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Mechanisms of EPO-Induced Hypertension: A Randomized Control Trial

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INTRODUCTION

Hypertension is a common but frequently overlooked and underreported adverse effect of erythropoietin (EPO) therapy [1-6]. Although EPO was approved in 1989 for treatment of anemia in patients with chronic kidney disease (CKD), only recently have trials noted substantial cardiovascular risks associated with normalization of hemoglobin [7-10]. In the largest of these randomized trials, Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT), the risk of stroke was increased twofold with EPO therapy [10]. Stroke risk is strongly related to poorly controlled hypertension. Blood pressure was not measured the way it usually is in hypertension trials, so we cannot be completely confident that the increased stroke risk in this large, randomized trial was not related to EPO-induced hypertension (EIH). Furthermore, new therapies, such as hypoxia-inducible factor (HIF) stabilizers, are on the horizon. It remains to be seen whether these new drugs would have a lower or a higher risk for hypertension compared to EPO. Accordingly, understanding the mechanism of EPO-induced hypertension is urgent [11]. Animal and human experiments suggest that endothelial dysfunction is central to the genesis of EPO-induced hypertension (EIH).

Mediated by a variety of molecules, there is an imbalance in the vascular tone favoring net vasoconstriction that mediates EIH. Animal studies unambiguously demonstrate the primary importance of CKD in the genesis of EIH. There is little evidence for EPO as a direct vasoconstrictor nor is there strong evidence for its effect on blood viscosity as a mechanism of EIH. However, preclinical studies show deranged regulation of NO, endothelins, prostanoids, and the sympathoadrenal and renin-angiotensin pathways as causes of EIH. Preliminary human studies suggest that EPO administration is also associated with enhanced vasoconstriction induced by catecholamines and angiotensin II; in addition, oxygen sensing by the vascular tissue may be deranged in those with EIH. EIH, at least in part, appears to be independent of an increase in Hgb because experiments show that Hgb may be raised by EPO without an increase in blood pressure (BP) by simply treating animals with EPO binding protein and that treatment with EPO in the setting of iron-deficiency may not raise Hgb but still increase BP. Finally, in rats with CKD, whereas EPO increases Hgb and BP, HIF stabilizers increase Hgb similarly but may actually reduce BP compared to placebo-treated animals. A review of the literature suggests an incomplete and poor understanding of the mechanisms of EIH. Better mechanistic designs are therefore needed, especially in patients with CKD, to dissect the precise mechanism of EIH.

OBJECTIVES AND HYPOTHESIS

Objectives

Primary Objective

To test the hypothesis that EPO treatment from baseline to 12 weeks in anemic patients with CKD and controlled hypertension will raise BP, we are performing a randomized control trial with a "waiting-list" design. After a 1-week run-in, we will randomize 160 participants into either the delayed start group, or the immediate start group.

If the time course, the magnitude, and the mechanisms of EIH, we will better be able to design studies to compare the vascular effects of EPO and HIF stabilizers in the future. Thus, this study has the potential of improving our understanding of a common side effect of EPO by precisely quantifying the

magnitude of BP change, its effects on endothelial function, and discovering the biomarkers of these adverse effects. Thus, we can in the future robustly compare these effects of EPO with HIF stabilizers.

Our study will focus on the potential mechanisms by which EPO induces an increase in BP. The time-course and magnitude of change in BP will be assessed using the gold-standard measurement of 24h ambulatory blood pressure monitoring (ABPM). More frequent clinic BP recordings using validated methods will better allow us to track changes in BP over time. Our lab is uniquely qualified to carry out these experiments due to a large experience with such types of studies [12-17]. We will examine endothelial function using a reference method—that of flow-mediated dilatation—which is established in our laboratory [18]. A select set of biomarkers examining endothelin, renin-angiotensin, and nitric oxide systems will enable the dissection of molecular mechanisms of hypertension in this novel application.

Our study has the ability to detect a smaller effect size (0.45%). Thus, we have adequate power to see the observed effects. Finally, the feasibility of randomizing in a timely manner of what appear to be large numbers is supported by screening through the VA Informatics and Computing Infrastructure (VINCI) databases

Hypothesis

We hypothesize that, compared to untreated controls, EPO therapy in anemic patients with CKD will raise diastolic blood pressure. The magnitude of the increase in diastolic BP at 12 weeks after treatment will be related to endothelial dysfunction and worsening of endothelial function from baseline to 4 weeks. The factors underlying endothelial dysfunction will be explored by interrogating the nitric oxide pathway (24h urine nitrate and nitrite and plasma ADMA), endothelin activation (plasma endothelin 1 concentration), and changes in the renin angiotensin system (seated plasma aldosterone, renin activity, and 24h urine sodium excretion rate). We will use a randomized controlled trial design with open-label administration of EPO to 80 patients in each group and comparison of the responses over 12 weeks of treatment. The dispensing of EPO will depend on which group the participant is randomized to. The immediate start group will be given EPO at randomization and diastolic BP will be assessed after the first 12 weeks of treatment. While the untreated "waitlisted" controls will be treated with EPO after 12 weeks. We then will examine the diastolic BP after an additional 12 weeks of treatment with EPO. Oral iron will be used in both groups to replete iron deficiency, if necessary given lab work. We will observe the relationship between diastolic BP and endothelial dysfunction using paired testing with each subject's baseline results serving as their own control. Preliminary data show that our sample size has the ability to detect 5 mmHg change in diastolic BP between groups. For endothelial dysfunction, most studies are powered to detect 1-2% change from baseline.

BACKGROUND

Hypertension is believed to be a common adverse effect of EPO therapy [1-6]. However, the incidence and severity of EIH is difficult to quantify because underreporting of hypertension is common. Underreporting is likely due to 3 reasons: (i) more aggressive management of hypertension through prescription of antihypertensive drugs, (ii) among dialysis patients, closer attention to dry-weight [19,20] and (iii) lack of precise quantification, such as by ABPM [21]. We have conducted a cross-sectional survey

of hemodialysis patients using 44-hour interdialytic ambulatory BP monitoring to conclusively demonstrate that EPO therapy is indeed an independent determinant of hypertension [22].

Importance of CKD in EPO-induced hypertension

The first study to show that CKD was an important determinant of EIH was reported in 1995 [23]. In this study, male Wistar rats underwent subtotal nephrectomy at the age of 6 weeks to induce CKD. After 6 weeks of recovery, they were treated for 3 weeks with either vehicle or EPO; sham-operated animals who received EPO or vehicle served as comparators. Treatment with EPO provoked a significant increase in Hgb regardless of nephrectomy. Interestingly, EIH occurred only in the nephrectomized group; systolic BP increased by 37.5 ± 11 mmHg. In contrast, confirmed by a study in 1997 [24], no significant increment was seen in systolic BP in the non-nephrectomized, sham- operated EPO-treated or vehicle-treated rats despite increase in Hgb.

