Mitochondrial and Chronic Kidney Disease
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1.0 Background

Cardiovascular complications in end stage renal disease (ESRD)

Patients undergoing maintenance hemodialysis (MHD) have a high risk of developing cardiovascular events. Cardiovascular death accounts for more than 40% of overall mortality in these patients. Depending on the age group, the risk of cardiovascular death is between 5 and 100 times higher than in the general population.[1, 2] Furthermore, the survival rate after myocardial infarction in patients on MHD is only 40% at one year.[3] Pharmacological interventions that reduce the risk of cardiovascular events in the general population are ineffective in patients undergoing MHD. Two different prospective, placebo controlled studies did not show a protective effect of statins in ESRD patients despite significant reduction in LDL cholesterol.[4, 5] A more recent study showed that the combination of simvastatin/ezetimibe, while effective in CKD patients not on dialysis, did not reduce the occurrence of atherosclerotic events in patients on MHD.[6, 7] Similarly, the only prospective clinical trial of an ACE inhibitor showed no protective effect on cardiovascular events in these patients.[8]

Inflammation and oxidative stress: risk factors for cardiovascular events in ESRD

The increased incidence of cardiovascular events and cardiovascular death in ESRD cannot be explained by the traditional risk factors, (*e.g.* age, male, diabetes, hypertension, hyperlipidemia, and smoking).[9] Other factors have been implicated in the pathogenesis of accelerated atherosclerosis in ESRD. Among them oxidative stress and systemic inflammation may play a central role (Figure 1).[10, 11] Chronic inflammation is commonly observed in ESRD patients.[12] Inflammatory markers, particularly interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNFa), are chronically elevated in patients with ESRD, even before initiation of MHD.[13] HD procedure itself also induces an acute increase in circulating concentrations of inflammatory markers.[14, 15] These data suggest that both, chronic uremia and recurrent HD contribute to inflammation.[16, 17] Inflammatory cytokines also predict cardiovascular endpoints in patients with ESRD. For instance, interleukin 6 (IL-6) predicts mortality and correlates with the severity of carotid atherosclerosis.[18, 19] Oxidative stress occurs when the reactive oxygen species (ROS) production is higher than the antioxidant capacity and is common in patients undergoing MHD. Markers of oxidative stress are increased in ESRD.[20-23] For example, F2-Isoprostanes levels are increased before, during, and after HD procedure. Other markers of oxidative stress correlate with mortality and morbidity in ESRD: plasmalogen is associated with increased mortality,[24] and malonaldehyde correlates with history of cardiovascular disease.[25] Inflammation and oxidative stress play a role in the pathogenesis of atherosclerosis and the interaction between these factors is complex. Regardless of the initial event (either oxidative stress or inflammation), ROS are present in the atherosclerotic wall.[26] In turn, ROS induce the activation of signaling pathways including the nuclear factor kappa β (NF-κβ) cascade. Activation of NF-κβ results in the expression of cytokines and cell adhesion molecules,[27] leukocyte recruitment, and more ROS production in the atherosclerotic lesion.

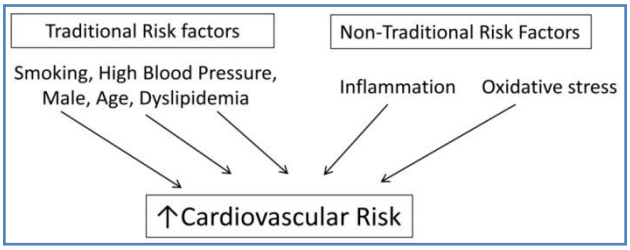

Figure 1. Risk factors for cardiovascular risk in ESRD

Mitochondrial dysfunction contribute to atherosclerosis, oxidative stress and inflammation in ESRD

The mitochondria are one of the main sources of ROS. Around 2% of oxygen is incompletely reduced by the mitochondria and converted into ROS. Superoxide is the main ROS produced by mitochondria, which is then converted by manganese superoxide dismutase into hydrogen peroxide (H_2O_2). H_2O_2 can then diffuse across the mitochondrial membranes or be decomposed by either glutathione peroxidase or catalase. ROS produced by the mitochondria can activate the NLPR3 inflammasome inducing the production of interleukins (*e.g.* IL-1β).[28] There is a clear association between atherosclerosis, inflammation, oxidative stress and mitochondrial dysfunction.[29, 30] Mitochondrial DNA mutations are more common in atherosclerotic aorta, with none or little damage in nuclear DNA.[30] Furthermore, mitochondrial DNA mutations are significantly more frequent in hearts from patients with coronary disease compared to age-matched healthy subjects.[31] Using magnetic resonance spectroscopy (MRS), previous studies have shown that patients undergoing MHD exhibit skeletal muscle mitochondrial dysfunction when compared to healthy individuals.[32-34] But no study has compared patients with different stages of CKD. It has also been suggested that mitochondrial dysfunction worsen years after HD initiation; [32, 35] however, this hypothesis has not been tested yet. Nevertheless, previous studies have shown abnormalities on mitochondrial metabolism in skeletal muscles biopsies[36-38] and in peripheral blood mononuclear cells (PBMCs) from patients with CKD.[39, 40] Mitochondrial dysfunction in skeletal muscle may also explain why patients with CKD exhibit poor exercise tolerance and rapid fatigability.[41, 42] It is still unknown if mitochondrial dysfunction is the result of intrinsic mitochondrial defects or changes in mitochondrial number. These two factors can be differentiated by measuring at the same time mitochondrial function and mitochondrial volume density, which has never done in patients with CKD. Mitochondrial volume density is usually measured with electron microscopy. Other methods that correlate with electron microscopy are fluorescent dyes, mitochondrial enzymes activities and mitochondrial DNA content (or mitochondria DNA copy number). The latter has recently been proposed as a potential marker of mitochondrial dysfunction.[43] Accordingly, a recent study showed an inverse correlation between mitochondrial DNA copy number in PBMCs and survival rate in patients undergoing MHD,[44] suggesting that mitochondrial dysfunction may play a role in all-cause mortality in ESRD.

