cytokines, chemokines, and growth factors involved in the pathogenesis of atherosclerosis including C-reactive protein (CRP)⁵ and osteopontin (OPN).⁶ Ang II also modulates vascular inflammation by regulating the expression of adhesion molecules such as soluble intracellular adhesion molecule-1 (ICAM-1), and (soluble intracellular adhesion molecule-1VCAM-1), leading to leukocyte activation and recruitment.⁷ Furthermore, a growing body of evidence indicates that a pro-thrombotic state can be induced by both the renin-angiotensin and bradykinin systems, through nitric oxide (NO) and downstream effects on proteins involved in, stimulated by, or associated with thrombosis including fibrinogen, D-dimer,⁸ von Willebrand Factor (vWF),⁹ and thrombin-antithrombin III complex (TAT).¹⁰

Thus, it has been suggested that ACE inhibitors may favourably modify inflammation and thrombosis, both of which are critical in the complications of atherosclerosis¹¹⁻¹³ and functional decline in PAD patients.¹⁴

In the current study, we conducted exploratory analyses of the potential mechanisms underlying the improvement in functional capacity observed following ramipril therapy, through assessment of circulating biomarkers related to angiogenesis, inflammation, leukocyte adhesion and thrombosis.

METHODS

This investigation is a sub-study of a previous trial for which primary endpoints were reported in February 2013.¹ While the original study involved 3 participating centres, the current sub-study was restricted to a single site (165 participants recruited from The Alfred Hospital, Melbourne, Australia). In the 47 participants recruited from The Townsville Hospital and Royal Women's Brisbane Hospital, Australia, blood collection for exploratory endpoints was not performed.

Participant Identification

The institutional review board of the Alfred Hospital approved the protocol. Participants provided written informed consent and the study was performed in accordance with the Declaration of Helsinki 2000. Data collection and interventions were performed at the Alfred Hospital, Melbourne, Australia between May 10, 2008 and August 23, 2011. Of the 165 patients completing the study, 105 were from general practice clinics in the metropolitan area of Melbourne, 22 from among patients diagnosed with PAD in the non-invasive vascular laboratory of the Alfred Hospital, and 38 participants were recruited from newspaper advertisements.

Inclusion and Exclusion Criteria

The inclusion criteria were: An ankle brachial index (ABI) of less than 0.9 at rest in at least one leg; history of intermittent claudication (unilateral or bilateral) (as defined by the Edinburgh Claudication Questionnaire¹⁵ and following clinical examination) which was stable for the previous six months, and a stable medication regime for at least six months. The exclusion criteria were resting brachial blood pressure $\geq$ 160/100 mmHg; use of either ACE inhibitors or angiotensin receptor blockers (ARBs) currently or within the prior 6 months; use of potassium sparing diuretics or potassium supplements currently or within the prior 6 months; renal failure (serum creatinine >0.2 mmol/L); renal artery stenosis; previous coronary and/or lower extremity revascularization procedures; myocardial infarction in the previous 3 months; major surgery planned during the following year; critical limb ischemia (ischaemic ulcer[s] or minor gangrene), and any condition other than PAD limiting walking ability including limiting coronary artery disease, chronic obstructive pulmonary disease and musculoskeletal conditions (assessed during physical examinations and medical history performed by the study physician).

Randomisation and Masking

Patients were randomly assigned to receive either ramipril (Ramace, sanofi-aventis) (10mg/d for 24 weeks) or matching placebo in a parallel-group, double-blind design as previously described.¹ Once the randomisation list was generated by the Epidemiology & Preventive Medicine Department of the Alfred Hospital, Melbourne, it was forwarded to the The Alfred Hospital Clinical Trials Pharmacy which prepared and supplied the medication. None of the investigators had access to the randomisation list. Ramipril and placebo tablets were identical and supplied in consecutively numbered drug packs. Each patient was assigned a randomisation number and received tablets from the corresponding drug pack. Investigators and patients were blinded to drug assignment. Investigators did not have access to baseline data when they performed follow-up measurements and patients were not asked which treatment they thought they were receiving. No patients assigned to placebo crossed over to ramipril during the trial or vice versa.

Treadmill Test

Pain-free and maximum walking times were assessed by a standard treadmill exercise test performed at a speed of 3.2 km/h and a grade of 12%.¹ All patients had a single baseline treadmill test and assessment was performed blinded to treatment assignment as previously described.¹

Ankle Brachial Index Measurement

ABI was measured and calculated by the same investigator for all patient visits, as previously described.¹

Duplex Ultrasonography

Scanning was performed by a single, qualified, experienced vascular technologist who was blinded to clinical data and treatment assignment as previously described.¹ All images were also assessed and analyzed by 2 independent experienced vascular physicians blinded to patient identity and treatment ($\kappa=0.94$ for interobserver reliability). Peak systolic velocity in lower limb vessel segments was measured to determine the grade of stenosis as previously described.¹⁶ In 111 patients with femoral disease (50 on placebo and 61 on ramipril) we also determined volume flow. Femoral disease was classified as $\geq 50\%$ stenosis but no occlusion in the common or superficial femoral arteries. Volume flow was calculated from the lumen cross-sectional area and the integrated mean velocity in the common femoral artery 5 cm proximal to the site of stenosis.

Blood Collection

A 21-gauge butterfly needle was inserted into an antecubital vein, and the tourniquet was removed immediately. Blood from participants was collected into appropriate anti-coagulant tubes and plasma immediately separated by centrifugation at 3000 rpm for 20 minutes in a refrigerated centrifuge. Blood was processed and stored at -80°C within 60 minutes of collection.

Laboratory Analysis

The plasma biomarkers studied can be broadly classified into four categories: angiogenic markers (vascular endothelial growth factor, VEGF-A; and fibroblast growth factor, FGF-2), thrombosis markers (D-dimer; von Willebrand Factor, vWF; and thrombin-antithrombin III, TAT), inflammatory markers (high sensitivity C-reactive protein, hsCRP and osteopontin, OPN), and leukocyte adhesion markers (soluble vascular cell adhesion molecule-1, sVCAM-1; soluble intracellular adhesion molecule-1, sICAM).