Clinical observations are consistent with animal data. Although EPO is well known to be abused by athletes to enhance performance, we have not observed hypertension or hypertensive crises in this population. Similarly, the incidence of hypertension in zidovudine-treated HIV positive anemic patients or among those undergoing cancer chemotherapy is low. However, EIH is commonly reported among those with CKD, including those on dialysis [6]. The implication of the above animal studies and human observations is that underlying CKD is necessary for EIH.

Hemopoietic versus hypertensionogenic effects of EPO

Animal experiments in general suggest that hypertension is not associated with an increase in Hgb [25]. If EPO is administered to anemic animals with CKD but Hgb is kept low by feeding an iron deficient diet, hypertension still occurs. Similarly, among patients on long-term hemodialysis, treatment with iron to increase Hgb is not associated with a parallel increase in BP [26]. The dose-response relationship of Hgb with EPO versus BP with EPO are different. For example, an increase in Hgb is dose-dependent, but an increase in BP is not [1,27]. Some studies, however, point to a direct relationship. For example, during the first 5 weeks of administration of erythropoietin, the change in Hgb concentration was directly related to an increase in diastolic BP ($r=0.42$, $p<0.001$) [3].

To understand the hypertensionogenic effects of EPO, consideration of the biology of the EPO receptor expressed in the hemopoietic and non-hemopoietic tissues is needed. Taken together, these data support the importance of the EPO receptor in the non-hemopoetic tissues in the genesis of EIH.

EPO-receptor and its relevance to EPO-induced hypertension

There is substantial evidence that blood vessels express the EPO receptor. In a 1994 report, mRNA for the EPO receptor was identified in human umbilical vein endothelial cells [28]. The functional importance of these receptors was demonstrated by the following: (i) proliferation of cultured endothelial cells in response to EPO [29,30] at concentrations as low as 1 U/mL 30, which is often achieved with intravenous administration of the drug [31]; and (ii) migration of human umbilical vein endothelial cells in response to EPO [30].

Tissue-specific knockout studies also provide evidence for the importance of the vascular EPO-receptor. Mutation of EPO-receptor is lethal in utero [32]. However, EPO-receptor null mice can survive

simply by expressing the EPO-receptor in the hemopoietic tissue only [32]. Despite the lack of the EPO-receptor in the endothelium, heart, and brain, these mice, somewhat surprisingly, develop normally. Using genetic engineering, mice were created with varying amounts of EPO-receptor expressed in the hemopoietic tissue only [32]. A series of studies among mice expressing EPO-receptor in only the hemopoietic tissue demonstrate the importance of EPO-receptor expression outside of the hemopoietic tissue [32], such as in the heart, lungs, and limbs [33-36].

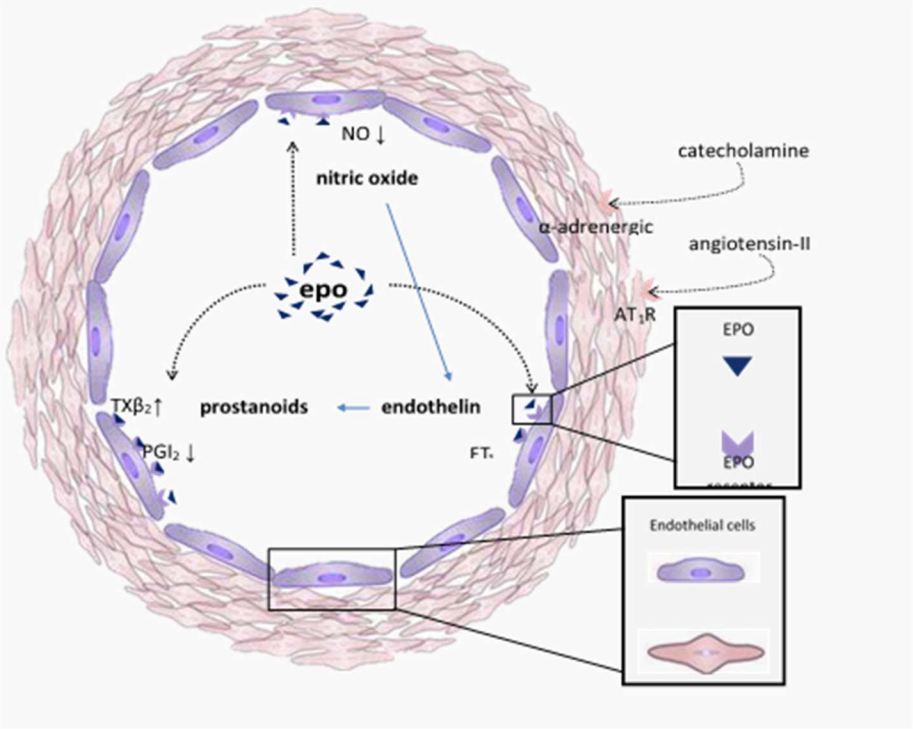
Experiments suggest that the effects of EPO on hemopoiesis and vasoconstriction may be mediated by different epitopes on the EPO molecule [37]. Rodents treated with EPO, when also treated with an EPO-binding protein, had an increase in Hgb but no change in BP. These data suggest that the hypertensionogenic and hemopoietic effects of the EPO molecule may be mediated by different parts of the same molecule.

EPO can also be engineered by targeting it more specifically to the hemopoietic EPO- receptor and reducing its ability to bind to non- hemopoietic tissues [38]. A synthetic EPO molecule has been created that interacts minimally with the EPO-receptor yet can specifically bind to the RBCs [38]. Compared to darbepoietin, this new molecule augments the reticulocyte response but minimizes the platelet effects [38]. The authors speculate that EPO receptor expression on maturing megakaryocytes may create an off target prothrombotic state.

Molecular mechanisms of EPO-induced hypertension

The mechanisms of EIH are incompletely understood. **Figure 1** summarizes putative mediators and their effects on BP.

Figure 1: Mechanisms of EPO-induced hypertension



Evidence against direct effect of EPO on vascular smooth muscle or endothelium

Among untreated patients with essential hypertension, serum EPO concentration correlates with both systemic vascular resistance and 24h ABPM [39]. This has led to speculation that EPO may have direct vasoconstrictive effects. In order to interpret the results of in vitro studies, it is important to compare the plasma concentrations of EPO achieved after a bolus dose in humans to that used in pre-clinical studies. A study describing the pharmacokinetics of EPO in hemodialysis patients notes that the peak concentration of EPO when given as an intravenous bolus injection was 0.768 U/mL with 50 U/kg EPO and 2.434 U/mL with 150 U/kg [31].

