ID: CARA-019-16F

Frequent, Low-Dose Erythropoietin A Mechanistic Approach to Mitigate Adverse Cardiovascular Effects of Erythropoietin

April 23, 2019

Protocol

1. Project Title: Frequent, Low-Dose Erythropoietin: A Mechanistic Approach to Mitigate Adverse Cardiovascular Effects of Erythropoietin Therapy in Patients with Chronic Kidney Disease

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3. Abstract: Erythropoietin (EPO) is the most widely prescribed cytokine, yet the benefits and potential side effects of different dosing regimens are poorly understood. It is now recognized that erythropoietin administered at high doses to patients with chronic kidney disease, results in an increased risk of morbidity and mortality from heart disease and stroke. However, the mechanisms that mediate this increased risk of cardiovascular disease is not known. There are two receptors for erythropoietin the homodimeric EPO receptor (EPOR) and the heterodimeric beta common receptor (βCR)/EPOR. We have demonstrated that activation of the heterodimeric βCR/EPOR only occurs with high doses of EPO. Our exciting, published, preliminary data also demonstrates that the βCR is in a complex with vascular endothelial growth factor receptor-2 (VEGFR-2) and that high doses of EPO activate VEGFR-2 through the βCR, resulting in the deleterious effects of VEGFR-2 activation on the cardiovascular system. This is particularly important in patients with kidney disease since they are already at a high risk of cardiovascular disease. Moreover in advanced kidney disease cyanate derived from the high urea levels can non-enzymatically form an amide bond with EPO. This carbamylated EPO (cEPO) has no effect on hemoglobin, but still activates the heterodimeric βCR/EPOR. To date there have been no studies that have directly measured levels of cEPO or activation of the βCR/EPOR in patients with kidney disease. Our hypothesis is that the administration of low-doses of EPO more frequently will result in lower levels of total and carbamylated erythropoietin decreased activation of VEGFR-2 via the heterodimeric βCR/EPOR and consequently decreased inflammation and atherosclerosis. We will directly test this hypothesis by randomly allocating 120 patients with chronic kidney

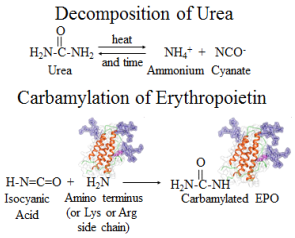
disease to either low- dose EPO given thrice weekly or the same cumulative dose, a high-dose, administered once every 2 weeks. Our hypothesis predicts that low-dose EPO will be as effective at correcting anemia, but will demonstrate less progression of carotid artery plaque, as assessed by non-contrast magnetic resonance imaging, as compared to the high-dose, EPO given every 2 weeks. To delineate how EPO affects blood vessels, we will isolate endothelial cells from blood vessels in 20 patients who are assigned to low-dose EPO and 20 allocated to high-dose EPO. Within these cells we will investigate the signaling pathways that are triggered by activation of βCR/EPOR. In a substudy of 20 subjects with kidney disease randomized to low-dose EPO or to high-dose EPO, as well as 20 healthy controls receiving a single dose of high- or low-dose EPO, we will determine how kidney function and dosing affects levels of total and carbamylated erythropoietin. Our study will not only provide us with a thorough understanding of the mechanism by which EPO mediates the increased risk of atherosclerosis, but a clinical strategy to avoid the side effects of EPO therapy and a tool to quantify the cardiovascular risk of EPO and newer erythropoiesis stimulating agents by assessing activation of the heterodimeric βCR/EPOR.

4. Background:

A.1. Anemia and CKD: Progressive loss of renal function is associated with a reduced ability to synthesize EPO and progressive anemia. The decline in hemoglobin begins to occur when the creatinine clearance falls below 70 ml/min/1.73 m^2 in men and 50 ml/min/1.73 m^2 in women,[25] and worsens with further loss of renal function. In a cross-sectional, multicenter survey of patients with CKD, 9% had a hemoglobin of less than or equal to 10 g/dL, a level that qualifies for EPO treatment.[25] Compared to those with mild CKD, the relative risk of having a hemoglobin of less than 10 increased from 2.6-fold in those with moderate CKD to 7.6-fold in those with severe CKD.[25] Thus, anemia is common in CKD and with progression of renal disease, the majority of patients will require EPO therapy.

A.2. Cardiovascular disease (CVD) and CKD: Approximately 13% of the general population of the US and 20% of Veterans have CKD.[26] CKD is associated with exponential increases in morbidity and mortality and patients with advanced CKD, especially those with end stage kidney disease (ESKD), carry annual mortality rates 5-25 times higher than the age and gender matched general population.[27] CVD is the major contributor to mortality in patients with CKD and ESKD, with nearly half of all deaths attributed to CVD.[28] CKD and ESKD are also major risk factors for accelerated atherosclerosis[29] and increased risk of premature[30] and recurrent atherosclerotic CVD.[31] Patients with CKD are also at a substantial risk for non-atherosclerotic CVD, which is in large part believed to be related to vascular stiffness/endothelial dysfunction, and inflammation.[28, 32]

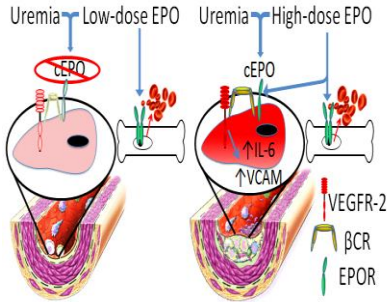
A.3. Erythropoiesis stimulating agents and increased risk of CVD and death from cancer recurrence: The landmark clinical trials, Correction of Hemoglobin and Outcomes in Renal Insufficiency (CHOIR),[33] The Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta (CREATE),[34] and the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT)[35] all demonstrated that targeting a higher hemoglobin, with increased doses of an erythropoiesis stimulating agent, led to an increase in the primary composite endpoint of death, myocardial infarction, and stroke in CHOIR, more angina and peripheral vascular disease in CREATE, and increased incidence of stroke and cardiac revascularization in TREAT. We demonstrated that mobilization of bone marrow derived angiogenic cells (BMDAC) by EPO is dependent on the heterodimeric βCR/EPOR signaling though the VEGFR-2.[1] Since the Kd of



the heterodimeric βCR/EPOR is ~9-times higher than that of the homodimeric EPOR, we believe the clinical trials targeting a higher hemoglobin level, using higher doses of erythropoiesis stimulating agents, led to activation of the heterodimeric βCR/EPOR and in turn VEGFR-2. Activation of VEGFR-2 leads to an increase in inflammation[36] and downregulation of VEGFR-2 diminishes the risk of atherosclerosis.[37] Thus activation of the heterodimeric βCR/EPOR may explain the cardiovascular side effects seen with high-doses of EPO. This is supported by a recent meta-analysis of 31-randomized control trials demonstrating that the dose of EPO correlated strongly with cardiovascular adverse events, even after controlling for the target hemoglobin.[38] Our proposal directly tests this hypothesis and if true would provide a mechanism to increase hemoglobin levels without increasing the risk of adverse cardiovascular events.

A.4. The benefit of higher hemoglobin levels on quality of life: In response to the CHOIR,[33] CREATE,[39] and TREAT[35] studies, in 2007 the FDA required a black box warning on erythropoiesis stimulating agents to avoid targeting a hemoglobin level greater than 11 g/dL. However, in a retrospective analysis, a time-averaged hemoglobin less than 12 g/dL in patients with CKD was associated with higher mortality and faster progression to ESKD.[40] This observation was confirmed in a subsequent retrospective study, specifically in Veterans, which demonstrated a slower decline in renal function after initiation of erythropoiesis stimulating agents.[41] Studies that have examined the quality of life and hemoglobin level have consistently demonstrated an improvement in the quality of life[42] as well as work capacity[43] with increasing hemoglobin. So limiting the use of EPO threatens to hasten the progression of kidney disease and need for dialysis, increase mortality, and decrease quality of life. If our hypothesis is correct, we would be able to obtain the benefits of a higher hemoglobin level on quality of life and slowing progression of CKD, while avoiding the increased risk of cardiovascular events.