Bradykinin may play a role in oxidative stress and mitochondrial dysfunction

Hemodialysis activates the kallikrein-kinin system and the production of bradykinin.[45] Under normal physiological conditions, bradykinin exerts its effect through B_2 receptors. Bradykinin has a dual effect over the cardiovascular system: it increases tPA release and inhibits platelet aggregation,[46, 47] but also increases oxidative stress and systemic inflammation.[48-51] Very few studies, however, have studied the effect of bradykinin on oxidative stress and inflammation in ESRD patients. Our group showed that endogenous bradykinin contributes to increases in plasminogen activator inhibitor-1 and monocyte chemo-attractant protein 1 following HD, consistent with a pro-inflammatory effect.[52] Thus, any

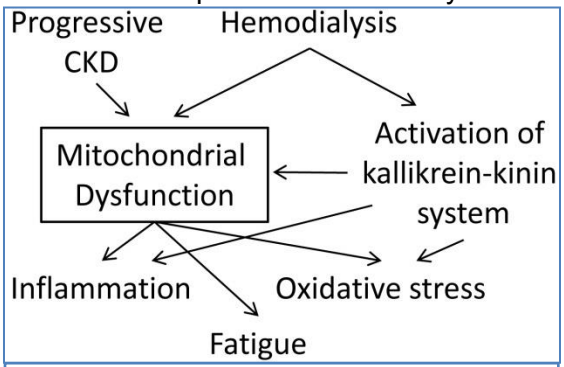

Figure 2. Relation among progressive CKD, HD, and activation of kallikrein-kinin system

intervention that increases bradykinin, such as ACE inhibitors, may increase the inflammatory response to HD. In fact, the candidate has observed that one week treatment with the ACE inhibitor ramipril elicits a greater pro-inflammatory response compared to ARB valsartan in patients undergoing MHD.[53] Bradykinin also increases ROS production in cardiomyocytes, endothelial cells, vascular smooth muscles and renal tubular cells.[51, 54, 55] Studies in human coronary endothelial cells showed that bradykinin vasodilator effects depended on ROS production by NADPH oxidase.[51] Other studies suggest that bradykinin increases the production of mitochondrial ROS.[54, 55] But little is known about the effect of bradykinin on mitochondrial function, especially in patients undergoing MHD.

Mitochondrial dysfunction in CKD.

There is strong evidence that oxidative stress and inflammation correlate with cardiovascular events in ESRD patients. Mitochondrial dysfunction may be the cause of both oxidative stress and inflammation. Although many previous studies have studied mitochondrial function in patients with CKD, they have not evaluated the effect of progressive kidney disease and the contribution of HD on mitochondrial function. We are planning to study for the very first time, mitochondrial function *in vivo* in patients at different stages of CKD not yet on HD compared to patients with ESRD. This approach will allow us to study the role of progressive CKD on mitochondrial dysfunction. Furthermore, no study has simultaneously performed *in vivo* measurements of mitochondrial function and skeletal muscle biopsies in patients with ESRD. This methodology will allow us to correlate in vivo mitochondrial function with biochemical and ultra-structural changes in mitochondria. But we will also evaluate the contribution of the HD procedure on mitochondrial dysfunction. HD activates the kallikrein-kinin system, which increases bradykinin levels and may contribute to the increased oxidative stress and inflammation observed in these patients. Our preliminary data suggest that bradykinin contributes to mitochondrial dysfunction, which is also present in ESRD. Little is known about the relation among hemodialysis, mitochondrial function and bradykinin, however. Understanding the interplay among these factors would help us to direct therapeutic interventions to patients with ESRD.

2.0 Rationale and Specific Aims

Approximately 500,000 people per year undergo maintenance hemodialysis (MHD) as renal replacement therapy for end stage renal disease (ESRD) in the United States (US). Patients undergoing MHD are more susceptible to cardiovascular morbidity and mortality. Factors such as systemic inflammation and increased oxidative stress may play a role in accelerated atherosclerosis in these patients. Mitochondria are the main energy machinery of the cell but also sources of reactive oxygen species (ROS) and oxidative stress. Mitochondrial dysfunction, by increasing oxidative stress and inflammation, has been implicated in the pathogenesis of atherosclerosis in the general population, and it has been described in patients on MHD. The etiology of mitochondrial dysfunction in ESRD is not clearly elucidated, but progressive chronic kidney disease (CKD) and MHD are the possible causes. While earlier studies have shown the presence of mitochondrial dysfunction in ESRD compared to healthy individuals, none has previously studied the role of progressive CKD on mitochondrial dysfunction. Several studies suggested that the decline in mitochondrial function occurs following initiation of MHD and that the hemodialysis (HD) procedure is a potential contributor to this process. HD activates the kallikrein-kinin system, which increases bradykinin formation. Bradykinin exerts cardio-protective effects such as vasodilatation and tissue plasminogen activator (tPA) release, but also increases inflammation and oxidative stress and eventually may lead to

mitochondrial dysfunction. In fact, our preliminary data suggest that bradykinin increases mitochondrial superoxide production in peripheral blood mononuclear cells (PBMCs) *in vitro,* and endogenous bradykinin increases plasma isofurans during HD. **The overarching goal of this study is to determine the role of progressive CKD and the activation of the kallikrein-kinin system during MHD on the development of mitochondrial dysfunction.** In order to achieve this goal, we propose the following specific aims:

Specific Aim 1: Test the hypothesis that mitochondrial function worsens with the progression of CKD and initiation of HD. We hypothesize that mitochondrial function gradually deteriorates with the progression of CKD. We also expect that MHD rather than preventing the decline worsens mitochondrial function. To test this hypothesis, we will measure *in vivo* mitochondrial function (half-time of phospho-creatinine recovery) using ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) in three groups of subjects: 1) patients with ESRD on MHD, 2) patients with CKD stages 3-5 but not on MHD and 3) control subjects without CKD. The subjects in each group will be matched by age, sex, race, diabetic status, and BMI. We will also evaluate changes in mitochondrial quantity by measuring markers of mitochondrial biogenesis and mitochondrial density in muscles biopsies. We anticipate that patients on MHD exhibit the worst mitochondrial function (*i.e*. the longest half time of phosphocreatinine recovery).

Specific Aim 2: Test the hypothesis that endogenous bradykinin promotes mitochondrial dysfunction in patients undergoing MHD. To test this hypothesis, we will conduct a randomized, double-blind, placebo-controlled, 2x2 crossover study, comparing HOE-140, a bradykinin B_2 receptor blocker, and placebo administered 1 hour prior to beginning of HD session and during HD. Mitochondrial function will be measured using ^{31}P-MRS. We anticipate that bradykinin B_2 receptor blockade with HOE-140 will improve mitochondrial function during HD.

Understanding the mechanisms leading to mitochondrial dysfunction is an important step in terms of developing new strategies to prevent cardiovascular morbidity and mortality in ESRD. The candidate is in unique position to accomplish this project based on his training in mitochondrial physiology, clinical pharmacology and clinical trials. The proposed studies will develop the candidate credentials to become an independent and successful physician scientist.

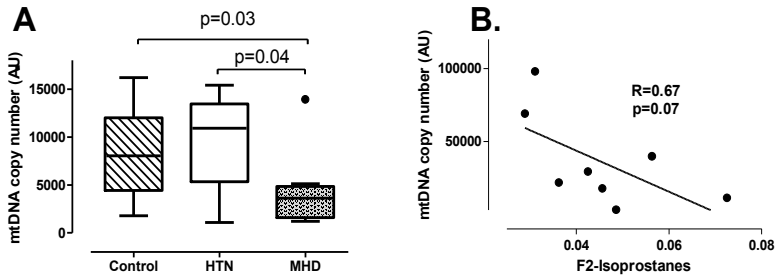

Figure 3. A. Mitochondrial copy number in healthy controls, hypertensive subjects without history of ESRD (HTN) and chronic HD patients (n=8 in each group, *p<0.05 compared to chronic HD group). **B.** Correlation between mtDNA copy number and F2-Isoprostanes in ESRD patients measured 2 hours after the end of HD (n=8).

3.0 Animal Studies and Previous Human Studies

3A *Mitochondrial DNA copy number in chronic hemodialysis*

Mitochondrial DNA (mtDNA) is susceptible to damage by oxidative stress due to the lack of histone protection.[56] mtDNA mutations and reduced mtDNA copy number are

commonly present in cells exposed to excessive concentration of ROS.[57] Using qPCR we measured mtDNA copy number in white blood cells from patients on MHD, as previously described[44] (Figure 3A). Copy number was significantly decreased in patients on MHD compared to healthy and hypertensive subjects, matched by age, sex, race and BMI. Furthermore, there was a trend toward negative correlation between mtDNA copy number and F2-Isoprostanes (Figure 3B), suggesting that oxidative stress may play a role in the observed reduction of mtDNA copy number.

3B *Markers of oxidative stress are increased in patients undergoing chronic hemodialysis*

F2-Isoprostanes and Isofurans, products of non-enzymatic oxidation of arachidonic acid, are reliable measures of oxidative stress in plasma. Formation of either F2-Isoprostanes or Isofurans depends on tissue oxygen tension. In conditions of high oxygen tension the formation of Isofurans is favored. This may occur due to increased oxygen delivered (breathing a high FIO2) or decreased oxygen consumption (as in mitochondrial dysfunction). Previous studies showed that F2-Isoprostanes are increased in patients undergoing MHD.[20] We also found that the levels of Isofurans were higher in patients undergoing MHD than in patients undergoing cardiac surgery (Figure 4A). Furthermore, the increased ratio of Isofurans to F2-Isoprostanes suggests a preferential production of Isofurans in patients undergoing MHD (Figure 4B). These findings may be a consequence of mitochondrial dysfunction, as previously suggested.[58]

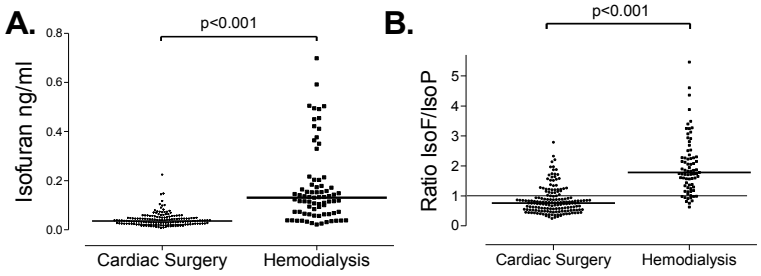

Figure 4. Isofurans levels **(A)** and ratio of Isofurans (IsoF) to F2-Isoprostanes (IsoP) **(B)** in patients undergoing chronic HD compared to a cohort of patients that underwent cardiac surgery.