Serum AngII levels were assessed by ELISA (Phoenix Pharmaceuticals INC, Burlingame, USA). Serum VEGF-A and FGF-2 concentrations were also assessed by ELISA (Quantikine® human VEGF; R&D Systems, Minneapolis, USA). The assay exhibits no significant cross-reactivity with other angiogenic factors and has a sensitivity of 7.0 pg/mL and 9.0 pg/mL respectively. The assay recognises human VEGF₁₆₅ and VEGF₁₂₁. VEGF₁₆₅ is the predominant human isoform comprising $\sim 90\%$ of VEGF-A in blood¹⁷ and is also the most potent in terms of stimulating angiogenesis.¹⁸ VEGF₁₆₅ and VEGF₁₂₁ interactions with their receptors (VEGFR₁ and VEGFR₂) and nonsignaling neuropilin-1 co-receptor have been extensively studied using validated computational models in the context of angiogenesis,^{17, 19-22} including models relevant to PAD.^{19, 22, 23} These models have demonstrated that in the absence of neuropilin-1, VEGF₁₆₅ and VEGF₁₂₁ behave similarly. When present, however, neuropilin-1 enhances the binding of VEGF₁₆₅ to VEGFR₂, which is the major mediator of the angiogenic effects of VEGF-A.²⁴

D-dimer was measured with a commercial assay (Technoclone, Vienna, Austria). Serum vWF and TAT levels were evaluated with the use of the IMMUBID® vWF ELISA, American Diagnostica, USA. hsCRP measurements were performed using an Architect ci16200 latex-enhanced immunoturbidimetric assay (Abbott Diagnostics, Abbot Park, IL, USA). OPN concentrations were assessed using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions and expressed as ng/ml (R&D Systems, Minneapolis, USA).^{25, 26} sVCAM-1 and sICAM-1 levels were also determined by commercially available ELISA kit (R&D System, Minneapolis, USA). The limits of detection of sVCAM-1 and sICAM-1 were 0.35mg/ml and 0.60ng/ml respectively and plasma samples were diluted to

ensure all samples fell below these limits. The interassay coefficients of variation for all above assays using 2 levels of control materials ranged from 2% to 7%.

Statistical Analyses

All analysts were blinded to treatment assignment. Baseline characteristics were compared using the χ^2 test for categorical variables and 1-way ANOVA for continuous variables. We compared 24-week changes from baseline in all parameters by using an analysis of covariance model with terms for treatment and baseline values. Data were expressed as means (SDs) or 95% confidence intervals (CIs). The Pearson correlation coefficient was used for univariate analysis comparing the change in maximum walking times with the change in biomarkers in the ramipril treated group only. All analyses were adjusted for age, sex, BMI, diabetes mellitus, hypertension, ABI, cigarette smoking, and medication use. Statistical analysis was performed using SPSS (Version 12.0). A *P* value less than 0.05 was deemed to be significant. For the 165 participants who completed the 6-month follow-up, no data were missing for any clinical variable measured.

RESULTS

Of 640 potential participants who were approached for recruitment, 45 declined and 425 met an exclusion criterion, leaving 170 eligible participants (**Figure 1**). Of those, 5 participants withdrew, leaving 165 participants who completed the 6 month follow-up (**Figure 1**). The ramipril (n=82) and the placebo (n=83) groups were similar in age, other cardiovascular risk factors, medication use, and PAD severity, as evidenced by clinical symptoms (walking times) and resting ABI (**Table 1**). Furthermore, groups did not differ in creatinine levels at baseline nor after ramipril therapy (**Table 2**) indicating no confounding effects of renal function. Finally, all plasma biomarkers examined were similar in the placebo and ramipril groups at baseline (**Table 2**).

Treadmill Test

Relative to placebo, ramipril was associated with an 82 second (95% CI, 65 to 99 seconds) increase in mean pain-free walking time ($P<0.001$), and a 273 second (95% CI, 229 to 317 second) increase in maximum walking time ($P<0.001$) (**Table 3**). When change in mean arterial blood pressure was included as a covariate, the effects on walking times were shown to be independent of the small change in blood pressure following ramipril treatment (systolic: -2.7 mm Hg [95% CI, -3.2 to -2.4 mm Hg], $P<0.01$ and diastolic: -3.0 mm Hg [95% CI, -3.4 to -2.6 mm Hg], $P<0.01$).

Duplex Ultrasonography

In 111 patients with femoropopliteal disease (50 on placebo and 61 on ramipril) we determined volume flow in the common femoral artery at both the site of stenosis and 5 cm proximal to the site of stenosis in the leg with the lower ABI (limiting leg). Volume flow was unaltered at the stenotic site both in the placebo and ramipril groups. However, relative to placebo, there was a significant increase in volume flow after ramipril therapy in the common femoral artery 5cm proximal to the site of stenosis (63 mL/min (95% CI, 55 – 71 mL/min); $P<0.0001$) (**Table 3**).

Blood Parameters

At baseline there was no significant difference between the placebo and ramipril group for any of the blood parameters measured (**Table 2**). Relative to placebo, ramipril was associated with a 29% (95% CI, 26 to 32%) reduction in AngII levels, demonstrating the efficacy of ramipril to inhibit ACE. Relative to placebo, parameters associated with angiogenesis were increased with ramipril. VEGF-A increased by 38% (95% CI, 34 to 42%) and FGF-2 increased by 64% (95% CI, 44 to 85%; $P<0.001$ for both; **Table 2**). With regard to thrombosis markers, relative to placebo, ramipril was associated with reductions in D-dimer by 24% (95% CI, -30 to -18%), vWF by 22% (95% CI, -35 to -9%), TAT by 16% (95% CI, -19 to -13%). Inflammatory and leukocyte adhesion markers were also reduced by ramipril relative to placebo, hsCRP by 13% (95% CI, -14 to -9%), OPN by 12% (95% CI, -14 to -10%), sVCAM-1 by 14% (95% CI, -18 to -10%), and sICAM-1 by 15% (95% CI, -17 to -13 %; all $P<0.001$; **Table 2**).

Following the 24-week intervention, the change in AngII levels correlated inversely in univariate analysis with the change in maximum walking time ($r=-0.45$, $P=0.001$) and the change in femoral volume flow ($r=-0.43$, $P=0.001$; **Table 4**).

The change in the angiogenic markers, VEGF-A and FGF-2 positively correlated with the change in maximum walking time ($r=0.51$, $P<0.001$ and $r=0.28$, $P=0.001$ respectively; **Table 4**), and the change in femoral volume flow ($r = 0.27$, $P=0.04$; and $r=0.25$, $P=0.02$ respectively; **Table 4**). The change in D-dimer, TAT, hsCRP, OPN, sVCAM-1, and sICAM-1, levels correlated inversely in univariate analysis with the change in maximum walking time (with r values ranging from -0.58 to -0.28; $P=0.001$ for all; **Table 4**). In addition the change in D-dimer, TAT, OPN, sVCAM-1 and sICAM-1 levels correlated inversely with the the change in volume flow in the common femoral artery proximal to the site of stenosis (with r

values ranging from -0.51 to -0.22 and *P* ranging from <0.001 to 0.02; **Table 4**). The significance of all correlations remained unchanged when age, sex, BMI, diabetes mellitus, hypertension, cigarette smoking, medication use, and ABI were included as covariates (**Table 4**).