A.5. Hypothesis to be tested and its significance: As previously discussed there are two EPO receptors: 1) the homodimeric EPOR that has a Kd of ~6 mIU/ml; and 2) the heterodimeric βCR/EPOR that has a Kd of ~50 mIU/ml. Activation of the heterodimeric βCR/EPOR only occurs with high-doses of EPO. The βCR is in a complex with VEGFR-2 and thus supraphysiologic doses of EPO activate VEGFR-2 through the βCR, resulting in all the deleterious effects of excessive VEGFR-2 activation. The erythropoietic effect of EPO is inactivated by carbamylation, a process in which the high urea levels in CKD patients leads to production of cyanate which modifies EPO through an amide bond can modify EPO (**Figure 2**). However, cEPO can still activate the heterodimeric βCR/EPOR.[19] **Our hypothesis is that frequent administration of low-doses of EPO, as compared to the administration of infrequent, high-dose EPO, will result in lower levels of total and carbamylated EPO, decreased activation of VEGFR-2 via the heterodimeric βCR/EPOR, and consequently decreased inflammation and atherosclerosis (Figure 3).** By understanding the complex interaction between EPO dosing, carbamylation, and half-lives of EPO and cEPO we will be able to develop optimal dosing strategies for EPO and erythropoietin stimulating agents, minimizing adverse events.



A.6. Relevance of hypothesis to a new generation of erythropoiesis stimulating agents: For the last thirty years, the basis of erythropoiesis stimulating agents has been on directly stimulating the homodimeric EPOR, with either EPO or modified EPO (darbepoetin alfa an engineered EPO with extra glycosylation to increase its half-life). EPO is regulated predominantly by oxygen sensing by hypoxia-inducible factor (HIF) prolyl hydroxylases. Mimicking hypoxia by inhibiting HIF prolyl hydroxylases, leads to HIF stabilization and leads to an increase in endogenous EPO production. Recent studies[3, 4] have demonstrated that at doses that lead to an increase in hemoglobin levels, prolyl hydroxylase inhibitors will result in EPO levels well above 50 mIU/ml, the Kd of the heterodimeric βCR/EPO. Our hypothesis predicts that endogenous EPO levels above the Kd of the heterodimeric βCR/EPOR, would lead to the same side effects as exogenous EPO. The cardiovascular and cancer risk of EPO was not recognized until 17 years after EPO came to market and only because of large clinical trials powered to look at cardiovascular endpoints. The new erythropoiesis stimulating agents which inhibit prolyl hydroxylase may have the same cardiovascular side effects, which if not dosed properly may go unrecognized. ***This study will not only provide us with a thorough understanding of the mechanism by which EPO mediates the increased risk of atherosclerosis, but will also offer a clinical strategy to avoid the side effects of EPO therapy and a tool to quantify the cardiovascular risk of EPO and newer erythropoiesis stimulating agents by assessing activation of the heterodimeric βCR/EPOR.***

A.7. Relevance to the Veteran population: The prevalence of CKD in the Veteran population is estimated to be 34% higher than in the general population due to demographic differences and the prevalence of co-morbidities, such as diabetes mellitus and hypertension. The Veterans Administration (VA) currently cares for over 600,000 Veterans with kidney disease and 15,000 Veterans receive dialysis treatments at the 70 VA dialysis units or in the community under VA contracted care. Of Veterans with CKD initiating dialysis, 36% are estimated to be receiving EPO and another 31% would benefit from EPO therapy.[44] However EPO use could increase the risk of CVD, which is the largest cause of morbidity and mortality among the aging US Veteran population and the top expenditure among chronic conditions for the VA healthcare system. A therapeutic strategy that will improve the quality of life of Veterans with kidney disease, slow down progression of kidney disease, and decrease the risk of heart disease will be of immense benefit.

B. PRELIMINARY STUDIES

The foundation for our hypothesis was our work on the mechanism by which EPO mobilized BMDAC. We had previously shown nitric oxide (NO) generation was critical in BMDAC mobilization and NO deficiency impaired BMDAC mobilization.[2] We confirmed the observation by others[45] that EPO mobilizes BMDAC and wanted to determine if EPO mobilization was NO dependent and which EPO receptor mediated mobilization.

B.1. EPO increases CD34+ BMDAC migration via an NO dependent mechanism: It has previously been demonstrated that EPO lea to an increase in NO within endothelial cells.[46] We have demonstrated that CD34+ BMDAC migration was dependent on NO.[2] We have also shown that EPO stimulates chemokine mediated CD34+ BMDAC migration (**Figure 4**). Importantly, the ability of EPO to stimulate CD34+ BMDAC migration is NO dependent. If CD34+ BMDAC are preincubated with 10 μM of a NO synthase inhibitor, N^G-Nitro-L-Arginine Methyl Ester (L-NAME), a concentration that inhibits the ability of CD34+ BMDAC to increase the level of NO, but does not alter basal levels of NO (data not shown), EPO's ability to stimulate CD34+ BMDAC migration is markedly reduced (**Figure 4**).

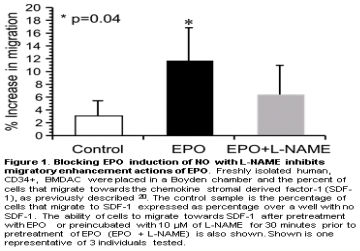
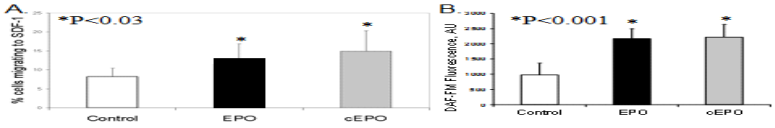


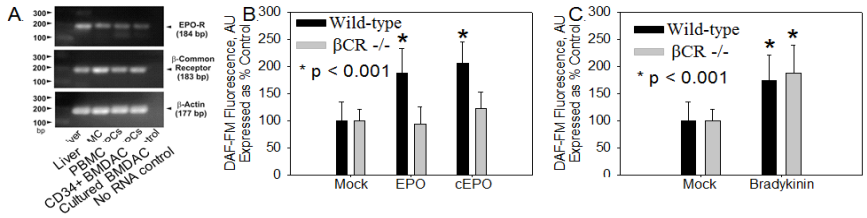
Figure 4. Blocking EPO induction of NO with L-NAME inhibits migratory enhancement actions of EPO. Freshly isolated human, CD34+, BMDAC were placed in a Boyden chamber and the percent of cells that migrate towards the chemokine stromal derived factor-1 (SDF-1), as previously described [46]. The control sample is the percentage of cells that migrate to SDF-1 expressed as percentage over a well with no SDF-1. The ability of cells to migrate towards SDF-1 after pretreatment with EPO or preincubated with 10 μM of L-NAME for 30 minutes prior to pretreatment of EPO (EPO + L-NAME) is also shown. Shown is one representative of 3 individuals tested.

B.2. EPO induction of NO within CD34+ BMDAC is likely to occur via the heterodimeric βCR/EPOR: cEPO is only able to stimulate the heterodimeric βCR/EPOR. However, cEPO is able to stimulate CD34+ BMDAC migration to an equal extent as EPO (**Figure 5A**) and cEPO is able to stimulate NO within CD34+ BMDAC as effectively as EPO (**Figure 5B**). The implication of these results is that the heterodimeric βCR/EPOR may be mediating EPO induced stimulation of BMDAC. To determine the mechanism of this stimulation in NO, we examined phosphorylation of eNOS, a major mechanism of control of eNOS activity, and found that EPO induces phosphorylation of eNOS on serine 1177.[1]

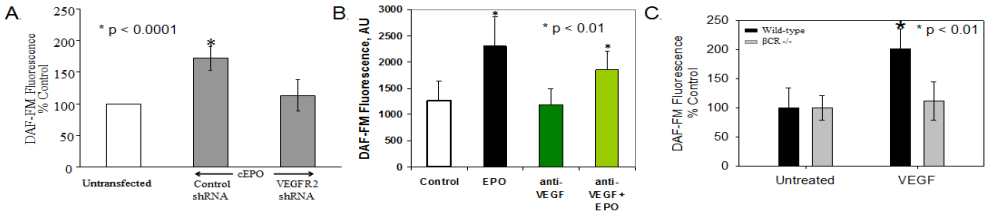


B.3. The heterodimeric βCR/EPOR is expressed in CD34+ BMDAC and mediates the increase in NO within BMDAC: By real time-PCR analysis, CD34+ BMDAC as well as human BMDAC colonies appear to express the βCR and the EPOR (**Figure 6A**). The CR is required for EPO/cEPO induction of NO, since mouse BMDAC colonies from a βCR knockout (-/-) mice did not increase their intracellular NO in response to EPO or cEPO (**Figure 6B**). Importantly,

bradykinin could stimulate NO in these mouse colonies as well as in BMDAC colonies isolated from wild-type mice, indicating the NO synthase system was intact (**Figure 6C**).