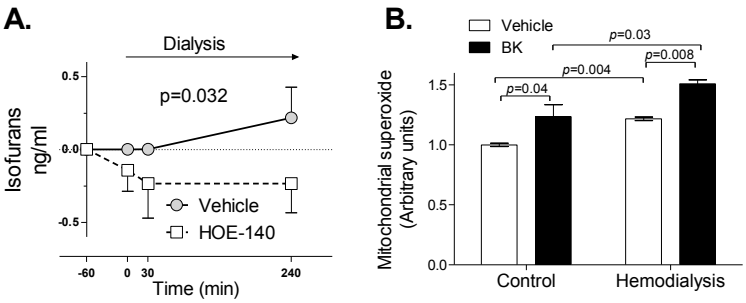

Figure 5. **A** Isofurans levels during HD in patients receiving HOE-140 or placebo. **B.** Mitochondrial superoxide levels, measured using MitoSOX™ in peripheral blood mononuclear cells (PBMCs) with and without bradyinin stimulation.

3C. *Role of bradykinin in oxidative stress during hemodialysis*

Our group has previously shown that, during HD, blockade of endogenous bradykinin, through the bradykinin B_2 receptor antagonist HOE-140, decreases markers of inflammation.[52] We also found that HOE-140 decreases the formation of Isofurans during HD, suggesting that endogenous bradykinin contributes to oxidative stress in patients undergoing HD (Figure 5A). Using isolated PBMC from healthy individuals and patients undergoing MHD, we found that bradykinin increased mitochondrial superoxide production (Figure 5B). These preliminary results suggest a role of bradykinin on mitochondrial ROS production.

3D. M*itochondrial ultrastructure in patients undergoing MHD*

Mitochondrion ultra-structure was also evaluated in muscle biopsy from 5 different patients undergoing MHD. Figure 6 shows some of representative electron micrographs of normal mitochondria (Figure 6A) and abnormal mitochondria with signs of cristae swelling (Figure 6B). There also were many double membrane formations compatible with auto-phagosomes, which are surrounding structures that resemble mitochondria (Figure 6C). This suggests that mitophagy, a particular type of authophagy, may be increased in patients undergoing HD. We also found many lipofuscin granules (Figure 6D), probably as result of oxidative damage of mitochondria and lysosomes.

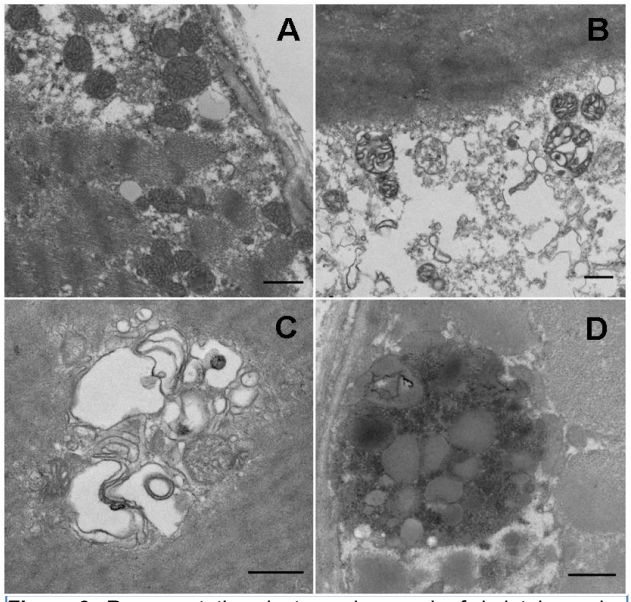

Figure 6. Representative electron micrograph of skeletal muscles from patients undergoing MHD. **A.** Normal sub-sarcolemmal mitochondria. **B.** Mitochondria with signs of swelling. **C.** Double membrane structure compatible with auto-phagosome. **D.** Lipofuscin pigment (scale bar=500µm).

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria

1. Age 18 years or older.
2. On thrice-weekly chronic hemodialysis for at least 6 months.
3. Clinically stable, adequately dialyzed (single-pool Kt/V > 1.2) thrice weekly, with polysulphone membrane for at least 3 consecutive months prior to study.
4. For Specific Aim 1 only: Chronic kidney disease stages 3-5 but not yet on hemodialysis (eGFR less than 59 mL/min per 1.73 m^2), or subjects with no history of CKD.
5. Negative serum pregnancy test

Exclusion Criteria –both Aims

1. History of functional transplant less than 6 months prior to study
2. Use of anti-inflammatory medications other than aspirin < 325 mg/d
3. Use of immunosuppressive drugs within 1 month prior to study
4. Use of anticoagulant medication
5. History of active connective tissue disease
6. History of acute infectious disease within one month prior to study
7. AIDS (HIV seropositivity is a exclusion criteria only for Specific Aim 1)
8. History of myocardial infarction or cerebrovascular event within 3 months
9. Advanced liver disease
10. Gastrointestinal dysfunction requiring parenteral nutrition
11. Active malignancy excluding basal cell carcinoma of the skin
12. History of ACE inhibitor-associated cough or angioedema
13. Ejection fraction less than 30%
14. Predialysis potassium repeatedly higher than 6.0 mmol/L (confirmed on a repeated blood draw)

15. Anticipated live donor kidney transplant
16. Use of vitamin E > 60 IU/d or vitamin C >500 mg/d
17. Pregnant or breast-feeding
18. History of poor adherence to hemodialysis or medical regimen
19. Inability to provide consent

Specific Aim 1

20. Subjects with cardiac pacemaker, artificial heart valve, any metallic implant, permanent tattoo, or any retained foreign metallic bodies will be excluded from the magnetic resonance spectroscopy (MRS) study (Specific Aim 1).
21. HIV infection
22. Albumin levels lower than 3.5 mg/dL.