DISCUSSION

The current study provides unique clinical insights regarding the actions of ramipril to improve functional capacity in the setting of a randomized controlled trial. Ramipril was associated with changes in nine circulating biomarkers which are linked with mechanisms including angiogenesis/arteriogenesis, inflammation, thrombosis and leukocyte adhesion. These mechanisms are plausible candidates contributing to the increased functional capacity and the long term cardiovascular risk reduction resulting from treatment with this ACE inhibitor in PAD patients.^{1, 27}

The most marked changes were in FGF-2 (64% increase) and VEGF-A (38% increase), factors associated with both arteriogenesis (growth of pre-existent anastomotic arteriolar branches to functional collateral arteries) and angiogenesis (formation of new capillary beds). The reduction in D-dimer (by 24%) is also notable and only moderately lower than reported for the established anti-thrombotic drug warfarin, for which the reported reduction in plasma D-dimer ranges from 32% to 58%.^{28,29} The changes observed contribute to a rational understanding of the functional improvements associated with ramipril in patients with intermittent claudication.

Increased muscle perfusion

The ramipril induced increase in functional capacity as assessed by maximum walking time was associated with an increase in femoral flow at a site upstream from the main stenosis, but no change at the sites of major stenosis. This observation is consistent with an elevation in total leg perfusion mediated via collaterals between the stenotic site and the site of blood flow measurement 5 cm upstream from the major stenosis. The mechanisms increasing collateral flow are likely to be multiple and include vasodilatation, arteriogenesis (formation of new collaterals) and angiogenesis (formation of muscle capillaries downstream from the major stenosis) and reduced thrombosis. Our data, while associative and indirect in nature, do provide support for these mechanisms and are consistent with the known mechanisms of action of ACE inhibitors.^{30, 31}

Vasodilatation

ACE inhibitor treatment can lead to vasodilatation through a reduction in Ang II, sympathetic inhibition, but also improvement in endothelial function through preservation of bradykinin (**Figure 2**). Increased levels of bradykinin promote the formation and release of nitric oxide (NO)³²⁻³⁴ which promotes vasodilatation through well described mechanisms³⁴ (**Figure 2**).

Angiogenesis/Arteriogenesis

Given that Ang II is angiogenic *in vitro* and *in vivo* via actions on the Angiotensin Type 1 receptor (AT1R), the assumption might be that inhibition of AngII synthesis via ACE inhibitors would lead to reduced angiogenesis.³ (**Figure 2**). However, studies support just the opposite demonstrating that ACE inhibitors increase vascular density in several models including the rat limb muscle model.³⁵⁻³⁷ Furthermore, it has been reported that treatment with an ARB was not associated with enhancement of the coronary collateral circulation,³⁸ while treatment with an ACE inhibitor promoted coronary collateral formation in patients with coronary artery disease.³⁹ These findings suggest that the difference between the effects of treatment with an ACE inhibitor and an ARB on the enhancement of collateral circulation are via BK signalling. Indeed, Li *et al* demonstrated that ACE inhibition enhanced NO formation and tissue VEGF levels through the Angiotensin Type 2 receptor (AT2R) in the setting of tissue ischemia in an AT1R knockout mice. These effects appeared to be mediated via elevation in BK and downstream effects on both the BK1 and BK2 receptors⁴⁰ (**Figure 2**). Thus when Ang II is reduced in consort with elevation in BK as is the case with ACE inhibition, it is the effects of BK which dominate with respect to promotion of angiogenesis.³

Further studies have implicated BK as the mediator of ACE inhibitor-induced arteriogenesis and angiogenesis via its actions on the BK2 receptor.⁴¹⁻⁴⁴ Stimulation of the BK2 receptor induces activation of eNOS and production of NO induces synthesis of the angiogenic factor VEGF⁴² (**Figure 2**). Consistent with these mechanisms, ramipril therapy was associated with a 38% increase in plasma levels of VEGF-A in our PAD cohort. In addition, BK acting via the BK1 receptor up-regulates the arteriogenic/angiogenic factor FGF-2 via the eNOS pathway.⁴⁵ (**Figure 2**). The marked, 64% increase in plasma FGF-2 levels associated with ramipril treatment in the current study suggests that this mechanism is an important target for ACE inhibition in the setting of intermittent claudication.

To our knowledge, this is the first randomised controlled trial to systematically examine plasma levels of VEGF-A and FGF-2 in PAD patients following ramipril therapy. Other previous studies in patients with either hypertension⁴⁶ or Type 1 Diabetes Mellitus⁴⁷ have been equivocal, owing to issues including small sample size, short intervention duration, and absence of a placebo group.

In terms of clinical evidence for angiogenesis in association with ACE inhibition, in the context of vascular disease ACE inhibitor treatment was associated with a progression of coronary collateral circulation in patients with coronary artery disease, likely via bradykinin BK2R induced NO synthesis and thus downstream effects on VEGF.³⁹ Further supporting the role of ACE inhibition in human angiogenesis, Min et al demonstrated that in patients with coronary artery disease 1-3 weeks of ramipril treatment increased levels, proliferation, migration, adhesiveness and tube formation of cultured blood-derived endothelial progenitor cells (EPCs), which play a central role in angiogenesis, possibly through an eNOS/VEGF mechanism.⁴⁸ Similarly in a more recent study ramipril therapy for 20 days increased circulating EPCs as measured by flow cytometry in patients with acute coronary syndrome.⁴⁹

In terms of peripheral ischemia, therapeutic angiogenesis has emerged as a potential strategy for the treatment of patients with PAD. In a meta-analysis of trials that included gene and cell-based therapies in PAD, the investigators concluded that these therapies have the potential for clinical benefit.⁵⁰ However, certain randomized clinical trials have shown that administration of angiogenic factors such as VEGF-A⁵¹ and FGF-1⁵² provided no clinical benefit in patients with debilitating PAD and critical limb ischemia respectively. Lack of efficacy may relate to administration of single growth factors where multiple agents may be necessary to activate endogenous pathways promoting angiogenesis.⁵³ In addition, these trials are conducted in either patients with disabling intermittent claudication or patients with critical limb ischemia, conditions where the capacity to stimulate new blood vessel growth may be extremely limited. Further clinical trials addressing these issues are required to determine the viability of these approaches.