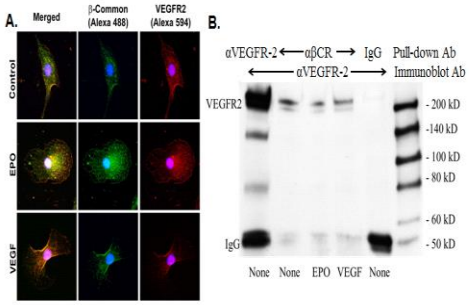


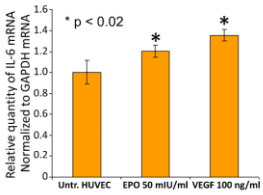
B.4. Vascular endothelial growth factor (VEGF)-induced NO production is dependent on the heterodimeric βCR/EPOR: If EPO is able to mobilize BMDAC and increasing BMDAC is beneficial,[47] why did the CHOIR,[33] CREATE,[41] and TREAT[33] studies demonstrate adverse events when a higher hemoglobin level was targeted? To investigate this question, we studied the downstream signaling of EPO. We found that EPO requires the presence of VEGFR-2 (**Figure 7A**) and that paracrine production of VEGF was not involved, since anti-VEGF antibodies added to the media did not prevent EPO mediated increase in NO (**Figure 7B**). In addition, VEGF stimulated an increase in NO that was dependent on the presence of the βCR, since bone marrow cells from βCR knockout animals had no increase in NO in response to VEGF treatment (**Figure 7C**). This suggested an interaction between the βCR with VEGFR-2.



B.5. The βCR and VEGFR-2 colocalize and co-immunoprecipitate: Since the heterodimeric βCR/EPOR required VEGFR-2 and the VEGFR-2 required the βCR for stimulation of NO, this

suggested an intimate interaction between the two receptors. When examined by immunofluorescence, the two receptors colocalized and stimulation with EPO or VEGF did not affect the colocalization (**Figure 8A**). When we immunoprecipitate the βCR from endothelial cell extracts and immunoblot with an antibody against VEGFR-2, VEGFR-2 was detected. Again stimulating with EPO or VEGF did not influence the interaction (**Figure 8B**)

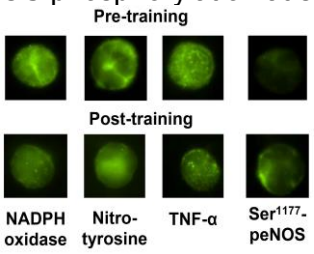




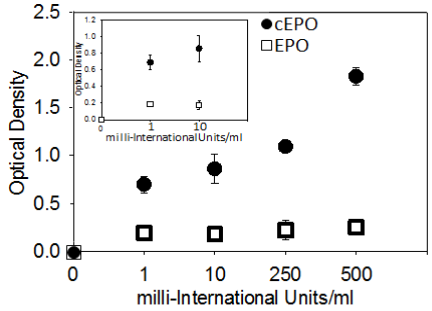
B.6. Activation of VEGFR-2 or the heterodimeric βCR/EPOR results in inflammation: Since a previous study demonstrated that activation of VEGFR-2 results in an increase in inflammation, as evidenced by an increase in IL-6[36], we hypothesized that treatment with EPO at a dose to stimulate the heterodimeric βCR/EPOR would also lead to an increase in IL-6. RNA was isolated from endothelial cells treated overnight with either 50 mIU/ml EPO, a dose that stimulates the hetero-dimeric βCR/EPOR, or 100 ng/ml of VEGF, and real-time PCR performed. EPO treatment resulted in an increase in IL-6 (**Figure 9**).

B.7. Ability to capture and analyze an individual's endothelial cells: In order to examine the effect of the different EPO dosing regimens on endothelial activation *in-vivo*, in **Specific Aim 2** we will use a translational, physiological approach to capture and study endothelial cells, as we have previously published.[48-50] Briefly, 2 sterile J-shaped guidewires will be sequentially advanced ~10 cm through an 18-gauge antecubital intravenous catheter and retracted. Cells will be recovered by washing the wires with a dissociation buffer and centrifugation. After fixing the cells with 4% paraformaldehyde, the cells will be washed, plated on poly-L-lysine coated slides, and stored at -80°C until immunofluorescence staining is performed. Based on studies on human subjects over a wide range of age and chronic medical conditions an adequate number of endothelial cells are obtained via venous biopsies to allow for quantification of protein levels by immunofluorescence.[48, 51, 52] For immunofluorescence staining, the fixed, vascular, endothelial cells will be rehydrated and the slides incubated with the appropriate primary antibodies followed with a corresponding Alexa Fluor 488 conjugated secondary antibody. Slides will also be incubated with a primary antibody for VE Cadherin (Abcam, Cambridge, MA) and corresponding secondary antibody conjugated with Alexa Fluor 555 (Life technologies, Grand Island NY) to allow identification of endothelial cells. Finally, slides will be mounted with Vectashield with nuclear DAPI stain (Vector Laboratories, Burlingame, CA). To minimize the

potential confounding of inter-batch variability in staining, 2 slides of control cells (i.e., human umbilical vein endothelial cells; HUVEC) will be stained in each batch and endothelial cell protein levels will be reported relative to average HUVEC intensity in that batch. For analysis, cells will be examined with a fluorescence microscope at 100 X magnification using a fixed exposure time (**Figure 10)**. We have previously demonstrated, in a pilot study, that exercise training resulted in a decreased expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), nitrotyrosine, and tumor necrosis factor alpha (TNF-α) and upregulation of eNOS phosphorylation at serine 1177 **(Figure 11)**.



B.8. Ability to detect cEPO: Although the concept of inactivation of the erythropoietic actions of EPO was first described by Mun and Golper in 2000,[53] to date, there has been no publication demonstrating the ability to directly detect cEPO. A key feature of our hypothesis is that cEPO can lead to activation of the heterodimeric βCR/EPOR, while not aiding erythropoiesis. To determine whether the levels of cEPO are affected by different dosing strategies (frequent, low-dose versus infrequent, high-dose), we need to determine the level of cEPO in blood and for the first-time determine its half-life. Using *in-vitro* cEPO, we were able to capture cEPO on a standard EPO ELISA plate (R&D Systems, Inc., Minneapolis, MN) and then using a primary biotinylated antibody that recognizes carbamoyl groups (MyBioSource, Inc., San Diego, CA) and streptavidin poly-horseradish peroxidase (Pierce), to amplify the signal, we were able to detect 1 mIU/ml of cEPO (**Figure 12**).



Implications of the preliminary data: We have demonstrated that EPO mobilization of BMDAC is dependent on the heterodimeric βCR/EPOR, whose activation requires the presence of VEGFR-2. EPO, like VEGF, induces IL-6 in endothelial cells. This preliminary data provides a mechanism by which higher doses of EPO can mediate atherosclerotic events via activation of

the heterodimeric βCR/EPOR, likewise lower, more frequent doses of EPO may be able to increase the hemoglobin levels without untoward side effects. In addition, we have demonstrated that we have the ability to monitor the direct, *in-vivo* effects of EPO on biopsied endothelial cells. Lastly, we can accurately detect and quantify cEPO and thus we will be able to determine whether the carbamylation and the half-life of cEPO are dependent on the dosing regimen of EPO.