5.0 Enrollment/Randomization

Subjects will be recruited from the Vanderbilt University Medical Center (including the Vanderbilt Nephrology Clinic and the Vanderbilt Outpatient Dialysis Clinic) the Nashville Veterans Administration (VA) Medical Center, and 10 outpatient hemodialysis units operated by Dialysis Clinics Inc (DCI) and DaVita Clinics. Written advertisements, which give the name and phone numbers of a contact Research Nurse, will be placed on bulletin boards in these locations. In addition, subjects may be informed of the study by their dialysis nurse or primary care provider. Subjects who call for more information will be given a brief description of the study protocol and, if interested, will be invited to set up a meeting with the Research Nurse. During this meeting, the Research Nurse or Investigator will describe the study protocol in detail. Interested subjects will be invited to read and sign an IRB-approved consent form and will be given a copy of that consent form.
Subjects will be randomly assigned to treatment order using a permuted-block randomization algorithm (for Specific Aim 2). The Vanderbilt Investigational Pharmacy will be responsible for the storage, preparation, and labeling of all investigational agents and for maintaining accurate drug storage and dispensing logs at Vanderbilt. A Clinical Research Pharmacist, will devise standard operating procedures for the pharmacy to follow with regard to preparing, labeling, blinding, and dispensing study drug.

6.0 Study Procedures

Specific Aim 1: Test the hypothesis that mitochondrial function worsens with the progression of CKD and initiation of HD

Population

Three different groups of patients will be evaluated: 1. Patients with ESRD and on MHD for at least 6 months, 2. Patients with CKD stages 3-5 (eGFR less than 59 mL/min per 1.73 m^2) not yet on MHD and who have visited a nephrologist at least 1 month prior to enrollment, and 3. Control subjects with no CKD. The subjects in each group (including the controls) will be matched by age, sex, race, diabetic status, and BMI. All participants will be adults 18-75 years of age.

General protocol
After obtaining informed consent, the investigators will perform a physical examination.

Physical Activity
We will measure physical activity using triaxial accelerometer. For this purpose, the monitor of the size of a small pager is clipped on clothing over the right hip. Activity will be recorded during waking hours. Physical activity will be examined over a 7-day period requiring at least 5 days of recorded activity to be included in the analysis. The accelerometer will provide information about total energy expenditure and energy expenditure of activity.

Prior to any study procedure, subjects will report to the VUIIS for a training session for the ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) as described below. Mitochondrial function will be evaluated using ^{31}P-MRS. Endurance will be also evaluated using the six-minute walk test as explained below. The subjects will report to the Vanderbilt University Institute of Imaging Science (VUIIS) in the morning on one of the non-dialysis days. During this day we will perform the six-minute walk test and a training session for the ^{31}P-MRS. Two days later, subjects will be asked to return to the General Clinical Research Center (CRC) at Vanderbilt in the morning under fasting conditions for blood sampling, ^{31}P-MRS, and skeletal muscle biopsy of the vastus lateralis (see below).

^{31}P Magnetic Resonance Spectroscopy
Mitochondria function can be evaluated using non-invasive techniques such as ^{31}P-MRS, which evaluates the concentration of PCr and other phosphate-energy carrier molecules in limb muscles. Dr. Bruce Damon and Dr. Theodore Towse, faculty members of the VUIIS, have successfully implemented this technique that will be used to evaluate *in vivo* mitochondrial function. Each subject will lie prone for approximately 30 minutes with a coil positioned over the belly of the vastus lateralis muscle. The position of the coil will be confirmed using scout localizer images and a reference positioned within the center of the coil (Figure 7A). After basal measurements, subjects will be asked to perform cycles of knee extension and rest. Measurements will be recorded during the exercise protocol and for additional 15 minutes (recovery period). Spectra will be used to calculate concentrations of inorganic phosphate (Pi), PCr, adenosine triphosphate (ATP). The primary end-point will be the half-time of PCr recovery (time to achieve half of basal concentration during the recovery period). A spectra obtained by the investigators is shown in Figure 7B. This method is reliable and reproducible and has been considered the "gold standard" to measure mitochondrial function.[59, 60] Subjects will report for an initial familiarization session (a non-dialysis day). During this session, subjects will be familiarized with the exercise protocol. The subjects will be asked to return 2 days later to