Thrombosis

Elevation in BK also has implications for thrombosis including the activation of plasminogen, and the inhibition of both platelet aggregation and leukocyte adhesion to the endothelium surface. Through these mechanisms, BK prevents or reduces the occurrence of thrombosis and may inhibit haemostasis,^{54, 55, 56} contributing to improved tissue perfusion. Some of the many proteins involved in, stimulated by, or associated with thrombosis include fibrinogen, D-dimer,⁸ vWF,⁹ and TAT complex¹⁰ (**Figure 2**). The significant reduction in plasma D-dimer, vWF and TAT levels associated with ramipril therapy, further support the anti-thrombotic action of ACE inhibitors which are likely mediated via the BK pathway. ACE inhibition reduces these plasma thrombotic markers to an extent comparable with conventional anticoagulant therapies such as warfarin and aspirin.^{28, 29, 57, 58} It should be noted that while factors affecting coagulation and blood rheology do play a role in intermittent claudication via impact on microvascular blood flow^{59, 60} there is little evidence that current anti-coagulant therapies improve walking ability.⁶¹ The use of anti-platelet agents either alone or in

combination with warfarin is the subject of ongoing research and may reveal specific PAD patient sub-groups benefiting from such therapy.⁶²

Interestingly, vWF has also been demonstrated to be a negative modulator of angiogenesis.⁶³ The molecular basis of this vWF-dependent effect is still not completely clear, however experimental data point to vWF as a negative modulator of VEGF dependent angiogenesis via multiple intracellular and extracellular pathways involving VEGFR-2, extracellular matrix proteins such as $\alpha_v\beta_3$ integrin and angiopoietin-2 (**Figure 2**).^{63, 64}

Skeletal Muscle Glucose Uptake

In combination with mechanisms which may enhance blood flow, ACE inhibition may improve walking ability by enhancing glucose uptake and utilization in skeletal muscle.⁶⁵ This effect has been attributed to BK and downstream effects on NO, which increases delivery of both insulin and glucose to muscle.⁶⁶ Finally, BK also directly stimulates insulin-dependent and insulin-independent glucose uptake into muscle.^{67, 68}

Inflammation and Adhesion

In addition to the mechanisms which may improve walking ability via effects on leg blood flow and glucose uptake/utilization,, ramipril therapy was also associated with a reduction in markers of inflammation and leukocyte adhesion which may reduce longer term atherosclerotic risk. AngII functions as a potent inflammatory stimulus and up-regulates the expression of many redox-sensitive cytokines, chemokines, and growth factors involved in the pathogenesis of atherosclerosis including CRP⁵ and OPN⁶ (**Figure 3**). CRP has been shown to induce leukocyte adhesion molecule expression in endothelial cells, suggesting a local pro-inflammatory function.⁶⁹ Ramipril therapy was associated with a 13% reduction in plasma hsCRP levels in our PAD cohort. In agreement with our findings, ACE inhibition has previously been shown to reduce hsCRP levels by up to 46% in patients with impaired cardiac function.⁷⁰

The effects of ACE inhibitor therapy on CRP and cardiovascular outcome are controversial. In the Prevention of Events With Angiotensin-Converting Enzyme Inhibition (PEACE) trial, there were no significant interactions between hsCRP levels and the effects of trandolapril on adverse cardiovascular events.⁷¹ However, the PEACE trial included patients with stable coronary artery disease with baseline hsCRP levels that were significantly lower compared to those in our PAD cohort; this may account for the lack of effect of trandolapril reported in PEACE.

On the other hand several studies have demonstrated a correlation between CRP reduction and improved outcome. Di Napoli et al. demonstrated that concomitant therapy with an ACE inhibitor at the time of an acute stroke was associated with lower CRP plasma concentration and a reduced 2-year cardiovascular risk.⁷² In other therapeutic contexts, statin therapy has also been associated with reductions in hsCRP ranging from 12% to 37% and associated reduction in the incidence of major cardiovascular events.⁷³

OPN can function as a chemotactic cytokine, regulating immune cell function and promoting the adhesion, migration, and activation of macrophages.⁷⁴ Several animal studies have shown that AngII up-regulates OPN expression, while the blockade of AngII by using an ACE inhibitor such as ramipril suppresses inflammatory cytokine OPN expression.⁷⁵ Our data demonstrate that ramipril was associated with a modest 12% reduction in plasma OPN levels.

Furthermore, Ang II also modulates vascular inflammation by regulating the expression of adhesion molecules such as ICAM-1, ICAM-2 and VCAM-1, leading to leukocyte activation and recruitment⁷ (**Figure 3**). In our PAD cohort, ACE inhibition therapy resulted in a 14% reduction in sVCAM-1 and 15% reduction in sICAM-1. Increased formation of NO through the BK2 receptor can, via reduction in vWF also reduces the adhesion of leukocytes to the

vascular endothelium⁷⁶ and inhibit platelet aggregation. vWF is also tightly associated with the inflammatory response. For example, reduced levels of vWF cause reduction in leukocyte recruitment⁷⁷ (**Figure 3**).

It is thus clear that the well documented reduction in AngII levels and increase in BK levels following ACE inhibition^{30, 31, 78} would mediate a variety of anti-inflammatory and anti-adhesion actions, which would reduce long term atherothrombotic risk.

Strengths and Limitations

The effects of ramipril were examined in the context of a robust randomized placebo-controlled study design within a well defined clinical population. While the absolute concentration of all measured biomarkers may vary between tissues and blood, there is evidence of significant correlations between concentrations in these different compartments.^{17, 23, 79-82} VEGF plasma concentration has been shown to vary approximately linearly with VEGF secretion rate in a biophysically-accurate compartment model.¹⁷ This model permits study of the distribution of VEGF isoforms including 165 and 121 in both tissue (matrix-bound, cell surface receptor-bound and free VEGF isoforms) and blood.¹⁷ Such models have also demonstrated that VEGF concentration in blood, but not in tissue is dependent on the vascular permeability of healthy tissue.^{17, 23, 83}

The net arteriogenic/angiogenic effects of the measured VEGF isoforms (165 and 121) would also be influenced by other VEGF isoforms and receptors not assessed in this study. These include the VEGF_{xxx}b isoforms, generated through alternative splicing, which are generally anti-angiogenic.^{84, 85} The soluble VEGF receptors were also not measured. Based on mouse models, the soluble VEGFR₁ (sVEGFR₁) would have been of particular interest as its overexpression in PAD may account for the blunted angiogenic response.⁸⁶

The reduction in plasma AngII demonstrates that ramipril elicited effective ACE inhibition. Although it was not possible to measure plasma BK levels in our cohort, there is substantial evidence in the literature showing elevation in circulating BK concentration after ACE inhibition.^{87, 88} Further, the evaluation of walking times and femoral blood flow permit interpretation of changes in circulating factors from a functional perspective. It must be acknowledged however, that the relationships between changes in functional and circulating parameters are associative, and that cause and effect cannot be directly established. In addition, since femoral blood flow was only assessed at rest we cannot determine whether ramipril increased blood flow during exercise. Finally, it is possible that our observations resulted from a combination of the effects of ramipril and increased physical activity. However, since physical activity was not assessed in the current cohort, it is not possible to determine whether this was the case.