5. Specific Aims:

Erythropoietin (EPO) is the most widely prescribed cytokine, yet the benefits and potential side effects of different dosing regimens are poorly understood. Unfortunately, in an effort to save nursing visits and administration costs, there has been a clinical shift in this country to give fewer doses of EPO, but at supraphysiologic doses. This was done without regard to the presence of two EPO receptors: 1) the homodimeric EPO receptor (EPOR) that has high affinity for EPO [Kd of ~6 milli-International Units per milliliter (mIU/ml)] and is responsible for erythropoiesis; and 2) the low affinity [Kd of ~50 mIU/ml] heterodimeric beta common receptor (βCR)/EPOR. We have demonstrated that activation of the heterodimeric βCR/EPOR only occurs with high doses of EPO. Our exciting, published, preliminary data demonstrates that the βCR is in a complex with vascular endothelial growth factor receptor-2 (VEGFR-2) and that supraphysiologic doses of EPO activate VEGFR-2 through the βCR, resulting in all the deleterious effects of excessive VEGFR-2 activation, such as increased inflammation and atherosclerosis. Clinically, it is now recognized that EPO administered at high doses to cancer patients results in an increased probability of cancer recurrence and patients with chronic kidney disease (CKD) randomized to a higher hemoglobin target and receiving elevated doses of EPO have an increased risk of morbidity and mortality from heart disease and stroke. We believe that our novel observation of VEGFR-2 activation by high doses of EPO explains these clinical findings. Importantly in patients with kidney disease, cyanate, derived from the high urea levels, can non-enzymatically form an amide bond with EPO. This carbamylated EPO(cEPO) lacks erythropoietic activity, but is still able to activate the heterodimeric βCR/EPOR. Consistent with this, levels of carbamylated albumin are increased in CKD and correlates with EPO resistance. However, no study has directly measured levels of cEPO or activation of the βCR/EPOR in CKD. The activation of the heterodimeric βCR/EPOR is dependent on a complex interaction between EPO dosing, carbamylation, and the half-lives of EPO and cEPO. **Our hypothesis is that frequent administration of low-doses of EPO, as compared to the administration of infrequent, high-dose EPO, will result in lower levels of total and carbamylated EPO, decreased activation of VEGFR-2 via the heterodimeric βCR/EPOR, and consequently decreased inflammation and atherosclerosis.** We will directly test this hypothesis by the following specific aims:

Specific Aim 1*: Determine if administration of more frequent, low-doses of EPO as compared to the same, cumulative dose given every 2 weeks, will be as effective at stimulating erythropoiesis, but have less progression of carotid artery plaque in subjects with CKD and anemia requiring EPO therapy.*

A total of 100 subjects with stage 3 or 4 CKD, estimated glomerular filtration rate between 15-59 ml/min/1.73 m^2, and requiring EPO therapy will be randomized to receive either low-dose EPO (25 IU/kg/dose) three-times a week, or the same, cumulative dose administered every two

weeks (i.e., 150 IU/kg/2 weeks; high-dose). The progression of carotid artery plaque will be determined by magnetic resonance imaging (MRI) at baseline and after 12 months of receiving EPO.

Specific Aim 2*: Determine whether receiving high-dose EPO results in VEGFR-2 activation and increased endothelial activation, as measured by phosphorylated VEGFR-2, vascular cell adhesion molecule (VCAM) and interleukin-6 (IL-6) expression, as compared to low-dose EPO.*

In 20 subjects randomized to low-dose EPO three-times a week and 20 subjects randomized to high-dose EPO every 2 weeks, we will capture endothelial cells using a vascular endothelial cell biopsy technique, at baseline and after 12 months. Endothelial cell activation will be quantified by immunofluorescence.

Specific Aim 3*: Determine the interaction of renal function and dosing of EPO on a) the rate of carbamylation; and b) the length of time that total and cEPO exceed the Kd of the βCR/EPOR.*

In a subset of 5 subjects randomized to low-dose EPO, 5 subjects randomized to high-dose EPO, as well as 5 healthy subjects who will receive a single, low-dose of EPO, and 5 healthy subjects who will receive a single, high-dose of EPO, we will determine the pharmacokinetics of EPO and cEPO. We predict that high-dose EPO given every 2 weeks will result in increased time with total serum levels of EPO exceeding 50 mIU/ml, the Kd of the βCR/EPOR.

The cardiovascular and cancer risk of EPO was not recognized until 17 years after EPO came to market and only because of large clinical trials powered to look at cardiovascular endpoints. Although new erythropoiesis stimulating agents which inhibit prolyl hydroxylase are being developed, they stimulate endogenous EPO production and may have the same cardiovascular side effects, which if not dosed properly may go unrecognized. This study will not only provide us with a thorough understanding of the mechanism by which EPO mediates the increased risk of atherosclerosis, but a clinical strategy to avoid the side effects of EPO therapy and a tool to quantify the cardiovascular risk of erythropoiesis stimulating agents.

6. Research Plan:

Please note that only some of the study subjects enrolled into Aim 1, will be asked to enroll into the sub-studies for aims 2 and 3. In addition, only aim 3 will have a healthy control group.

C.1. ***Specific Aim 1****: Determine if administration of thrice weekly, low-doses of EPO as compared to the same, cumulative dose given every 2 weeks, will be as effective at stimulating erythropoiesis, but slow progression of carotid artery plaque in subjects with CKD and anemia requiring EPO therapy.*

Rationale: Several well-designed, large, randomized, control trials have demonstrated that EPO therapy targeting a higher hemoglobin level, is associated with adverse cardiovascular outcomes in patients with CKD.[33-35] A recent meta-analysis of 31 prospective studies found that adverse effects are related to the dose of EPO, rather than targeted or attained hemoglobin levels.[38] Consistent with this observation, increased levels of endogenous EPO have been associated with adverse cardiovascular events in diverse patient populations.[54-56] While EPO has been associated with endothelial dysfunction in patients with CKD[57] and *in-vitro* EPO stimulates proliferation of vascular smooth muscle cells,[58] the mechanistic basis of accelerated atherosclerosis with EPO therapy remains unknown. Our preliminary data demonstrates that high-doses of EPO stimulates the heterodimeric βCR/EPOR and in turn VEGFR-2. Persistent activation of VEGFR-2 has been associated with inflammation[36] and atherosclerosis.[37] The

stimulation of the heterodimeric βCR/EPOR does not occur with low-doses of EPO. Thus our novel observation not only suggests an underlying mechanism for atherosclerosis associated with EPO therapy, but also provides an inexpensive way to mitigate cardiovascular side-effects of EPO therapy by administering more frequent, lower-doses of EPO.

Study design: This is an open label, randomized, clinical trial comparing progression of carotid atherosclerosis in subjects with stage 3 -5 CKD (estimated glomerular filtration rate of <30 ml/min/1.73 m^2) and anemia requiring EPO therapy assigned to thrice weekly, low-dose EPO or to same, cumulative high-dose of EPO administered every 2 weeks.

Primary outcome measure: Change in carotid total plaque volume from baseline to approximately 1 year, as assessed by non-contrast MRI.

Secondary outcome measures: Severity of maximal stenosis, percentage of total plaque area, and the characteristics of plaques (soft or fibrous and stable or unstable) at baseline and upon follow-up.

Investigators and sites: Mark S. Segal, MD/PhD is an expert in molecular biology particularly of endothelial cells and EPO signaling and has been the principal investigator of several National Institute of Health funded clinical trials. Rajesh Mohandas, MD, MPH has training in epidemiology and is an expert in clinical trials and data analysis. Drs. Segal and Mohandas are physicians at the Malcom Randall VA Medical Center (VAMC), a large, academic VAMC within the North Florida/South Georgia Veterans Health System (NF/SGVHS) where they study will be conducted. The NF/SGVHS consists of two VAMCs, three large multi-specialty outpatient clinics, and eight small community-based primary care outpatient clinics. In 2013, NF/SGVHS was the busiest health care system in the VA system, treating 131,000 unique patients, with 1.6 million outpatient visits. It currently has more than 300 active research projects and is home to four VA Research Centers.

Study population: We will enroll Veterans who fulfill the following criteria: **1**) age greater than 18; **2**) stage 3, 4, or 5 CKD (estimated glomerular filtration rate of less than 60 ml/min/1.73 m^2) on at least two separate occasions greater than 3 months apart; and **3**) candidates for EPO therapy as per the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative guidelines (hemoglobin < 10 gm/dL and anemia of CKD). For specific aim 3, we will also enroll age- and sex-matched control Veterans. These subjects can have chronic medical conditions, like hypertension, that are treated, but will be required to have an estimated glomerular filtration rate of greater than 60ml/min/1.73 m^2.