In 1991, a group of investigators from Muenster, Germany demonstrated vasoconstriction in resistance arterioles from the rodent kidneys and intestine when exposed to EPO doses ranging from 10 to 200 U/mL [40]. In 1993, the same group demonstrated that intracellular calcium is increased in the cultured vascular smooth muscle cells in a dose dependent manner upon incubation with EPO [41]. The vascular smooth muscle cells were incubated with 100 U/mL or 250 U/mL EPO. It should be noted that these concentrations of EPO are approximately 100-200 times higher than the peak concentrations achieved with IV EPO administration [31]. The physiological and clinical relevance of these observations is therefore unclear. In humans, EPO by itself does not appear to have any effect on vasoconstriction. In a double-blind cross-over study in 9 hemodialysis patients, Hon et al. administered either EPO or saline intravenously [42]. BP was measured every 5 minutes for 60 minutes following treatment. Between treatments, no differences were seen [42]. Other studies in humans have limitations in that they do not have appropriate controls[43] or use suprapharmacologic ex-vivo exposures to EPO [44].

EPO, through its receptors on the endothelial cells, especially in the setting of CKD, can trigger endothelial dysfunction and vasoconstriction. The nitric oxide pathway is severely deranged in response to EPO.; ADMA concentrations increase and cGMP release with NO-donors is impaired. Inhibitors of NO synthase severely elevate BP and provoke mortality in rodents with excess EPO expression. NO can also trigger endothelin-1 release, which itself is a potent vasoconstrictor. Prostanoids are downstream to endothelin and there is a net imbalance favoring vasoconstriction. The effects on the renin-angiotensin system are complex, but in preliminary studies vasoconstriction is to angiotensin II is enhanced when patients are given EPO. Similarly, compared to patients not on EPO, vasoconstriction to catecholamines is enhanced when patients are treated with EPO.

Endothelin-1(ET-1)

In rats with CKD, increased ET-1 expression is seen in the aorta, mesenteric artery, and renal cortex [45,46]. In addition, they have increased 24h urinary excretion of ET-1 [47]. EPO induces release of ET-1 from endothelial cells in culture [29,48]. The expression of ET-1 provoked by EPO is organ-specific. CKD rats treated with EPO have no increase in ET-1 in the mesenteric artery or renal cortex, yet the aortic content of ET-1 is increased [49,50]. The increase in aortic ET-1 is further augmented by the nitric oxide synthase inhibitor L-NAME, indicating that nitric oxide is an upstream process [49]. Tempol, an oxidative stress inhibitor, reduces tissue levels of ET-1, indicating that it is also an upstream process [51]. However, prostanoids are downstream to ET-1 as discussed in **Figure 1**. The critical importance of endothelial expression of ET-1 is demonstrated in mice specifically overexpressing ET-1 in the endothelium. These

mice, when treated with EPO—even without nephrectomy—have impairment of endothelial function, resistance artery remodeling, aortic inflammation, and an increase in BP [52].

Compared to wild-type mice, those heterozygous for the ETB receptor have a greater increment in BP with EPO [53]; mice heterozygous for the ETA receptor have BP responses that are similar to those of wild- type mice. In rats with subtotal nephrectomy and EIH, treatment with a selective ETA receptor antagonist is more effective than placebo in abrogating the rise in BP [54]. In contrast, the non-selective ETA / ETB receptor antagonist bosentan had no effect in preventing EIH [54].

In patients with CKD treated chronically with EPO, ET-1 concentration is increased [55-58]. In a cross- sectional study of 44 ESRD patients, of whom half were on EPO therapy, plasma ET-1 concentration directly correlated with systolic BP [59]. However, acute administration of EPO does not increase ET-1 concentration [42]. Forearm vasoconstriction induced by the infusion of ET-1 is similar in patients with CKD before and after treatment with EPO; thus, the vasoconstriction dose-response relationship to ET-1 is not altered with EPO- treatment [60].

Prostanoids

Prostanoids play an important role in maintaining vascular tone and in renal sodium handling. Thromboxane A_2 (TXA_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have vasoconstrictor activity, whereas PGI_2 is vasodilatory. TXB_2 is a stable metabolite of TXA_2, whereas 6-keto-$PGF_{1\alpha}$ is a stable metabolite of PGI_2 [48]. Rats with CKD have increased vascular and renal concentrations of TXA_2 and PGI_2[61]. In these animals, treatment with EPO further aggravated hypertension and stimulated vascular and renal TXA_2 and PGI_2 [61]. TXB_2 was increased but 6-keto-$PGF_{1\alpha}$ remained unchanged in the aortas of rats with CKD treated with EPO [62]. An antagonist of thromboxane, ridogrel, abrogated EIH but did not alter the plasma concentration of ET-1 [62]. An antagonist of ET-1, ABT-627 was even more effective than ridogrel in treating hypertension; moreover, it reduced the concentration of TXB_2 in the aorta [62]. Thus, prostanoids are a downstream mediator of vasoconstriction in response to ET-1 activation.

The imbalance in the ratio of vasoconstrictor to vasodilatatory prostanoids may lead to a net increase in vascular resistance and therefore hypertension. The use of antiplatelet therapy has been postulated to prevent the development of hypertension among patients treated with EPO [63], however the mechanism of its putative antihypertensive effect remains unclear.

Nitric oxide

Nitric oxide (NO) is a potent endothelium-derived vasodilator and plays an important role in the genesis of hypertension in CKD. NO has important effects on the endothelium, blood vessels, kidneys, and BP. In the context of EIH, each of the effects are discussed below.

Endothelium: In uremia, NO synthesis in the endothelium is blunted. In vivo studies reveal that, compared to saline-treated controls, Balb/c mice with intact kidneys injected with clinically relevant doses of EPO (such as 30 U every other day for 10 weeks) showed 46% higher ADMA concentrations [64]. In comparison, symmetric dimethyl arginine (SDMA) concentrations were unchanged. Of note, there is no mechanism of direct synthesis of ADMA; free ADMA is generated by proteolysis of methylated

proteins [65]. Free ADMA can inhibit endothelial cell NOS and cause endothelial dysfunction and has been linked to poor outcomes in CKD.

Blood vessels: Among rats with CKD, in aortic rings, the release of cyclic GMP by nitrate donors is augmented. However, this augmentation is blunted when rats are treated with EPO [66]. Among rats with CKD, in the aorta, the endothelial and inducible NO synthase protein mass is reduced regardless of EPO treatment, but when treated with felodipine the protein mass is restored, suggesting an upstream role of calcium channels.

Kidney effects: Rats with intact renal mass upon treatment with EPO display an increase in renal NO; this is noted by an increase in urinary cyclic GMP [67] and urinary nitrates [68]. Rats with CKD have a reduction in urinary nitrate excretion rate that is further reduced with EPO treatment [69].