Conclusion

Ramipril therapy was associated with an increase in markers of angiogenesis and a reduction in markers of thrombosis, inflammation and leukocyte adhesion suggesting that multiple inter-related mechanisms are likely to contribute to the beneficial effects of ramipril in PAD patients. Collectively we observed positive correlations between the change in maximum walking time and change in angiogenic markers, and negative correlations with markers of adhesion, thrombosis and inflammation. Not only does this study inform potential mechanisms by which ramipril mediates improved functional capacity, but importantly it provides guidance for future therapeutic strategies directed to improving functional capacity in PAD patients with intermittent claudication. Specifically the most marked changes were in VEGF, FGF-2 and D dimer suggesting that approaches targeting these mechanisms could be targets for intervention in future randomized clinical trials. New strategies are needed to improve mobility and reduce disability among individuals with PAD⁸⁹ in the context of the current shift in global disease burden to chronic disease and more years lived with disability.

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DISCLOSURES

None to report

NOVELTY AND SIGNIFICANCE

What Is Known?

- 24 weeks therapy with the angiotensin-converting enzyme (ACE) inhibitor ramipril increases maximum walking time by 134% beyond placebo in peripheral artery disease patients with intermittent claudication.
- This is associated with a highly significant improvement in quality of life.
- Leg blood flow changes suggest that ramipril increases collateral blood flow, however the mechanisms contributing to this effect are unknown.

What New Information Does This Article Contribute?

- In peripheral artery disease patients with intermittent claudication, ramipril induces changes in circulating biomarkers, which include increases in markers of angiogenesis/arteriogenesis and reduction in markers of inflammation, thrombosis and leukocyte adhesion.
- The largest effects were with respect to increases in circulating angiogenic/arteriogenic markers suggesting that ramipril may induce growth of new blood vessels which may contribute to improved leg perfusion and walking ability.
- Whether the changes in circulating biomarkers are primary effects of ACE inhibition, or secondary to vasodilatation and possibly increased physical activity requires further investigation.

Peripheral artery disease affects 200 million people worldwide and is currently under-diagnosed and under-treated, despite the 30% 5 year mortality rate associated with this condition. Approximately one third of peripheral artery disease patients experience intermittent claudication which substantially limits mobility and quality of life. Ramipril has recently been shown to dramatically improve walking ability and quality of life in peripheral artery disease patients with intermittent claudication. However, for widespread uptake and rational prescription of ramipril, it is important to understand its mechanism of action with regard to these effects. While vasodilatation is an expected mechanism associated with ACE inhibition, the current study implicates angiogenesis/arteriogenesis and reduction in inflammatory, thrombotic and leukocyte adhesion processes amongst additional actions of ramipril which may contribute to improved leg perfusion and walking ability. Of these, the largest effects were with respect to increases in circulating angiogenic markers suggesting that ramipril may contribute to growth of new blood vessels. This assertion will require further substantiation in future animal and clinical studies. The current study provides preliminary mechanistic insight into an inexpensive and highly effective therapy for intermittent claudication which also has benefit for global cardiovascular risk reduction.

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Table 1. Baseline Characteristics of the Study Population*

	Placebo (n=83)	Ramipril (n=82)	P†
Age, y	65.1 (7.5)	65.5 (5.8)	0.73
Male, No. (%)	73 (88)	64 (78)	0.11‡
BMI, kg/m ²	25.8 (3.7)	25.6 (3.7)	0.84
Diabetes Mellitus, No. (%)	19 (22.9)	20 (24.4)	0.86‡
Creatinine, umol/L	76.5 (25.8)	74.3 (22.6)	0.78
Hypertension, No. (%)	37 (44.6)	38 (46.3)	0.88‡
Smoking History, No. (%)			
Current	24 (29)	32 (39)	0.25‡
Former	37 (45)	34 (42)	0.75‡
Never	22 (27)	16 (20)	0.36‡
Brachial Blood Pressure, mmHg			
Systolic	138 (9)	139 (8)	0.67
Diastolic	82 (11)	81 (10)	0.65
Medication, No. (%)			
Antiplatelet Agents			
Aspirin	53 (64)	51 (62)	0.87‡
Clopidogrel	32 (39)	20 (24)	0.07‡
Cilostazol	19 (23)	29 (35)	0.08‡
Statins	9 (11)	5 (6)	0.40‡
β-blockers	49 (59)	51 (62)	0.75‡
β-blockers	10 (12)	14 (17)	0.39‡
Limiting leg ABI at rest	0.54 (0.15)	0.57 (0.14)	0.16
Pain-free walking time, s	138.4 (54.4)	136.6 (65.1)	0.85
Maximum walking time, s	231.0 (72.9)	226.3 (97.0)	0.72

Data are presented as mean (SD) unless otherwise indicated.

BMI, body mass index; ABI, ankle brachial index; hsCRP, high sensitivity C-reactive protein; OPN, osteopontin; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM, soluble intracellular adhesion molecule-1; vWF, von Willebrand Factor; TAT, thrombin-antithrombin III; FGF-2, fibroblast growth factor; VEGF, vascular endothelial growth factor.

*77.8% of the original full cohort is included in the current report. The baseline characteristics of the 165 patients presented do not differ to those of the 47 patients that were not included in the current report.