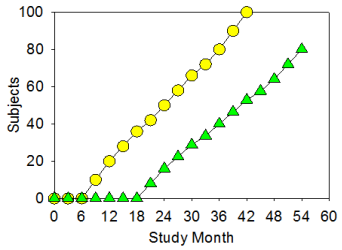
Exclusion criteria: We will exclude any Veteran who meets any of the following criteria: **a**) pregnant, planning to become pregnant in the next year, or breast feeding; **b**) uncontrolled hypertension (blood pressure > 180/100 mm Hg despite optimal antihypertensive medications); **c**) active gastrointestinal bleeding (visible blood or positive tests for stool occult blood accompanied by a decrease in hemoglobin); **d**) likely to have EPO resistance (serum ferritin < 200 ng/ml, iron saturation < 20% despite iron replacement, intact PTH ≥ 600 pg/ml, alkaline phosphatase ≥ 160 IU/L or serum albumin < 3.0 g/dL); **e)** an adverse cardiovascular event in the prior three months; **f**) active or recent (within the last 3 months) severe, systemic infection; **g**) active inflammatory disease such as lupus, rheumatoid arthritis, or vasculitis requiring immunosuppressive or immunomodulatory medications; **h**) history of solid organ transplantation; **i**) expected off-dialysis survival of less than one year (as determined by the estimated glomerular filtration slope and the treating physician; **j**) active cancer (undergoing chemotherapy or radiation within the last 3 months) or primary bone marrow disease such as

myelofibrosis; or **k**) a contraindication for an MRI or individuals who cannot comply with the study protocol. We will exclude healthy subjects you meet a, b, f, g, h, or j.

Rationale for the study population: Five percent of patients with stage 3 CKD and almost half (44%) of patients with stage 4 CKD have anemia, which often requires EPO therapy.[25] Cross-sectional studies have shown that patients with CKD stage 3, 4 or 5 have significant carotid atherosclerosis.[59] Since EPO therapy increases the risk of adverse cardiovascular events by 30%,[33] it is likely these atherosclerotic lesions will progress during the proposed 1-year follow-up period. Thus, this patient population is uniquely suited to study the effect on EPO on atherosclerosis. We excluded those on dialysis and those expected to require dialysis during the follow up period, to avoid confounding by factors that are inherent to renal replacement therapy, such as access, infection, adequacy of dialysis etc.

Recruitment strategy: Any new patient referred to the EPO clinic at NF/SGVHS will be eligible for screening for recruitment. Veterans that are referred to the clinic will be mailed the recruitment letter and will be followed up as below.

We will also utilize a 'source cohort strategy' to identify and enroll subjects that have a hemoglobin of less than 10 gm/dL and CKD stage 3, 4, or 5 who may not have been referred to the EPO clinic. This technique has been successfully employed by a number of clinical researchers at NF/SGVHS and the University of Florida (UF) with excellent enrollment rates. If found eligible, the Veteran will receive a letter from our research team, signed by the Veteran's primary care provider, informing them that a study coordinator will contact them by phone in approximately 2 weeks to discuss the possibility of participation. A toll-free number will be provided for the Veteran to call in to "opt-out" of participation. Those who do not opt-out will be contacted by telephone. We will ensure that subjects are not coerced and know they have the option of not participating if they so desire. Once patients agree to participate, we will carry out a phone screen to make sure our EMR screen did not miss any exclusion criteria. All Veterans in the source cohort will be evaluated consecutively for eligibility by chart review. This process will continue until the target enrollment of 100 is achieved.



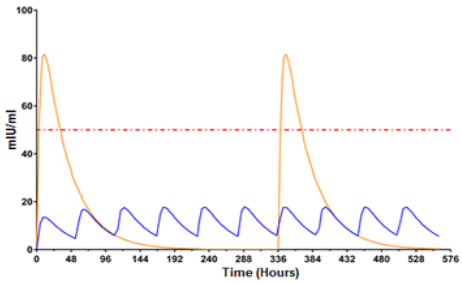
Control subjects for specific aim 3 will be via a flyer as well as we will ask those participants in the study if any of their friends of the same sex and same age, who are Veterans, who have normal kidney function would want to participate.

Recruitment feasibility: CKD is prevalent and it is estimated that 4-5% of the general population has CKD. The prevalence is higher (greater than 2-fold) among Veterans.[60-62] The NF/SGVHS has one of the largest populations of CKD patients within the VA and has a proven track record of supporting clinical and basic research. In preparation for this study, we have obtained the 'numbers' of our source cohort available through a de-identified query of 'VINCI' and found that we have a total of 1,568 patients with stage 3 or 4 CKD, within our VAMC, who have a hemoglobin of less than 10 gm/dL. All have at least one outpatient visit

over the last 18 months and appear to fulfill our inclusion and exclusion criteria. Even with a conservative enrollment rate of 7%, we should have an adequate number of subjects to achieve our recruitment goals. A number of ongoing or recently concluded clinical studies performed at the NF/SG-VAMC (Ongoing Institutional Review Board -protocols # UF142-2009, 148-2012) have consistently demonstrated recruitment rates in the 10-15% range. **Figure 13** is a graph of our recruitment goals and expected rate of subject retention over the life of the study. We will assess the enrollment and targets, in our monthly milestone meetings. In the unlikely scenario that recruitment is lagging, we will consider extending recruitment to the Bay Pines VAMC.

Randomization: Studies have demonstrated that cardiovascular outcomes are worse in patients with proteinuria regardless of the cause of underlying kidney disease.[63] Thus, we will employ block randomization stratified by albuminuria >300 mg/gm of creatinine. A complete list will be maintained by the investigators and will be periodically reviewed by the Data Safety and Monitoring Board (DSMB), per protocol. The pharmacy will dispense EPO and maintain records.

Intervention: Subjects will be randomized to a low-dose of EPO administered thrice weekly or the same cumulative dose of EPO administered as a high-dose of EPO every 2 weeks. The starting dose of EPO will be approximately 25 IU/kg given subcutaneously thrice weekly or approximately 150 IU/kg given every 2 weeks. These dosing regimens have been validated in large clinical trials and have been shown to be equally efficacious in treating anemia.[11] While titrating the EPO dose, hemoglobin measurements will be performed approximately every 2 weeks. However patient or clinical circumstances may dictate obtaining hemoglobin more or less frequently and approximately every 4 weeks, however patient or clinical circumstances may dictate obtaining hemoglobin more or less frequently, once therapeutic levels of hemoglobin are attained. The pharmacists at the EPO Clinic will follow the hemoglobin level and titrate the dose per a clinical protocol.



Rationale for EPO dosing regimens: To test our hypothesis we have chosen two clinically utilized, well-validated, EPO dosing regimens with the same cumulative dose of EPO. We used pharmacokinetic modeling to determine if the serum concentrations differed. The results of our modeling, shown in **Figure 14**, demonstrated that 25 IU/kg/dose of EPO administered subcutaneously every 48 hours will result in a peak serum EPO level of only 17.8 mIU/ml.[64] This level is well below the Kd of the heterodimeric βCR/EPOR. In addition, we determined that any EPO dose below 67 IU/kg administered subcutaneously every 48 hours would not attain serum EPO levels above 50 mIU/ml. Alternatively, subcutaneous dosing of EPO at 150 IU/kg every other week, results in peak levels of EPO of 81.7 mIU/ml, a concentration above the Kd of heterodimeric βCR/EPOR (50 mIU/ml).[65] In addition, the area under the curve of EPO dosed at 25 IU/kg three times a week is only 736, nearly **six-times less**

than the area under the curve for EPO dosed at 150 IU/kg every other week of 4,413. In fact, a subject dosed at 150 IU/kg every other week will have an EPO level above 50 mIU/ml for 60 hours a month, compared to 0 hours spent above 50 mIU/ml when EPO is dosed at 25 IU/kg three times a week. Thus we are confident that these dosing regimens will afford us the ability to study the effects of activation of heterodimeric βCR/EPOR on carotid atherosclerosis.

Duration of intervention: Each subject will be treated with low- or high-dose EPO for approximately 12 months or until they reach ESKD or death. In some circumstances the subject may be on EPO for over 14 months until the MRI could be scheduled at a time convenient for the subject. In previous studies, of comparable sample sizes,[66, 67] this duration has proven to be sufficient to detect a clinically significant change in progression of atherosclerosis. Subjects who develop ESKD as well as subjects in the low-dose EPO group who require EPO doses above 65 IU/kg subcutaneously thrice weekly will be censored for analysis. If they have completed at least 6 months of therapy, we will perform a carotid MRI and endothelial cell sampling prior to censoring.