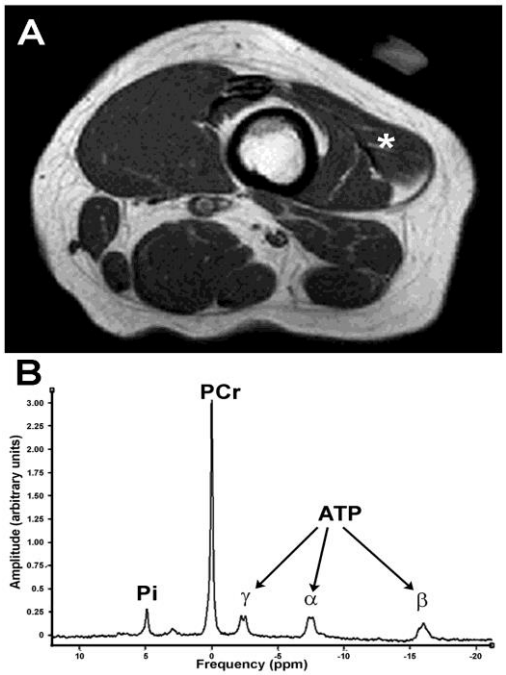

Figure 7 - A. MRI of the thigh. The white dot above the thigh marks the center of the ^{31}P MRS coil and aids in positioning the coil over the vastus lateralis (*). B. ^{31}P MRS spectra acquired at rest form the vastus lateralis of a healthy 37 y.o. female.

perform the ^{31}P-MRS. During the 2 days prior to testing, we will ask that subjects 1) perform no moderate or heavy intensity exercise within 1 day of testing; 2) not consume alcohol within the 1 day prior to testing; 3) not consume caffeine or use tobacco within 6 hours prior to testing; and 4) do not eat a meal 3-6 hours before testing.

Six-minute walk test

This test will be performed as previously described.[61, 62] Briefly, patients will be instructed to walk during 6 minutes on a 20 meter corridor. The distance in meters will be recorded. Patients will not be encouraged to perform the test, but they will be able to judge the time remaining from the large clock in the corridor. In this specific aim the test will be performed in a non-dialysis day. Dr Ikizler (co-mentor of the PI) has standardized the test in his lab and shown that physical activity is lower on dialysis days.[63]

Muscle biopsies

Biopsies will be obtained from the vastus lateralis skeletal muscle. Patients receiving anticoagulant medication will be excluded. Aspirin will be discontinued for 14 days before the procedure. This procedure will be performed under aseptic conditions and local lidocaine anesthesia in the CRC at Vanderbilt, by percutaneous needle biopsy. After removing subcutaneous fat, biopsies will be divided in 3 pieces, one will be immersed in fixatives for electron microscopy; the other 2 pieces will be immediately frozen in liquid nitrogen and stored at -80 ºC for DNA and RNA isolation and for future studies (***e.g.*** mitochondrial proteomics). The PI has already completed 5 biopsies in patients undergoing chronic HD.

Transmission electron microscopy to assess mitochondrial mass and morphology

Muscle biopsies will be dissected and cut into small pieces and fixed in glutaraldehyde (4%) and paraformaldehyde (2%) for 2 hours at room temperature. Samples will be prepared for electron microscopy as previously described.[64] Briefly, samples will be post-fixed in osmium tetraoxide (1%) for 1 hour, dehydrated and embedded for further sectioning. Thin (80 nm) fiber transverse sections will be stained with uranyl acetate and lead citrate and examined with a transmission electron microscope. Mitochondrial volume density (% of muscle fiber volume occupied by mitochondria) will be determined using a standard point counting method.[65] Representative electron micrographs from patients with ESRD and obtained by the PI are shown in Figure 6.

Table 1. Variables measured in Specific Aim 1

Variable	Specimen
Primary end-point:	
• Half time of PCr recovery (^{31}P-MRS)	Non-invasive
Secondary end points:	
• Six-minute walk test	Non-invasive
• Mitochondrial volume density	Muscle biopsy
• Mitochondrial biogenic factors	Muscle biopsy
• Mitophagy factors	Muscle biopsy
• Lactate-to-pyruvate ratio	Plasma

Markers of mitochondrial biogenesis and mitophagy

Markers will be measured using quantitative RT-PCR in skeletal muscles biopsies. For this purpose, muscle tissue will be homogenized and RNA isolated using RNeasy® Fibrous Tissue Mini Kit RNA (Qiagen, Chatsworth, CA), according to the manufacturer's protocol. Mitochondrial biogenic factors will include PPAR gamma coactivator 1-alpha (PGC-1α), nuclear respiratory factors (NRF) 1 and 2, and mitochondrial transcription factor A (TFAM). Among the mitophagy markers we will evaluate autophagy gene 8 (ATG-8 or LC-3), 5 (ATG-5) and 7 (ATG-7), BCL-2/adenovirus interacting protein 3 (BNIP-3) and BNIP-3 like protein (BNIP-3L). After performing RNA reverse transcription, PCR analyses will be performed in triplicate, using SYBR Green and 18S rRNA as the calibrator

housekeeping gene. Fluorescence signal will be detected using ABI PRISM 7500 Sequence Detection System.

Lactate-to-pyruvate ratio

Lactate-to-pyruvate ratio in blood samples has been used as another marker of mitochondrial function. Blood samples will be obtained and mixed with 8% perchloric acid (3 to 1 volume to volume ratio). Lactate will be measured using a colorimetric method (Sigma-Aldrich). Pyruvate will be measured using high performance liquid chromatography (HPLC). Table 1 summarizes the variables that will be measured in Specific Aim 1.

Specific Aim 2: Test the hypothesis that endogenous bradykinin promotes mitochondrial dysfunction in patients undergoing chronic HD.