†By 1-way analysis of variance unless otherwise specified

‡By χ^2 test

Table 2. Changes in Blood Parameters of the Study Population

	No. of Participants	Baseline	6 Months	Within-Group Changes	Between Group Changes†	P*
Creatinine, umol/L						
Placebo	83	76.5 (25.8)	79.0 (23.2)	2.5 (11.0)		
Ramipril	82	74.3 (22.6)	78.7 (21.9)	4.1 (9.8)	1.6 (1.4 to 4.8)	0.06
AngII, pg/ml						
Placebo	83	18.3 (2.4)	18.9 (2.9)	0.6 (1.9)	-5.1 (-5.6 to -4.5)	<0.001
Ramipril	82	17.7 (2.4)	13.2 (2.5)	-4.5 (1.6)		
VEGF-A, pg/ml						
Placebo	83	187(71)	186(69)	-1(11)		
Ramipril	82	184 (60)	247 (65)	63(24)	64 (58 to 70)	<0.001
FGF-2, pg/ml						
Placebo	83	41 (21)	39 (20)	-2(4)		
Ramipril	82	41 (20)	56 (22)	16(24)	18 (13 to 23)	<0.001
D-dimer, µg/ml						
Placebo	83	1.00(0.19)	1.05(0.23)	0.05(0.18)		
Ramipril	82	1.10(0.26)	0.89(0.23)	-0.21(0.18)	-0.26 (-0.32 to -0.20)	<0.001
vWF, ng/ml						
Placebo	83	808(296)	835(301)	28(17)		
Ramipril	82	910(304)	634(419)	-276(57)	-304 (-317 to -291)	<0.001
TAT, ng/ml						
Placebo	83	3.19(0.97)	3.31(0.97)	0.11(0.19)		
Ramipril	82	3.68(1.06)	3.18(0.79)	-0.46(0.52)	-0.57 (-0.69 to -0.45)	<0.001
hsCRP, mg/L						
Placebo	83	2.43(0.69)	2.48(0.67)	0.05(0.17)		
Ramipril	82	2.08(0.79)	1.88(0.77)	-0.20(0.15)	-0.25 (-0.30 to -0.20)	<0.001
OPN, ng/ml						
Placebo	83	73.7(12.9)	73.5(11.8)	-0.1(3.2)		
Ramipril	82	74.2(11.7)	65.6(8.8)	-8.7(5.5)	-8.6 (-9.9 to -7.2)	<0.001
sVCAM-1, ng/ml						
Placebo	83	801(154)	830(157)	29(35)		
Ramipril	82	788(163)	697(134)	-102(13)	-131 (-209 to 53)	<0.001
sICAM-1, ng/ml						
Placebo	83	244(75)	253(74)	9(15)		
Ramipril	82	248(72)	226(71)	-23(11)	-32 (-36 to -28)	<0.001

Data are presented as mean (SD) unless otherwise indicated.

VEGF-A, vascular endothelial growth factor; FGF-2, fibroblast growth factor; vWF, von Willebrand Factor; TAT, thrombin-antithrombin III; hsCRP, high sensitivity C-reactive protein; OPN, osteopontin; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM, soluble intracellular adhesion molecule-1

* By analysis of covariance with terms for treatment and baseline values.

† Expressed as Mean (95% Confidence Interval)

Table 3. Walking Times and Volume Flow Measurements of the Study Population

	No. of Participants	Baseline	6 Months	Within-Group Changes*	Between Group Changes*	P†
PFWT, s						
Placebo	83	138 (54)	143 (50)	5 (-2 to 12)	82 (65 to 99)	<0.001
Ramipril	82	137 (65)	225 (95)	87 (71 to 103)		
MWT, s						
Placebo	83	231 (73)	246 (77)	15 (8 to 22)	273 (229 to 317)	<0.001
Ramipril	82	226 (97)	517 (236)	288 (244 to 332)		
Volume Flow, limiting-leg ABI, mL/min						
Site of Stenosis						
Placebo	50	602 (95)	633 (96)	31 (17 to 45)	-2 (-27 to 22)	0.85
Ramipril	61	503 (140)	536 (148)	33 (14 to 53)		
Patent Site						
Placebo	50	599 (118)	577 (109)	-22 (-29 to -16)	63 (55 to 71)	<0.001
Ramipril	61	497 (67)	538 (72)	41 (36 to 46)		

PFWT, pain free walking time; MWT, maximum walking time; ABI, ankle brachial index.

Data are mean (SD) unless otherwise specified.

* Expressed as Mean (95% Confidence Interval)

† By analysis of covariance with terms for treatment and baseline values.

Table 4. Univariate correlations with % Δ Maximum Walking Time and % Δ Volume Flow in patients who received ramipril therapy

		<i>r</i> value	<i>P</i>	<i>r</i> value*	<i>P</i> *
% Δ Maximum walking time, s	% Δ AngII, pg/ml	-0.45	0.001	-0.43	0.001
	% Δ VEGF-A, pg/ml	0.51	<0.001	0.47	0.001
	% Δ FGF-2, pg/ml	0.28	0.001	0.24	0.001
	% Δ D-dimer, μ g/ml	-0.37	0.001	-0.35	0.001
	% Δ vWF, ng/ml	-0.11	0.17	-0.12	0.18
	% Δ TAT, ng/ml	-0.31	0.001	-0.29	0.01
	% Δ hsCRP, mg/L	-0.28	0.001	-0.25	0.02
	% Δ OPN, ng/ml	-0.49	0.001	-0.48	0.001
	% Δ sVCAM-1, ng/ml	-0.44	0.001	-0.43	0.001
	% Δ sICAM-1, ng/ml	-0.58	0.001	-0.46	0.001
% Δ Volume flow, limiting-leg ABI, mL/min	% Δ AngII, pg/ml	-0.43	0.001	-0.36	0.01
	% Δ VEGF-A, pg/ml	0.27	0.04	0.23	0.05
	% Δ FGF-2, pg/ml	0.25	0.02	0.24	0.02
	% Δ D-dimer, μ g/ml	-0.22	0.02	-0.19	0.04
	% Δ vWF, ng/ml	-0.10	0.29	-0.09	0.35
	% Δ TAT, ng/ml	-0.30	0.02	-0.24	0.02
	% Δ hsCRP, mg/L	-0.01	0.36	0.01	0.89
	% Δ OPN, ng/ml	-0.51	<0.001	-0.39	0.001
	% Δ sVCAM-1, ng/ml	-0.25	0.02	-0.22	0.03
	% Δ sICAM-1, ng/ml	-0.49	0.001	-0.40	0.001

AngII, angiotensin II; VEGF-A, vascular endothelial growth factor; FGF-2, fibroblast growth factor; vWF, von Willebrand Factor; TAT, thrombin-antithrombin III; hsCRP, high sensitivity C-reactive protein; OPN, osteopontin; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM, soluble intracellular adhesion molecule-1

* Adjusted for age, sex, BMI, diabetes mellitus, hypertension, cigarette smoking, medication use, and ABI were included as covariates