Blood pressure effects: Inhibitors of NO synthase can exacerbate hypertension in EPO-treated animals even when the renal mass is not reduced [67]. The critical importance of NO in EIH has been studied in a transgenic mouse model [70]. Transgenic mice overexpressing human EPO have hematocrit levels of 80%. Despite such a high hematocrit, adult transgenic mice did not develop hypertension or thromboembolism because of a compensatory increase in endothelial NO synthase levels and NO-mediated endothelium- dependent vasodilatation. Administration of L-NAME provokes vasoconstriction, an increase in vascular resistance, hypertension, and death in transgenic mice; their wild-type siblings developed hypertension but did not show increased mortality.

A translational research study elucidated the mechanism of endothelial dysfunction in 56 hemodialysis patients treated with EPO [71]. Endothelial progenitor cells, which the investigators state reflect endothelial cell function, were isolated from these patients and mRNA levels for both the full EPO receptor and a spliced, truncated EPO receptor were measured. The investigators noted that activation of the full EPO receptor triggers a signaling cascade that ultimately terminates in cyclic-GMP and NO production and subsequent vasodilatation. However, it was observed that the endothelial progenitor cells in patients with EIH displayed the spliced, truncated variant of the receptor. This led to the conclusion that the truncated variant of the receptor serves as a dominant negative regulator of the cyclic-GMP/NO cascade, thus blunting vasodilatation; blunted vasodilatation would provoke hypertension.

Catecholamines

Norepinephrine concentrations were increased following 12 weeks of EPO treatment in one study [72] but decreased in another study of Japanese hemodialysis patients [73]. Forearm vasoconstriction induced by infusion of norepinephrine was increased in patients with CKD after treatment with EPO; thus, the vascular sensitivity to norepinephrine was increased in human studies [60,74]. Furthermore, white blood cell α-2 receptor density was reduced following EPO treatment [55,75]. These preliminary data suggest that both an elevation of catecholamines as well as enhanced arterial sensitivity to catecholamines may provoke EIH.

Renin angiotensin aldosterone system (RAAS)

In Wistar rats, upon treatment with EPO, Eggena et al. evaluated the effects on RAAS at the molecular level [76]. In the kidney, both renin-mRNA and angiotensinogen-mRNA were increased by EPO.

In the heart, no alterations in the mRNAs were seen. In the aorta, angiotensinogen-mRNA, but not renin-mRNA, was elevated. In both the aorta and the kidney, a significant correlation was observed between angiotensinogen- mRNA and BP. Barrett et al., in cultured rat vascular smooth muscle cells, show that EPO induces an increase in both types of angiotensin II receptors even in the presence of enalapril or losartan [77]. Among hemodialysis patients, infusion of angiotensin II leads to excess vasoconstriction, suggesting that angiotensin II sensitivity is increased with EPO treatment [60]. Taken together, these data suggest that vasoconstrictive potential is enhanced with EPO treatment.

In isolated perfused rodent kidneys, EPO treatment results in RAAS-stimulated sodium retention [78]. This is in sharp contrast to normal human volunteers, wherein EPO treatment caused reduction in plasma volume, plasma renin activity and aldosterone [79]. However, in rats with subtotal nephrectomy treated with EPO, in terms of treatment there is no unique effect on BP lowering of RAAS blockade. This is because the reduction in systolic BP was similar when rats were treated with traditional triple therapy (reserpine, hydralazine, hydrochlorothiazide) or with the RAAS blockers captopril or losartan [47]. In CKD patients treated with EPO, the evaluation of RAAS remains incomplete.

Blood viscosity

Blood viscosity rises in parallel with blood Hgb and has been cited as a mechanism of EIH. However, not all patients who have correction of anemia get hypertensive. Thus, the change in blood viscosity by itself appears to be insufficient to account for EIH.

Hypoxia Inducible Factors (HIF) Prolyl Hydroxylase Inhibitors

HIF stabilizers stimulate not only erythropoiesis but also many other genes responsible for angiogenesis, tumor growth, cell proliferation, and metabolism [80]. HIF stabilizers are in Phase III clinical trials for the treatment of anemia in CKD. It remains unclear whether HIF stabilizers will protect or predispose people with CKD to hypertension. HIF stabilizers may aggravate hypertension by several mechanisms. For example, chronic intermittent hypoxia through HIF signaling in the carotid body is thought to provoke systemic hypertension [80]. A 2016 study showed in both in vitro and in vivo rodent models that hypoxia provoked inorganic phosphorus-induced vascular smooth muscle calcification. In this rodent model, roxadustat, an oral HIF stabilizer, in fact enhanced vascular calcification. Vascular calcification is common in CKD and contributes to arterial stiffness. Increased arterial stiffness is strongly associated with elevated interdialytic ambulatory BP [81]. The downstream effect of long-term use of HIF stabilizers may therefore be hypertension.

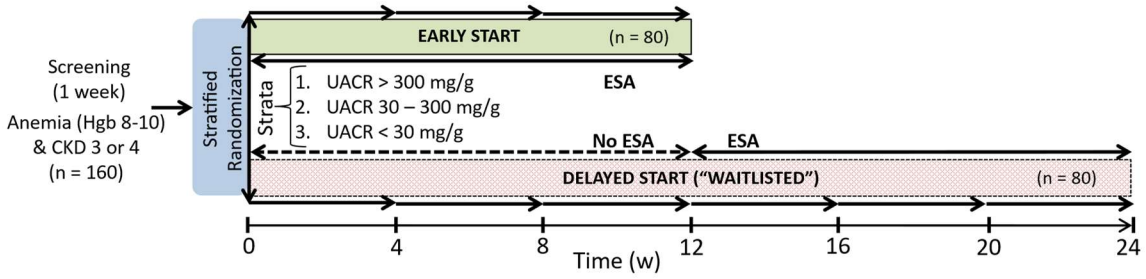
On the other hand, HIF stabilizers may raise EPO to more physiologic levels, reduce crosstalk with vascular EPO-receptors, and mitigate hypertension. A 2014 study demonstrated in a rodent model that compared to EPO, treatment with HIF-stabilizer molidustat corrected anemia associated with subtotal nephrectomy. However, in contrast to EPO, molidustat reduced systolic BP in a dose-dependent manner[82]. The authors postulate that anti-inflammatory and anti-fibrotic effects of the drug on the kidney may be operative. This study further illustrates that increment in Hgb can be dissociated from an increment in BP. Human data are available but are thus far inadequate to address the question of risk for or protection from incident hypertension with HIF stabilizers [83-86].