Population

Adult (18 years or older) patients undergoing MHD or will be considered for the study. Inclusion and exclusion criteria will be the same as specified in section 4B and outlined in detail in Protection of Human Subjects section. During the screening period we will test each dialysis subject for Hepatitis B if it has not been done within 1 month. This is done as standard of care for the dialysis procedure.

General protocol

In a randomized placebo-controlled 2X2 crossover study, subjects will receive either HOE-140 or placebo in the first study day. Prior to the first study day, subjects will report to the VUIIS for a training session for the ^{31}P-MRS. The first study day will occur at least 2 days but not more than 2 weeks after the training session. Participants will report to the CRC for the study day. HOE-140 (IND # 49,049) or placebo will be infused for 30 minutes prior to the initiation of dialysis at a rate of 100μg/kg/h and at 50 μg/kg/h for the duration of hemodialysis. We have previously found that this dose of HOE-140 blocks the vasodilator effect of bradykinin.[66] The study procedures will be performed in the CRC. Participants will be asked to perform the six-minute walk test, as described in Specific Aim 1. Subjects will then undergo HD in the CRC. Serial blood samples will be collected from the arterial tubing of HD 30 minutes before the beginning of HD, at the beginning of HD, 30 minutes and 1 hour after the initiation of HD, and at the end of HD. The patient will receive the same dialysis treatment prescription as their standard-of-care treatment. The investigator has experience performing this protocol.[53] At the end of HD, patients will remain in the CRC for 2 hours; the last blood samples will be collected and subject will be asked to perform the six-minute walk test again. The subject will be then transported to the VUIIS to evaluate mitochondrial function by ^{31}P MRS and finally discharged home.

One week later, the participants will return to the CRC for the second study day. The procedures will be the same as on the first study day, receiving the other study medication.

Markers of oxidative stress and inflammation

Table 2. Variables measured in Specific Aim 2

Variable	Pre-HD	Post-HOE	30 min HD	60-min HD	End HD	120 min post HD
Primary endpoint:						
• Half time of PCr recovery (^{31}P-MRS)						X
Secondary endpoints:						
• Six-minute walk test	X					X
• F2-Isoprostanes	X	X	X	X	X	X

• Isofurans	X	X	X	X	X	X
• Inflammatory cytokines	X	X	X	X	X	X
• Lactate-to-pyruvate ratio	X	X	X	X	X	X

In the blood samples we will measure circulating markers of mitochondrial function (lactate-to-pyruvate ratio), oxidative stress (F2-Isoprostanes and Isofurans) and inflammation. Table 2 summarizes the variables that will be measured in Specific Aim 2. Blood will be centrifuged immediately after collection and stored at -80ºC degrees until processing. F2-Isoprostanes and F2-Isofuranes are products of lipid peroxidation and markers of oxidative stress. Both will be measured in plasma using negative ion gas-chromatography mass spectroscopy as previously described.[67] Serum IL-1β, IL-6, IL-8, IL-10 and C-reactive protein will be measured using Luminex immunoassay technology. Monocyte chemo-attractant protein 1 concentrations will be measured by ELISA using commercially available kits (Linco Research, St Charles MO).

7.0 Risks

Potential risks

1. Insertion of venous catheters may cause bleeding, bruising or infection.

2. Frequent blood draws can lead to anemia.

3. HOE 140/Icatibant is a drug approved by the FDA for acute attacks of hereditary angioedema. We plan to use HOE-140 for an off-label use. We have held an IND for HOE 140 (#49,049) since 1995 and have had extensive experience with its safe administration. HOE 140 may cause increased blood pressure or heart rate, though we have not observed this in prior studies. In addition, we have not seen an effect of HOE 140 on blood pressure or heart rate during hemodialysis in a previous study. While HOE 140 has a short half-life and there have been no apparent adverse effects to date in studies in subjects undergoing cardiopulmonary bypass or hemodialysis, blocking bradykinin could promote ischemia by decreasing fibrinolysis and increasing blood pressure. In Specific Aim 2, we will monitor subjects for ischemia throughout the study.

4. ACE inhibitors can cause birth defects, cough, angioedema, hyperkalemia, or hypotension.

5. AT_1 receptor blockers can cause hyperkalemia or hypotension.

6. The collection and storage of DNA for genotyping creates the risk of release of information that could link subjects to stored samples and genotyping results.

7. MRS does not use ionizing radiation; therefore there are no harmful side effects with temporary exposure. But some situations limit the use of MRS, such as in subjects using pacemaker or similar metal device, where magnetic field may lead to malfunction. Prosthetic metal device or any other metal pieces (*e.g* aneurysm clips, copper intrauterine device) may heat up during the study causing skin or tissue irritation. Skin irritation may also result in subjects with tattoos or medication patches.

8. Muscle biopsy may cause muscle soreness, bruising, infection, a hematoma, or a slight chance of bleeding at the incision site. Soreness at the biopsy site may last 48 hours.

Adequacy of protection against risks

Recruitment and informed consent

Written advertisements approved by the Vanderbilt University Medical Center IRB will be placed on bulletin boards in the dialysis centers. The advertisement will the name and phone numbers of a contact Research Nurse and Investigators. In addition, subjects may be

informed of the study by their dialysis nurse, primary care provider, or nephrologist. Subjects who call for more information will be given a brief description of the study protocol and, if interested, will be invited to set up a meeting with the Research Nurse. During this meeting, the Research Nurse or Investigator will describe the study protocol in detail. Interested subjects will be invited to read and sign an IRB-approved consent form and will be given a copy of that consent form to take home.