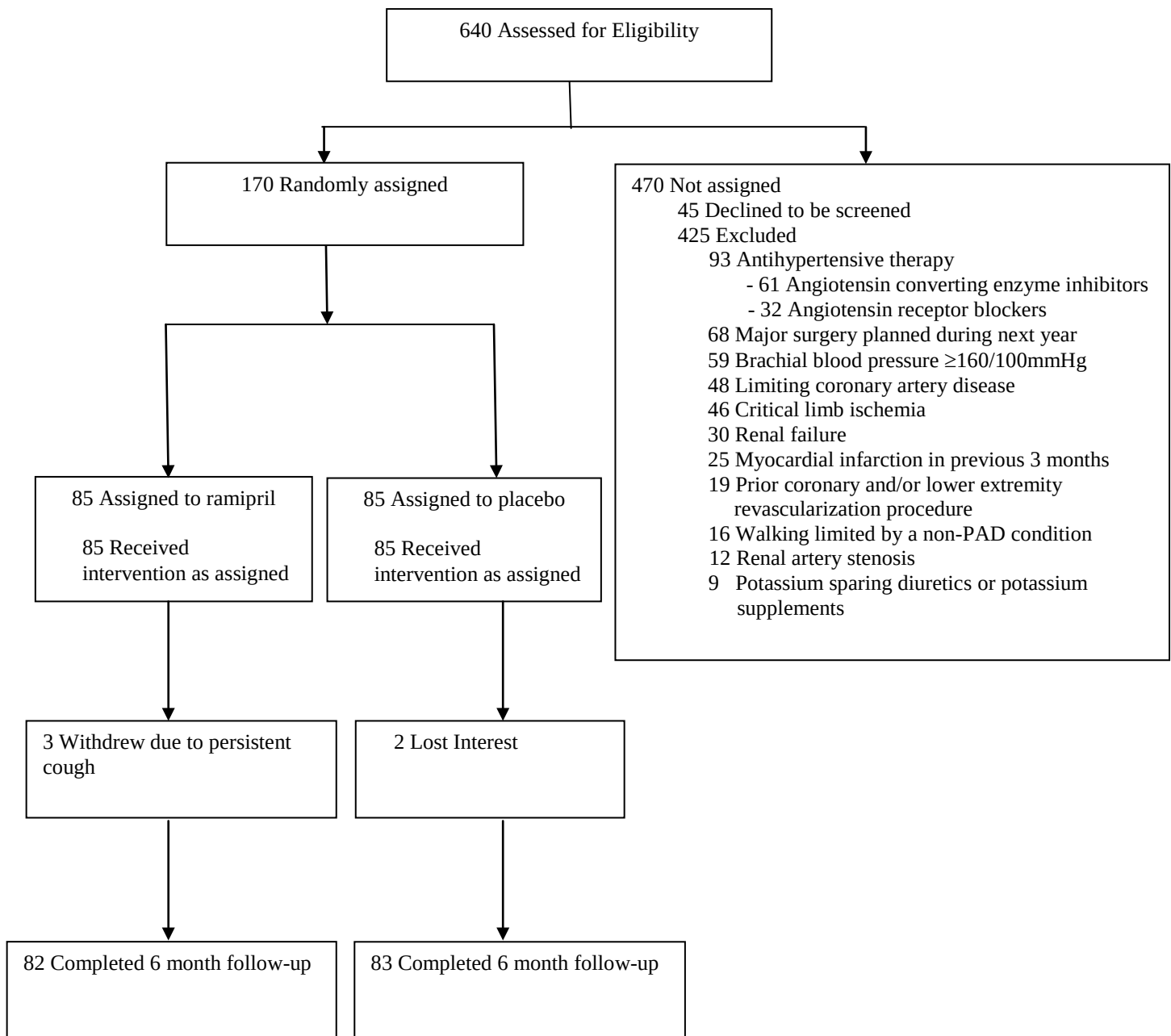


Figure 1. Study Flow. PAD indicates peripheral artery disease.