RESEARCH PROTOCOL AND TRIAL DESIGN

Overall trial design and plan

To test the notion that EPO treatment in anemic patients with CKD and stage 1 or less hypertension that is treated or untreated with antihypertensive medicationswill raise BP, we propose a randomized controlled trial with a "waiting-list design" as shown in **Figure 2**. In this design, half of the patients are treated immediately with EPO and the other half are put on a waiting list for 12 weeks, after which they begin treatment. Ultimately, the waitlisted patients are exposed to the intervention, thus serving as their own controls. The design allows between-group comparison by comparing responses in the parallel groups over 12 weeks and within-group comparison for the waitlist group in the second 12 weeks compared to the first 12 weeks. Titration of the dose of EPO will occur every 4 weeks to manage anemia as detailed below. The effect of EPO on BP will be assessed by change in diastolic ambulatory BP measured over 24 hours at baseline and again at 12 weeks in both groups and at 24 weeks in the waitlisted group. At baseline and every 4 weeks thereafter, we will also evaluate changes in clinic BP and albuminuria. At baseline and 4 weeks after intervention, we will measure endothelial function, and collect blood for biomarkers. The biomarkers we will be analyzing are renin, aldo, ET-1, catecholamine, ADMA, and urine nitrates and nitrites.

Figure 2: Trial design and study process



Visit		1	2-3	4	5	6-7	8	9	10
Measurement		Consent	Baseline	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24
PROC	FMD		x	x		DS	DS		
HTN	24h-ABPM		x			x			DS
	Clinic BP	x	x	x	x	x	DS	DS	DS
Labs	24h-urine		x			x			DS
	Blood labs*	x**	x	x	x	x	DS	DS	DS
	UACR (x3)		x	x	x	x	DS	DS	DS
	Biomarkers		x	x		DS	DS		
Group	Immediate Start		x	x	x				
	Delayed Start		x	x	x	x	x	x	x

*CBC, Renal Panel, Ferritin, TSAT ** V1 if consent: CBC & eGFR panel DS = Delayed Start ONLY

This is a two-arm, open-label, single-center, randomized controlled trial to understand the mechanisms of EIH. Participants will be randomized based on stratum of baseline level of urine albumin/urine creatinine ratios (UACR) measured on three consecutive days. The primary analysis will compare the 80 participants in the early start group to the 80 in the delayed start (DS) or waitlisted group over the first 12 weeks. All procedures will be performed as listed in the table based on study visit. Confirmatory analysis will compare the first 12 weeks to the next 12 weeks in the DS group. Accordingly, the study procedures labeled DS will be performed only in the delayed start randomized participants.

Study Intervention

Participants with CKD and anemia who meet the inclusion and exclusion criteria will be randomized to either the immediate or delayed start group. There are at least 7 study related visits in the early start group, and 11 in the waitlist group. Two consecutive days are needed to perform 24h ambulatory BP monitoring, which will be done twice in the immediate start group and 3 times in the delayed start group. Patients will receive oral iron (ferrous sulfate, 324 mg orally twice daily) if serum ferritin concentration is <100 ng/mL or transferrin saturation is <20%. If clinic BP exceeds either 179 mmHg systolic or 109 mmHg diastolic or if clinic BP raises by >20/10 mmHg compared to baseline, antihypertensive medication dose will be increased or a new antihypertensive will be added.

Participants will be identified in the renal clinic or the electronic medical record system based on estimated GFR and Hgb. At baseline, all participants will have 24-h urine Na measured to assess dietary Na intake.

Treatment Adherence

We recognize the limitations of syringe count, but we will assess treatment compliance with the study drug by performing a syringe count at each of the study visits.

Protocol for the management of anemia

Darbepoetin will be self-administered subcutaneously at a starting dose of 0.45 µg/kg once weekly. Hgb will be targeted between 10-12 g/dL with a goal of at least 2 g/dL increment from baseline. If a patient's Hgb increases by 2 or more g/dL during any 4-week period, the darbepoetin dose will be decreased by 25%. If Hgb rise over 4 weeks is < 1g/dL and patient is not in target Hgb range the dose of darbepoetin may be increased by 25%. If Hgb change from baseline is between 1 - 2 g/dL the dose of darbepoetin will be maintained. If Hgb concentration increases to >12.0 g/dL, the drug will be withheld until Hgb concentration falls to <12 g/dL, at which time it may be restarted at a dose 25% lower than the previous dose.

In a Phase III registration trial of darbepoetin in CKD,[87] the median response time to increase in Hgb was 7 weeks. The response was defined as an increase in Hgb of ≥1 g/dL from baseline and Hgb ≥11 g/dL. The peak response was achieved at 13 weeks. Since we want to magnify the difference between groups, a 12-week treatment period appears appropriate. We expect about half of the participants to be treated for 24 weeks since the waitlisted group will be treated after 12 weeks.

Stop points

1. Stop point for severe anemia: If Hgb drops to <8 g/dL, waitlisted patients (delayed start group) will be started on EPO therapy without further delay. In the TREAT trial, where the rescue threshold was 9 g/dL, less than half (46%) of the patients in the placebo group needed at least one dose of EPO over the life of the trial [10]. The target for rescue therapy is even lower in this trial and we do not expect many patients to be taken off the waitlist.

2. Stop point for uncontrolled hypertension: If clinic BP exceeds either 179 mmHg systolic or 109 mmHg diastolic, or rises by >20 mmHg/10 mmHg, antihypertensive medication dose will be increased, or a new medication added.

Safety Assessments and Variables

Harms will be assessed by monitoring changes in weight, eGFR, CBC, clinic BP, and ABPM. For more definitive assessments, including hospitalizations, serious adverse events will be properly completed and sent to the VAMC DMC as requested. Safety of continued participation will be assessed at each study visit. The stop-points will be treatment-emergent severe adverse events that precludes further exposure to the drug, such as an allergic drug reaction, initiation of renal replacement therapy, or death. If the study drug is stopped, the subject will be continued to be studied so as to not violate the intention-to-treat principle.

Participants

Inclusion Criteria

- Stage 3 or 4 CKD defined as eGFR <60mL/min/1.73m^2 for ≥3 months or persistent albuminuria for ≥3 months; (i) urine albumin to creatinine ratio of more than 30 mg/g; (ii) urine protein to creatinine ratio of more than 150 mg/g; or (iii) at least 1+ dipstick positive proteinuria. [88]
- 24h ABPM <140/90 mmHg at baseline in the presence or absence of antihypertensive treatment.
- Hgb between 8 and 10 g/dL.
- No treatment with ESA within the previous 3 months.
- Adults, age 18 and older.

Exclusion Criteria

- Need for packed RBC transfusion in the previous 2 months.
- Myocardial infarction, stroke, or hospitalization for heart failure in the past 2 months.
- In the assessment of the investigator, have hematologic, inflammatory, infectious, or other conditions that might interfere with the erythropoietic response.