Subject follow-up and retention: We will ensure compliance by having the study staff contact the subjects approximately every 2-4 weeks. This frequent contact will reinforce medication compliance, make sure that any subject's concerns are answered, and ensure all side effects are reported. Study visits will occur at enrollment and at the conclusion of the study at 1 year (**Table 1**). Subjects will be reimbursed for their travel costs and their time and effort. We will use pharmacy refill data (medication possession ratio)[68] to categorize subject adherence. As per consensus in the literature, subjects with a ratio greater than 80% will be considered adherent.[69]

Table 1. Schedule of Visits and Procedures

VISIT	Visit 1 (Baseline)	Visit 2 (12 months)
Screening Medical Records	X	
Informed Consent	X	
Physical Examination	X	X
Routine Laboratory Data	X	X
MRI of Carotid vessels	X	X
Endothelial Cell Harvest (n=20)	X	X
Pharmacokinetic study (n=10)	X	

Labs: Reticulocyte count, vitamin B12, folate, iron profile (serum iron, iron binding capacity and ferritin) thyroid stimulating hormone levels, intact parathyroid hormone levels will be performed at baseline. Iron profile will be repeated approximately every 3 months, as per standard-of-care.

Subject safety and monitoring for adverse events: EPO will be prescribed as per current guidelines for clinical use. Adverse events will be recorded locally and serious adverse events will be communicated with the DSMB within 24 hours of occurrence. The DSMB will meet quarterly, as per the Institutional Review Board requirements, and will have full access to all adverse events and randomization codes. Outcomes will be ascertained by examination of VA medical records approximately every 6 months during the duration of study. In addition, if during the routine phone calls an adverse event is noted, an experienced nurse or physician will contact the subject to capture care that might have been received outside of the VA system. We will ascertain the following outcomes: death, major cardiovascular event (hospitalization for myocardial infarction or angina, congestive heart failure or stroke), and composite of death or major cardiovascular events. The frequency of death is expected to be low in this population for the proposed follow-up period. However, when a subject is identified as deceased, all information on the circumstances surrounding death, including best estimate of cause of death, will be documented and classified as cardiovascular or non-cardiovascular related. For subjects

admitted to the VA system for cardiovascular reasons, we will collect documentation from the emergency room and inpatient chart to classify hospitalizations as myocardial infarction, angina, congestive heart failure or stroke. Events will be adjudicated by a committee that is blinded to the randomization. If a Veteran has a non-fatal cardiac event, they will continue to be followed according to the protocol. While we are not powered to see differences in adverse events, for safety, we will perform an interim analysis by an independent statistician blinded to the treatment allocation when 50% subjects have been randomized and completed 1 year of follow up. We will compare the adjusted event-free times to death, non-fatal myocardial infarction or major cardiovascular event using the using a two-sided significance test with the O'Brien–Fleming spending function and a type I error rate of 5 percent. If there is a statistical difference between the two arms, the study will be terminated by the DSMB.

Rationale for carotid MRI: Non-contrast MRI of carotid for total plaque area has numerous advantages compared to available alternatives, particularly in the CKD population *(***Table 2***).* MRI is non-invasive, provides superior images and minimizes inter-observer variations.[70, 71] The use of non-contrast MRI to identify and follow the progression of atherosclerotic plaques is well established both in the general population and in CKD patients.[72, 73] Several studies have validated that carotid MRI provides excellent distinction of normal from pathological carotid arterial walls,[74, 75] allows quantification of plaque size and type,[73, 76] and assessment of integrity of the fibrous cap.[77] Recently, morphological features such as fibrous cap thinning or rupture on MRI has been linked to clinical outcomes such as transient ischemic attacks and stroke.[77] Thus MRI not only quantitates the severity of stenotic lesions and their spatial distribution, but the varying composition and stability of the plaques as well.

Table 2: Comparison of Different Imaging Modalities for Atherosclerosis

	Location	Intimal Lesions	Plaque	Contrast	Invasive	Reproducibility
Ultrasound-Carotid	Carotid	Intermediate	+	None	No	Poor
Intravascular Ultrasound	Coronary	Detailed	+++	Yes	Yes	Very good
MRI	Carotid	Detailed	+++	None	No	Very good

Statistical considerations:

Power Analysis: The primary objective of Specific Aim 1 is to test the hypothesis that change in total plaque volume in patients randomized to high-dose EPO is significantly different from that of those in the low-dose EPO group. Previous studies, which have investigated changes in plaque volume, have shown an average standard deviation of 3%.[66, 72-77] We will recruit a pragmatic sample of 100 patients. Even with a generous 30% subject attrition, we expect to have a total of 70 subjects or 35 in each group complete the study. Using a conservative assumption that the repeated measures correlation is at least 0.50 (explains 25% of variance or less), the study of 35 evaluable per group has 95% power to detect a difference of 0.87 standard deviations or 2.6% difference in plaque volume. With 30 evaluable per group, we will have 95% power to detect 0.95 standard deviations (2.85% difference) in plaque volume and with 25 evaluable per group, we will have 95% power to detect 1.04 standard deviations (3% difference in plaque volume). These changes, although small, are associated with a clinically, significant increase in adverse cardiovascular outcomes.[19, 22, 67, 73]

Initial Data Analysis: An initial data analysis will be performed to detect any differences in distribution of characteristics measured at baseline and at the end of the study between groups. Measures of central tendency and variability will be reported for continuous variables and frequency distributions for categorical variables. This preliminary analysis will provide us with insight into data, distribution of variables and refine further analysis.

Statistical Analysis Plan: We will use analysis of covariance with baseline plaque volume as the covariate, treatment groups as the independent variable, and change in plaque at 12 months as the dependent variable. Even though in this randomized controlled trial study, the simple analysis of variance (ANOVA) model would offer unbiased treatment effect estimate, the analysis of covariance models are in general more efficient than ANOVA models. We will approximate intent-to-treat as closely as possible in the following ways: subjects with data at only one time-point (baseline or 12 months) will contribute their data to the model. The MRIs of subjects that adhered to the study protocol as well as those who did not will be included in the group they were randomized to for analysis.

Sensitivity analysis & incomplete data: While the analysis presumes a completely at random model for missing variables, we shall conduct a sensitivity analysis that will impute a final result from the pooled baseline verse the 12-month result for those with complete data, and use the predicted final value as the dependent variable (missing values behave as null hypothesized).

Model assumptions and alternate analysis: Model assumptions will be checked using validated informal (inspection of residuals) and formal methods (score tests for extra parameter or likelihood displacement). The influence of outliers will be evaluated using influence diagnostic methods, comparing estimates from models with and without outlying values. If the data is not normally distributed, non-parametric methods or log transformation of variables will be considered. To determine heterogeneity of the effect in response to EPO, a subgroup analysis will be performed on those who were on EPO prior to randomization and those with and without pre-existent atherosclerosis. We will also analyze the effect of EPO on composition of plaques (fibrous cap, lipid core, hemorrhage and calcification). These additional analyses will be considered exploratory.

Data management: The McKnight Brain Institute (MBI) at UF routinely performs MRIs for Veterans enrolled in VA studies and so there are established Institutional Review Board protocols for obtaining and storing MRI data securely at this facility. In brief, all MRI data will be collected anonymously and subjects will be identified only by the study ID. The images will be stored in a DICOM format to enable analysis to be performed blinded to randomization and to protect all protected health information. A secure ftp site has been constructed and resides behind firewalls of the Computer Information Technology (IT) section at the MBI and passwords will be provided only to the MRI personnel. Two computers will be dedicated for the sole use of this project at the MRI Core Laboratory. One will be dedicated to the plaque analysis processing of MRI data sets, while the other will serve as a secure ftp and PACS server. VA IT security director will have oversight of the protocols to ensure data security and regulatory compliance. All data will be available to the DSMB for quality control.

Expected results: Successful completion of this aim will determine if we can use more frequent low-doses of EPO to mitigate cardiovascular adverse effects associated with EPO therapy. Our hypothesis predicts that subjects randomized to low-dose, thrice weekly EPO will demonstrate less progression of carotid plaque volume by MRI, have less carotid stenosis, plaque area and more stable plaques as compared to those randomized to high-dose, biweekly EPO.