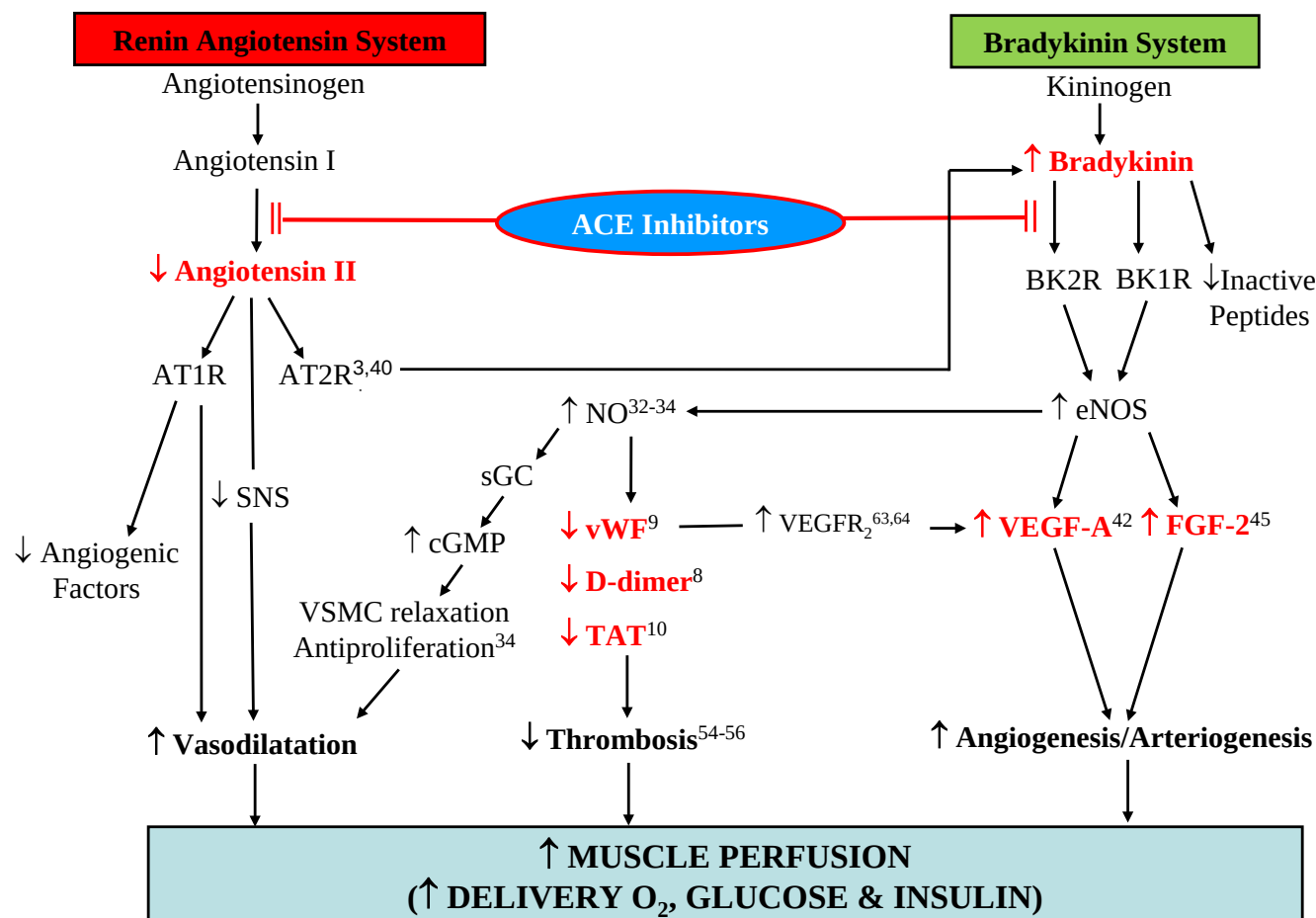


Figure 2. Potential mechanisms by which ramipril increases lower limb perfusion. Improved blood flow may be mediated via a) angiogenesis/arteriogenesis through both the Renin Angiotensin system (via AT2R) and the Bradykinin system, b) reduction in thrombosis via NO mechanisms mediated by preservation of bradykinin, and c) vasodilatation through reduction in angiotensin II. Parameters measured in the current study are shown in red. Abbreviations: AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; BK1R, bradykinin 1 receptor; BK2R, bradykinin 2 receptor; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; FGF-2, fibroblast growth factor; NO, nitric oxide; sGC, soluble guanylyl cyclase; SNS, sympathetic nervous system; TAT, thrombin-antithrombin III; VEGF-A, vascular endothelial growth factor-A; VEGFR₂, vascular endothelial growth factor receptor 2; vWF, von Willebrand Factor.

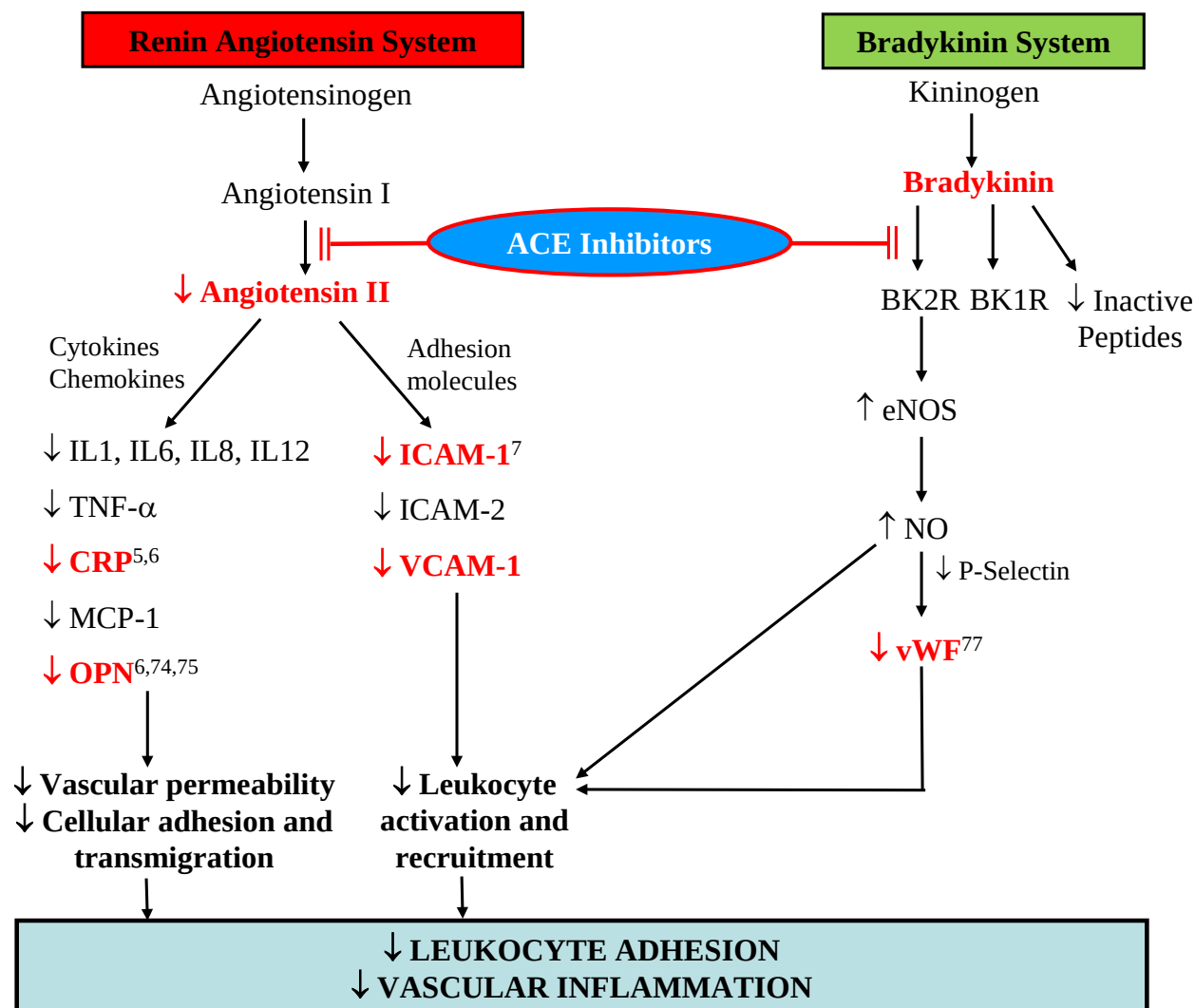


Figure 3. Potential mechanisms by which ramipril reduces vascular inflammation. A reduction in angiotensin II levels results in reduced expression of adhesion molecules, chemokines, cytokines and leukocyte margination (rolling and adhesion) and transmigration through the vascular wall. Increased bradykinin levels following ACE inhibition, also leads to improved endothelial function and reduced leukocyte adhesion. Parameters measured in the current study are shown in red. Abbreviations: BK1R, bradykinin 1 receptor; BK2R, bradykinin 2 receptor; CRP, C-reactive protein; ICAM, intracellular adhesion molecule; IL, interleukin; MCP-1, monocyte chemotactic protein-1; OPN, osteopontin; TNF-a, tumor necrosis factor; VCAM, vascular cell adhesion molecule; vWF, von Willebrand Factor.