Retention of Participants

For half of the participants, only 7 visits are needed. For the others, at least 11 visits over 24 weeks of the trial are needed. We will offer $20 per visit for participation in the trial to offset travel and time costs.

METHODS

Method of Randomization

Participants will be randomized in a 1:1 ratio to either early or delayed treatment ("waitlisted group") with EPO according to UACR strata. A random permuted block design will be used to avoid imbalance in assignment to the treatment groups over time. A randomization sequence will be generated by the statistician and individually concealed in sequentially numbered opaque envelopes. After confirming eligibility with the principal investigator, the study pharmacist will dispense EPO according to the randomization sequence.

Measurement of clinic BP

Clinic BP measurements will be obtained by a trained observer in triplicate in both arms after applying an appropriately sized cuff using a digital oscillometric sphygmomanometer (Model HEM-907,Omron Healthcare) following the recommendation of the European Society of Hypertension [89]. The arm and the forearm will be supported at the level of the heart (mid-sternum) and oscillometric BP will be measured after 5 min of seated rest without an observer in the room. Measurements averaged over three readings at 1 min intervals will be used to define clinic visit BP. The left arm will be used for all measurements, unless between arm reading exceed 5 mmHg systolic. If so, the arm with the higher BP will be designated the "BP arm" and will be used for clinic BP measurements. If a subject has dialysis access (AV fistula or AV graft), we will use their non-access arm for all BP measurements.

Measure of 24-hour ambulatory BP

Ambulatory BP monitoring will be performed in all participants at baseline prior to randomization and at the end of the treatment period as indicated in **Figure 2**. Appropriately sized cuffs will be used with bladder sizes that encircle 80–100% of arm circumference and widths that are at least 40% of arm circumference. We will measure ABP every 20 minutes from 06:00 – 22:00 and every 30 minutes from 22:00 – 06:00 based upon a prior protocol using the use the Spacelabs 90207 monitor [17]. Participants will be asked to record their wake and sleep times into diaries; the latter are used to calculate diurnal ambulatory BP. At least 16 recordings will be required to call 24h BP adequate (excluding readings taken for monitor validation during ambulatory dispensing). All ambulatory BP measurements will be taken on a subject's non-dominant arm. If a subject has dialysis access, all BP measurements will be completed on the non-access arm.

Measurement of endothelial dysfunction

We will instruct the participants to fast for eight hours, refrain from caffeine, alcohol and tobacco and avoid exercise for 8 hours prior to testing. Endothelial function testing will be performed using flow-mediated dilatation (FMD) in a quiet, temperature-controlled room with dimmed lights after the participant has been resting for at least 15 minutes in the recumbent position using international recommendations [90].

Using an uSmart NexGen Ultrasound 3300 (Terason, Burlington, MA) equipped with two-dimensional imaging, color and spectral Doppler, an internal electrocardiogram (EKG) monitor and a

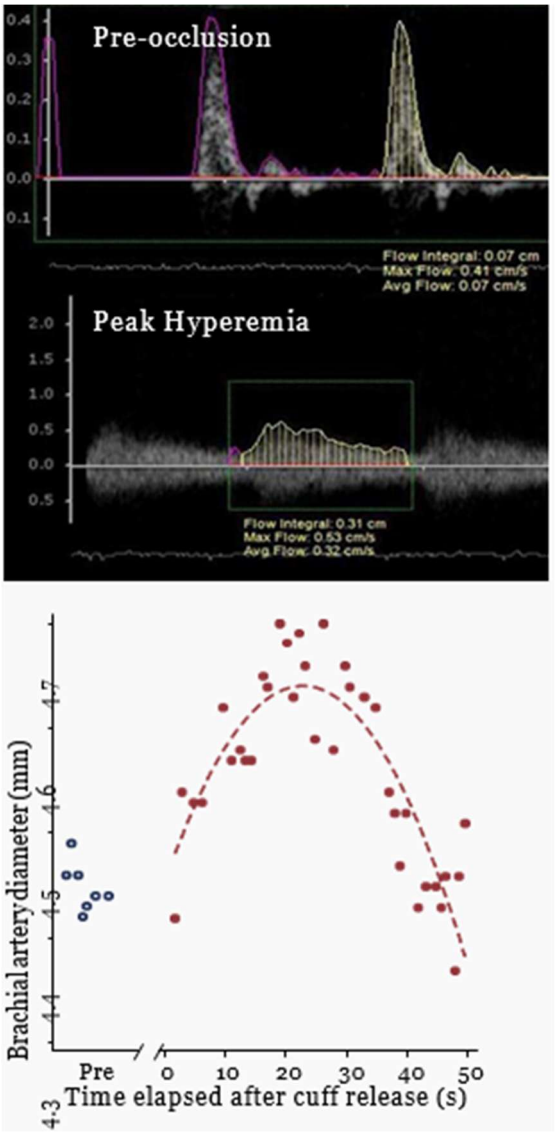


Figure 3: Example of flow-mediated dilatation in a healthy volunteer from 24 Aug 2017

high frequency vascular transducer (16 to 5 mHz, 16L5 probe), ultrasonography will be performed by a trained technician using a standardized protocol [91,92]. Participants will be positioned supine with the arm placed in a comfortable position to image the brachial artery in a longitudinal plane. A segment of brachial artery 5-10 cm above the elbow with clear anterior and posterior intimal interfaces between the lumen and the vessel wall will be selected. A sphygmomanometeric cuff placed on the forearm will be rapidly inflated to suprasystolic pressure (250 mmHg) for 5 minutes to produce ischemia using an air source coupled to a rapid cuff inflator (Hokanson E 20 Rapid Cuff Inflator; E.C. Hokanson, Inc).

Longitudinal resting images will be acquired before inflation. Blood flow estimated by the pulsed Doppler velocity signal using the outer envelope technique to calculate the velocity time integral will be obtained from a mid-artery sample volume at baseline and upon immediate cuff release to assess hyperemic velocity. Peak hyperemic velocity will be determined from the first complete velocity envelope after cuff release. Higher values of hyperemic velocity represent better microvascular dilatation [93]. Longitudinal images of the artery in B mode will be continuously recorded for 3 min after cuff deflation. Images will be recorded digitally using an R-wave gated digitizing interface external to the machine (MIA- LLC, Coralville, IA).

A few minutes after deflation, another image will be acquired to reflect a reestablished baseline. The above procedure is repeated to acquire the images in duplicate. The change in post-stimulus diameter at peak dilation as a percentage of the baseline diameter will be reported as endothelial function.