Methods:

MRI protocol: Subjects will be scanned using a 3T Philips Achieva MRI system located in the MBI at UF. Following successful screening, a double inversion fast spin echo imaging sequence will be used to acquire thirty contiguous image planes centered about the carotid bifurcation on each side of the neck. Slice thickness will be 3 mm, field of view 25 x 25 cm, and matrix of 1024

x 1024. T_1-weighted images will be acquired with TR 1 sec and TE 11 ms; while T_2-weighted images will utilize TR 500 msec and TE 40 msec. Proton density images will be acquired with a TR 5 sec and TE 11 msec. In our experience total exam time is ~20 minutes.

Sensitivity to changes in plaque volume has been reported at 7% using a 1.5T MRI platform.[73, 76] By moving to a higher field (i.e., 3T) we can increase the spatial resolution by a factor of 4 (in-plane resolution at 1.5T was 475 μm^2, while at 3T we routinely achieve 250 μm^2), with only a modest increase in acquisition time. Thus, we will be able to detect changes in plaque volume of 1.75%. Field of view and matrix size will be adjusted to provide an in-plane resolution of 250 μm^2. Three complete data sets will be acquired, comprised of T_1-weighted, T_2-weighted, and proton density weighted images. In addition to estimating atherosclerotic lesion size, these data sets will also permit the characterization of the plaque that comprises the lesion.[73, 76] Although these are the parameters we expect to need, the precise spatial dimensions and imaging parameters may need to be modified to provide the best image quality.

Carotid total plaque volume estimation: Studies will be performed only after passing a pre-study quality control. All studies will be read by a single, experienced observer (Dr. Forder) at UF. The plaque characteristics will be analyzed by MRI-PlaqueView™ (VP diagnostics Inc., WA). Planimetry will be performed on the image data sets, using histogram equalization to improve edge detection for plaque, arterial wall and lumen. Differences in image contrast between T_1-weighted, T_2-weighted, Time of Flight, and proton density will be used to characterize the plaque as fibrous, stable, or unstable.

Complicated plaque will be defined by areas of hyperintensity within the plaque (defined as intensity exceeding that of the surrounding muscle), and will be quantitated using thresholding and planimetry.[78] Volume areas for lipid-, necrotic-, and hemorrhagic-cores, luminal diameter, and fibrotic cap thickness will be determined for each of the slices. Vulnerable plaque will be assessed by progression from baseline exam to follow-up exam. Stability will be determined by thickness of fibrotic cap over the lipid or necrotic core. Although these are the parameters we expect to need, the precise spatial dimensions and imaging parameters may need to be modified to provide the best image quality.

C.2. *Specific Aim 2**: Determine whether receiving high-dose EPO results in VEGFR-2 activation and increased endothelial activation, as measured by VCAM and IL-6 expression, as compared to low-dose EPO.*

Rationale: While there is a preponderance of clinical trial data linking EPO to adverse cardiovascular events,[33-35] particularly in high doses,[38] the basic mechanism underlying this increased risk remains unknown. This has hampered development of therapeutic options to mitigate the cardiovascular risk associated with EPO therapy. Our novel, preliminary, *in-vitro* data demonstrates that EPO activates endothelial cells via the βCR/EPOR and this is dependent on VEGFR-2 tyrosine kinase activity.[1] Published studies have demonstrated that administrating EPO to healthy volunteers at doses that would result in serum levels markedly above 50 mIU/ml (levels that would activate the βCR/EPOR) leads to an increase in circulating levels of E-Selectin[79] and P-selectin,[80] suggesting endothelial cell activation. However, signaling within endothelial cells, particularly with long-term administration of EPO has not been studied. We will, for the first time, use our ability to capture endothelial cells to elucidate the exact, molecular, downstream effects of elevated EPO levels.

Study design: We will attempt to recruit 30 subjects in the low-dose, EPO group and thirty subjects in the high-dose, EPO group, who have not previously received EPO. We will attempt to isolate endothelial cells from each subject before initiation of EPO therapy and at some point

near the conclusion of the study. For the collection after the initiation of EPO therapy the subjects will be asked to come to the VAMC approximately 18-24 hours after administration of their EPO dose, coinciding with peak serum levels of EPO after subcutaneous administration.[81] We will use an intravenous catheter and guidewire to collect venous endothelial cells. Approximately 30 cells will be fixed and stained with an antibody to VCAM, phosphorylated VEGFR-2, or IL-6 and counterstained with appropriate fluorescent secondary antibodies. Fluorescence intensity will be quantified after correcting for background fluorescence and normalized to fluorescent intensity of HUVEC to normalize for inter-batch variability in staining.

Sample size and feasibility: We will recruit a pragmatic sample of 30 patients in each arm for this aim. Since the procedure is brief, low risk, and we are selecting from motivated subjects who have already enrolled in the study we do not anticipate difficulties in meeting recruiting goals for this aim. The power calculations are based on the mean and standard deviation from our own preliminary studies. The percentage detectable change for a power of 80 and 90%, as well as beta error of 5 or 1%, is summarized in **Table 3**. Assuming a dropout rate of 30% or even 50%, we will have 21 or 15 subjects per group, respectively with complete data for analysis. The detectable differences with 14 or 10 subjects are shown in parenthesis. Based on our previous studies as well as that of others differences of the magnitudes presented below are attainable and clinically significant.

Table 3: Power Calculations for Specific Aim-2 (Detectable Change in %)

Outcome	Mean	SD	80% Power		90% Power	
VEGFR2	802	221	25 (30,36)	30 (39,47)	30 (39,47)	16 (44,52)
VCAM	1012	115	10 (13,15)	13 (16,19)	13 (16,19)	15 (18,22)
IL-6	0.274	0.021	7 (8,10)	8 (11,13)	8 (11,13)	10 (12,15)

Data analysis and interpretation: The primary analysis is to determine the difference in endothelial cell activation (levels of VEGFR-2 phosphorylation, VCAM and IL-6) between subjects randomized to low- or high-dose of EPO using analysis of covariance adjusted for baseline levels of phosphorylated VEGFR2, VCAM and IL-6. Fluorescence intensity will be compared using procedures with appropriate transformation or nonparametric alternatives when normality assumptions are not met. Missing values will be addressed as outlined in **Specific Aim 1**.

Additional analysis: While Aim 1 has an analysis that has large enough sample sizes for the central limit theorem to apply, making the analysis virtually assumption-free, the small sample size for Aim 2 requires a sensitivity analysis. All of the data will be pooled to obtain a least squares line of month 12 data on baseline (ignoring for the moment the treatment group). The resulting residuals from the line (observed minus fitted) will be analyzed by a 2-sample permutation test to compare the treatment group. We will also perform additional analysis adjusting for covariates such as age and renal function.

Expected results: This aim will elucidate the basic mechanism underlying the increased cardiovascular risk associated with EPO therapy. Our hypothesis predicts that baseline levels of IL-6, VCAM, and phosphorylated VEGFR-2 will be the same in both groups and subjects randomized to high-dose EPO will have significantly higher levels of IL-6, VCAM, and phosphorylated VEGFR-2 at the end of the study.

Methods:

Harvesting endothelial cells: As previously described by our group and others, endothelial cells will be collected from an antecubital vein by sequentially advancing and retracting two

sterile, ~10cm, J-shaped guidewires (Daig, Inc., Minnetonka, MN) through an 18 gauge intravenous catheter, as previously described by our group and others.[49, 82, 86] Cells will be recovered from the wires by washing with a dissociation buffer and centrifugation. Cells will be fixed with 4% paraformaldehyde (USB corporation, Cleveland, OH), washed thoroughly with PBS, plated on poly-l-lysine coated slides (Sigma Chemical, St. Louis, MO), and stored at -80°C until the immunofluorescence staining will be performed.

Immunofluorescence: Fixed vascular endothelial cells will be rehydrated with PBS containing 50 mmol/L glycine. After blocking non-specific sites with 5% donkey serum (Jackson Immunoresearch, West Grove, PA), slides will be incubated with a primary antibody (rabbit) for phosphorylated VEGFR-2 (Santa Cruz, Dallas, TX) or IL-6 (Abcam, Cambridge, MA) or VCAM (Abcam) followed with a secondary antibody with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). To allow identification of endothelial cells, slides will also be stained with a primary antibody for VE cadherin (Abcam) and a secondary antibody with Alexa Fluor 555 (Life technologies, Grand Island, NY). Finally, to determine nuclear integrity, slides will be mounted with Vectashield containing the nuclear stain DAPI (Vector Laboratories, Inc., Burlingame, CA). To minimize the potential confounder of inter-batch variability in staining, 2 slides of HUVEC will be stained in each batch and endothelial cell protein levels will be reported relative to average HUVEC intensity in that batch.