Analysis will be automated using a validated software (Vascular Analysis Tools 6, MIA-LLC, Coralville, IA). We have reported a clinical trial using FMD from our laboratory [18]. **Figure 3** shows the results of FMD from our laboratory in a normal healthy volunteer using the above software. Although FMD requires an ultrasound machine, high frequency transducers, and a trained technician capable of measuring the diameter of the brachial artery before and after ischemia in a reproducible manner, we use the FMD technique because it is the reference standard for endothelial function [94,95]. We have previously reported FMD to be 12.9% in normal healthy volunteers with within-subject standard

deviation of 0.93% and between-subject standard deviation of 1.77%. Thus, the test-retest coefficient of variation was 7.2% in our laboratory.

Biomarker measurements

We may use specimen collected as part of this study for analysis of DNA which may include whole genome sequencing. Plasma endothelin-1 will be measured using validated ELISA kits (R&D systems, Inc., Minneapolis, MN). 24h urinary nitrite and nitrate and total nitrates will be measured using the Greiss reaction using kits from R&D systems, Inc. Renin and aldosterone will be measured using a commercial laboratory (LabCorp, Burlington, NC). Plasma ADMA will be measured using LCMS in our biochemistry core lab. The usual labs such as blood counts, chemistries, urine albumin and Na will be measured in the hospital laboratory. Renin + aldosterone lab testing will require the participants to be resting in seated position for at least 15 minutes prior to blood draw.

STATISTICAL METHODS

Outcome Variables

The outcome variables are as follows:

1. Change from baseline to 12 weeks in diastolic BP in EPO treated patients compared to delayed start controls. In the waitlisted group, we will measure the change from 12 weeks to 24 weeks compared to the change from 0 to 12 weeks.
2. Change from baseline to 12 weeks in systolic BP in the comparisons above. Between group change in hypertension status from baseline to 12 weeks. Worsening of hypertension at any time point will be defined either as an increase in BP medication or an increase in seated clinic diastolic BP of ≥10 mmHg or systolic BP ≥20 mmHg. Within-group change in hypertension status from 12w to 24 w will be compared to the control period of 0 to 12w in the delayed start group.
3. The primary outcome variable in this aim is to examine the change in FMD from baseline to 4 weeks in those treated with EPO compared to the waitlisted group. The hypothesis being tested is that EPO will cause impairment in endothelial function. Accordingly, those on EPO will have worsening of FMD.
4. The primary outcome variable in this aim is to examine the predictors of change in FMD from baseline to 4 w in those treated with EPO compared to time controls. Predictors of this change will be baseline values of the following variables: ADMA, urine nitrate and nitrite, renin, aldosterone, and plasma endothelin-1 and change from baseline to 4 w in the above variables. This analysis will answer the question: which of the pathways independently is predictive of endothelial dysfunction?

Sample Size

The primary objective of the study is to assess the change from baseline to 12 weeks in diastolic BP in EPO treated patients compared to controls. In the waitlisted group, we will measure the change from 12 weeks to 24 weeks compared to the change from 0 to 12 weeks. We have measured 24h

ambulatory BP in 183 CKD patients 4 weeks apart. The mean diastolic BP at baseline was 68.3 mmHg and at 4 weeks 68.1 mmHg. The mean difference was 0.14 mmHg and the standard deviation of the difference was 10.3 mmHg. With these assumptions, two-tailed tests and 80% power, the required sample size to detect a between-group difference of 5 mmHg is 68 subjects completing the study in each group. Assuming 85% of those randomized complete the trial, we would need to randomize 68/0.85 = 80 subjects per group, or 160 subjects in total.

The standard deviation of the difference in systolic BP is 16.7 mmHg and we can detect an 8.4 mmHg between-group difference using the above assumption. The assessment of endothelial function is another major goal of this application. Position papers on FMD studies 90 state the following: "Typically, significant improvement in FMD can be seen with 20 to 30 patients in a crossover design study and 40 to 60 patients in a parallel-group design study. In studies of this size, the minimal statistically significant improvement that can be detected with intervention is an absolute change in FMD of 1.5% to 2%." The SD of absolute change within subjects in our lab is 0.93%. The sample size proposed (160) will allow us to detect a change of as little as 0.45%. Notably, this change is much more sensitive— approximately one quarter of what is commonly seen with typical sample sizes in conventional FMD studies.

Analysis

The analyses will begin with descriptive statistics. Overall and by-group descriptive statistics will be calculated and examined graphically. Tests for differences in demographics between the early and delayed start groups and characteristics of dropouts will be computed using t-tests, chi square (or Fisher's exact tests if n<6 per cell) to understand if potential differences exist, although such differences are not expected. Comparison of the three UACR strata will be done to assess if there are differences in the populations recruited. Randomization will be within UACR group so that these differences should not bias the trial.

Primary Analysis

To test the hypothesis that early start EPO will worsen the 24-hour DBP assessed by change from baseline to 12 weeks, a mixed model will be used to analyze the results.

We will fit two separate models:

- model 1: The outcome variable will be 24h ambulatory diastolic BP. The predictors will be indicator variables for group (early or late start), time (baseline and 12 weeks), stratum (UACR: none, high, very high) and all interactions. The random component of the mixed model will include subject and time. Unstructured covariance will be used with estimation of marginal means by maximum likelihood. We expect the interaction between group by time to be significant. The random coefficient analysis does not require baseline systolic BP as a covariate. If the 3-way interaction term (group x time x stratum) is significant, then results will be reported within each stratum. Confirmatory analyses will utilize change from baseline in clinic diastolic BP.
- model 2: The outcome variable will be 24h ambulatory diastolic BP. The predictors will be indicator variables for period within the delayed start group (early or late), time (baseline and 12 weeks from start of period), stratum (UACR: none, high, very high) and

all their interactions. The random component of the mixed model will include subject and time. We expect the interaction between period by time to be significant. If the 3-way interaction term is significant, then results will be reported within each stratum.

Accounting for missing data

Ambulatory BP recording, the gold-standard to assess BP, is carried out only twice during the trial in the parallel group analysis, but clinic BP measurements occur every 4 weeks. Patients who drop out but have some post-baseline measurements of clinic BP will be included in the analyses, thus minimizing loss to follow up. We will impute ambulatory BP as follows: we will use the complete data to build a model that relates the follow-up mean 24-hour ambulatory diastolic BP from the baseline 24-hour ambulatory diastolic BP and the clinic measures using repeated measures Mixed Model regression - then use the expected value from the model for the missing value - and use PROC MI (which generates maybe 5 replicate values to insert variability into the imputation) so that there is a price to pay for imputing.

Comparison of the cohort with these measurements will also be done to understand if the inferences based on the ambulatory BPs accurately reflect the differences between the treatment groups using the clinic BPs. Repeated measures mixed models (PROC MIXED in SAS) will be utilized to assess the overall differences between the treatment groups using the clinic BPs. Additional analyses will explore the nocturnal patterns between the groups in the ambulatory BPs as well as the pattern over time using circadian models of BP in each group.