For analysis, cells will be examined with a fluorescence microscope (Eclipse 80i, Nikon Instruments, Inc., Melville, NY) at 100 X magnification using the same exposure time. Images of endothelial cells (identified by the presence of von VE cadherin staining) with intact nuclei (confirmed by DAPI staining) will be digitally captured by a coolSNAP ES2 camera (Photometrics, Tun, AR). Endothelial cell VEGFR-2, Il-6 or VCAM levels will be measured by quantifying Alexa Fluor 488 intensity while correcting for background fluorescence (NIS Elements software (version 3.2), Nikon Instruments, Inc., Melville, NY) and reported as intensity normalized to the intensity of the HUVEC fluorescence.

C.3. ***Specific Aim 3:*** *Determine the interaction of renal function and dosing of EPO on a) the rate of carbamylation; and b) the length of time that total and cEPO exceed the Kd of the βCR/EPOR.*

Rationale: Our hypothesis predicts that administration of low-doses of EPO three times weekly, as compared to the same cumulative dose administered as a high-dose every 2 weeks, will result in lower peak EPO levels and less activation of the heterodimeric βCR/EPOR. Key to our hypothesis is that the Kd of heterodimeric βCR/EPOR is ~50 mIU/ml, 10-fold higher than that of the homodimeric EPOR. This hypothesis is supported by a recent meta-analysis of all prospective EPO trials that found that adverse cardiovascular effects from EPO were strongly associated with EPO dose even when controlled for target hemoglobin levels.[38] Studies have also found a strong association between endogenous EPO levels and cardiovascular events.[54-56] Complicating the situation is that EPO, in CKD patients, is carbamylated, a process where cyanate derived from elevated urea levels can non-enzymatically bind to proteins. cEPO lacks erythropoietic activity, but can still bind and activate the heterodimer βCR/EPOR. Levels of carbamylated albumin, a surrogate for cEPO, are increased in patients with kidney disease and levels correlate with EPO resistance.[87] However protein levels and amino acid composition affects levels of carbamylation[88] and the carbamylation of albumin could be vastly different from that of EPO. In addition, cEPO may have a very different half-life than EPO itself. We have devised a novel ELISA that will enable us to directly measure total and cEPO levels simultaneously. Our ability to directly measure cEPO gives us the ability to determine how EPO levels and CKD affect the kinetics of carbamylation. *This has not been previously described!*

Study design: We will perform a pharmacokinetic study to assess the total levels of EPO and cEPO in approximately 10 subjects with CKD randomized to low-dose EPO administered three times a week and 10 subjects randomized to approximately the same cumulative dose of EPO administered as a high-dose once every 2 weeks. In subjects given low dose EPO, we will perform the pharmacokinetic studies after at least 2-4 weeks of continuous EPO therapy, ensuring ensure steady state EPO levels and stable hemoglobin levels. Previous studies have shown that those assigned to high-dose EPO every 2 weeks will never be in steady state, since EPO levels return to baseline by 7-10 days.[89] Since we are unsure about the half-life of cEPO, we will study these patients after at least 6-8 doses of EPO. We will exclude smokers, since smoking has been has been shown to increase cyanate and carbamylation through myeloperoxidase-catalyzed oxidation of thiocyanate.[90] To isolate the effects of renal function on carbamylation and EPO levels, we will also include approximately 20 age- and sex-matched, nonsmoking, healthy subjects, 10 of whom will be given a single low-dose of EPO and 10 given a single high-dose of EPO. Short-term administration of EPO to healthy controls is safe and has been used in multiple studies.[64, 65, 81, 89, 91]

Subjects will be brought into the VAMC for the pharmacokinetic study. Venous blood (3 ml) will be attempted to be collected in heparinized tubes before and at approximately 1, 3, 6, 9, 12, 15, 24, 36, and 48 hours after subcutaneous administration of EPO. Those subjects receiving EPO chronically will receive their regular EPO dose, those healthy subjects will receive approximately 25 IU/kg or 300 IU/kg. Since the time that the blood will be collected will be recorded, the time of the blood draws are not critical and are just approximate and clinical circumstances may necessitate missing some of the blood draw times or delaying others. Subjects will be housed near the research unit to allow for the timed blood draws. In some circumstances, the subjects may need to be taken to the Clinical Research Center at UF to complete the lab draws. Serum will be separated and stored at -80°C till analysis. Carbamylated and total EPO will be measured simultaneously using a sandwich ELISA as described in detail in the methods section.[64, 89]

Sample size and feasibility: The aim is to determine a) the length of time that total and cEPO exceeds the Kd of the βCR/EPOR and b) the interaction of renal function (normal and CKD) and dosing of EPO (low vs. high dose) on the rate of carbamylation. With 20 subjects we will have 80% power to detect a 36 or 47% difference in AUC > 50 between groups and at 90% power a difference of 54 or 69% at =0.05 and 0.01 respectively. Our modeling studies predict an AUC > 50 of 4,413 and 736 in the high and low EPO groups. While we will have enough power to detect the effect of dosing regimens on EPO levels that exceed 50 mIU/ml, the power to detect an interaction will be insufficient and analysis will be exploratory. This will be informative in terms of variance estimation for future studies. Because these observations are outlier prone, we shall use natural logs.

Data analysis and interpretation: A one compartment, open model, assuming first order absorption and a constant endogenous production of EPO, will be used to determine the plasma concentration-time profile of EPO and cEPO as per the published literature.[92] The pharmacokinetic parameters and the proportion of AUC above 50 mIU/ml will be calculated for each subject using WinNonlin software. The key interaction estimate is the AUC above 50 mIU/ml in patients with kidney disease (low-dose vs.high-dose) and the response for controls (low-dose vs. high-dose). Because these observations are outlier prone, we shall use natural logs.

Expected results: This aim will characterize the pharmacokinetics of EPO and cEPO. Our hypothesis predicts low-dose EPO will significantly decrease total and cEPO levels and the length of time that the total serum levels of EPO are above the Kd of the heterodimeric EPOR/βCR.

Methods:
EPO ELISA: EPO levels will be determined by a solid phase sandwich ELISA (R&D). This assay has a sensitivity of 0.6 mIU/ml and an assay range of 2.5-200 mIU/ml.

cEPO ELISA: We will perform a sandwich ELISA using a primary, capture, anti-EPO antibody (R&D biosystems), a secondary biotinylated anti-carbamylated lysine antibody (MyBioSource, Inc.) and Streptavidin conjugated poly horse radish peroxidase (Pierce) to increase the sensitivity of the assay. cEPO levels will be determined using a standard curve using known amounts of cEPO made from known amounts of EPO. We will confirm complete carbamylation by the lack of any erythropoietic activity, as previously described.[1]

7. Possible Discomforts and Risks:

The potential risks to subjects include:
a) *Risks related to EPO*: EPO has been associated with high blood pressure, swelling, fever, dizziness, nausea and pain at the site of the injection. However, we will use EPO as per standard clinical standard guidelines issued by Kidney Dialysis Outcomes Quality Initiative (KDOQI). As such the risks relate to that of EPO and no excess risk should result from participating in this study.
b) *Risks of MRI:* we will not use contrast. Non contrast MRI is not associated with serious untoward effects. Some subjects may feel claustrophobic.

c) *Risks of endothelial cell capture:* Endothelial capture can result in endothelial dysfunction, vessels spasm and bleeding. However, in all the collections done to date, in multiple clinical conditions there is no report of any serious adverse effects.

d) *Risks related to venipuncture*: There may be minimal risk of bruising, bleeding or infection at the site of puncture or psychological stress associated with the lab draw itself. However we will utilize an experienced phlebotomist for blood draws and we do not anticipate any problems.

e) *Risks related to potential breach of confidentiality*: There is minimal risk of breach of confidentiality.

8. Possible Benefits:

There is potential for study subjects to benefit from this study. Subjects enrolled in this study may benefit from diagnosis of subclinical carotid atherosclerosis that may otherwise have been missed. These results will be communicated to the primary physician caring for the subjects.

9. Conflict of Interest:

None.

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