Between-group changes in hypertension status from baseline to 12 weeks will be tested using logistic regression. Worsening of hypertension at any time point will be defined either as an increase in BP medication or an increase in seated clinic diastolic BP of ≥10 mmHg or systolic BP ≥20 mmHg. The McNemar test will be used to compare within-group change in hypertension status from 12w to 24 w to that in the control period of 0 to 12w.

To test the hypothesis that EPO is causal to EIH via worsening of FMD, we will evaluate changes from baseline in FMD. That is, we will test if the worsening is a mediation effect using the technique described by Judd and Kenny [96] and elaborated by MacKinnon [97]. The technique, expanded here for more than one potential mediator employs three regression equations:

A. $Y = \beta_0 + \tau X_p + \varepsilon$

B. $Xi = \beta_0 + \alpha_i X_p + \varepsilon$

C. $Y = \beta_0 + \tau' X_p + \beta_1 X_1 + \ldots + \beta_i X_i + \ldots + \beta_k X_k + \varepsilon$ for all mediators together,
or $Y = \beta_0 + \tau' X_p + \beta_i X_i \varepsilon$ for each mediator separately

Y is the outcome result (e.g., change in 24-hour diastolic systolic BP), X_p is the group (early vs delayed start) and X_i represent the k purported mediators. The total effect of the treatment on the outcome (measured by the coefficient, τ) is the sum of direct effect (τ') of the treatment and the mediated, or indirect, effects ($\alpha_i\beta_i$). Mediation is established when four conclusions are met: (1) the treatment predicts the outcome variable (supported by a test of statistical significance of τ in equation [A]); (2) the treatment causes the potential mediator (α_i is significant in [B]); (3) the mediator causes the

outcome variable controlling for the group assignment (β_i is significant in [C] and $\tau' < \tau$, which provides evidence for mediation rather than suppression); and (4) the mediated effect is statistically significant. For mediators that satisfy conclusions 1 through 3, the significance of mediation (conclusion 4) will be determined via interval estimation of the mediated effect using the asymptotic variance derivation of Sobel [98] since the outcomes are the continuous measures. Additional analyses that look at thresholds of change (binary: worsening of hypertension or not) will utilize bootstrapping methods and logistic regression. The proportion of the effect mediated is calculated as the ratio of the indirect effect to the total effect, or $\alpha\beta/\tau$.

The primary outcome for flow-mediated dilatation is change in FMD from baseline to 4 w in those treated with EPO compared to time controls. Predictors of this change will be baseline values of the following biomarkers: plasma ADMA, urine nitrate and nitrite, and plasma renin, aldosterone, and endothelin-1 and change from baseline to 4 w in the above biomarkers. In addition, the baseline FMD will be an independent variable. This analysis will use regression models similar to the above-listed analyses.

Pre-specified sub-group analyses for BP response

- Effect of age: The effect of age (e.g., 65 or more and <65) will be tested using interaction effects.
- Effect of volume overload: Volume overloaded subjects are more likely to show worsening EIH with EPO therapy. In people with volume overload, renin and aldosterone levels are expected to be low and 24-hour urine Na high. We will dichotomize the baseline levels of these markers and examine the outcome on ambulatory BP using interaction effects.

Safety analysis

The number of subjects who experience hypokalemia, hypercalcemia, gout, hospitalization for diabetes mellitus, transient elevations in serum creatinine concentration, and symptomatic orthostatic hypotension will be counted for each group and compared using a Poisson regression model using terms for treatment and treatment x stratum. The statistical significance of the treatment term will be used to indicate differences between CTD and placebo. If the interaction term is significant then the event rates will be reported for each stratum. The mean change from baseline in serum potassium, calcium, estimated GFR, uric acid, HgbA1C, PTH intact, and orthostatic BP will be calculated using mixed models as noted above.

Alternative study design considerations and clarifications

Choice of non-dialysis patients. Dialysis patients are challenging to study because it is more difficult to determine hypertension due to confounding by dry weight. Angioaccess makes it difficult to perform FMD. Furthermore, the number of dialysis patients—in contrast to CKD patients not on dialysis—is limited.

Timetable/Milestones

We expect to recruit and randomize a total of 160 participants in the first 3.5 years and complete the study in 4 years. This will require approximately 1 participant to be randomized per week. See table below.

Table 1: Timeline for randomization in the trial (t = 4y, n = 160)

	Year 1				Year 2				Year 3				Year 4			
Quarter	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Patients	x	16	16	16	16	12	12	12	12	10	10	10	10	8	x	x

Feasibility

A feasibility assessment was performed using data from the Veterans Administration's Corporate Data Warehouse (CDW), a national repository of data compiled from the electronic medical record (EMR) system used by the Veterans Health Administration (VHA). These data include current and historical values from multiple domains such as patient medications, vitals, labs, procedures, and diagnoses, and are available from October 1st, 1999 forward. Of note, these data are updated on a daily basis and are neither filtered nor validated prior to entry into the CDW. We will use these values to screen potentially eligible participants. It is only possible to uniquely identify potential participants by accessing their EMRs using real social security numbers.

To determine the number of prospective subjects meeting eligibility criteria for the study, a query of all patients seen at the Roudebush VA Medical Center (VAMC) in Indianapolis was performed by the VA Informatics and Computing Infrastructure (VINCI) staff. The query sought to identify adults (at least 18 years of age) seen at the Roudebush VAMC in the past 4 weeks with CKD 3-4 (eGFR 15-60 mg/dL/1.73m2), anemia (Hgb 8-10 g/dL), and controlled clinic BP (<140/90) without receipt of an erythropoiesis-stimulating agent (ESA) within the past 12 weeks. The initial query returned 140 eligible prospective subjects. The following month, the same query returned 28 new eligible prospective subjects.

It is estimated that approximately 25% of eligible prospective subjects will consent to the study visits and, of these, 67% will randomize to the treatment ((e.g., early vs. delayed start of EPO). Since we require ambulatory BP monitoring as an inclusion to participate in the trial, we expect approximately 33% of the patients to fail this criterion. After a robust recruitment in the early quarters, we expect the number of eligible participants to drop and therefore we expect fewer patients in the later quarters. Extrapolating from the feasibility data, we project that recruitment will begin in the second quarter of the first year and end by the third quarter of the fourth year from receipt of the grant, which gives us the following table.

Data and safety monitoring plan (DSMP)

We have prepared a DSMP to specify the process by which the PI will review the progress of the trial and the safety of its participants. Periodic assessments of these data will be done in addition to mandatory reporting requirements of the IRB

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