Protection against risk

In Specific Aim 1 to minimize risk of pain during the biopsy, lidocaine/prilocaine (5%) cream will be applied to the site of biopsy 1 hour prior to the procedure. The area will be also anesthetized with lidocaine (1%). We will make sure that the subject only feels pressure, but not pain, prior to starting the biopsy and during the procedure. To minimize the pain after the procedure, patients will be asked to take two acetaminophen tablets (325 mg) immediately after the procedure.

To avoid the risk of infection during the biopsy, the site of incision will be clean with povidone-iodine swabs followed by alcohol swabs (70% isopropyl alcohol), and the procedure will be performed under aseptic conditions.

At the end of biopsy to avoid the risk of swelling, a bag of ice will be place over the biopsy area wrapped with an elastic band to keep it in place. To avoid the risk of bleeding, pressure will be applied to the biopsy area for 10 minutes. Thin adhesive strips will be placed over the skin to close the small incision.

After the biopsy procedure, subjects will be discharged home with instruction of contacting the investigators immediately if they experience intense pain, signs of inflammation, bleeding or any kind of drainage from the biopsy site.

To avoid any possible risk derived from the MRS study, patients will be inquired about the use of any metal device, tattoos, or the presence of any retained metal foreign body.

In Specific Aim 2, blood pressure and heart rate will be measured continuously throughout dialysis. We will obtain an electrocardiogram before and after the study.

Subjects will also be discontinued from the study if they develop low blood pressure as defined by symptoms of hypotension or a systolic blood pressure <95 mmHg.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

The principal investigator will be responsible for ensuring the data integrity and the safety of all the study participants. All the adverse events will be recorded and reported to the IRB. Study progress report will be prepared every six months to describe: the screening and recruitment process, demographic of the participants, status and occurrence of adverse events (AE), drug toxicities, treatment adherence, and any incident of non-compliance with the protocol. These reports will be prepared by the principal investigator and reviewed with his mentor. The reports will be presented to the Data and Safety Monitor Board (DSMB). AE will be classified using a 0-5 scale (0-no AE, 1-mild AE and no treatment required, 2-moderate AE that respond to treatment, 3-severe AE that require hospitalization or limit daily activities, 4-disalbling or life-threatening AE, 5-death). Any AE greater than or equal to 3 will be reported to the IRB and DSMB within 10 business days of the PI's notification of the event. All other AEs will be reported at the time of the annual report to the IRB. Protocol changes will be reported to the IRB and will not be implemented until approved.

The DSMB will objectively review the treatment results as they relate to human safety and data quality. Drs. Italo Biaggioni, Kerri Cavanaugh and Aihua Bian have agreed to be members of the DSMB. Dr Biaggioni, who will serve as the DSMB chair, is Professor of Medicine and Pharmacology and Associate Director of the Vanderbilt CRC. Dr. Cavanaugh is Assistant Professor in the Division of Nephrology who focuses on patient education and awareness regarding chronic kidney disease. Ms. Bian is a biostatistician with experience as a consultant with the Department of Medicine. A statement from the members of the DSMB is included. None of the members of the DSMB have any conflict of interest, whether financial or intellectual with the investigator or the study. The DSMB will regularly meet twice a year; in case of an unanticipated or serious adverse event, an extraordinary meeting will be scheduled.

9.0 Study Withdrawal/Discontinuation

Blood pressure and heart rate will be measured continuously throughout dialysis. We will obtain an electrocardiogram before and after the study.Blood pressure will be measured during the study. If at any time during the washout period, the systolic blood pressure exceeds 160 mmHg or the diastolic blood pressure exceeds 100 mmHg, the subject's usual anti-hypertensive medications will be maximized. If at any time the systolic blood pressure exceeds 189 mmHg or DBP exceeds 114 mmHg, the subject will be discontinued from the study and his or her antihypertensive medications will be adjusted appropriately. Subjects will also be discontinued from the study if they develop low blood pressure as defined by symptoms of hypotension or a systolic blood pressure <95 mmHg.
Serum electrolytes will be measured prior to each study day. For a serum potassium from 5.5-5.9 mmol/L, dietary restriction will be emphasized and the potassium content of the dialysate will be adjusted to decrease serum potassium. A predialysis serum potassium of 6.0-6.4 mmol/L, if confirmed and not corrected with adjustment of dialysate potassium concentration, will result in discontinuation of the study day. The subject may be rescheduled to complete the study at a later date. A confirmed predialysis serum potassium of 6.5 mmol/L or greater will result in immediate discontinuation. All potassium measurements will be obtained prior to dialysis, which is the most effective treatment for hyperkalemia.

10.0 Statistical Considerations

Statistical Analysis

a. Sample size

- *Specific Aim 1*: We plan to enroll 3 groups: patients with ESRD on MHD (group 1), patients with CKD stages 3-5 but not on MHD (group 2), and control subjects without CKD (group 3). In previous studies,[32, 35] the half-time of PCr recovery in HD patients following exercise was 1.1 minutes (m) and the standard deviation was 0.35 m. Normal values are 0.6 m with a standard deviation of 0.24 m.[32] Assuming the same coefficient of variation, we anticipate the mean±SD of the half-time of PCr recovery would be 1.1±0.35m, 0.85±0.27m, and 0.6±0.19m, for groups 1, 2 and 3, respectively. Using a 2-sample t-test, we will need 26 subjects per group to have 80% power to detect the difference between groups 1 and 2, and 15 subjects per group to detect the difference between groups 2 and 3 with a type I error rate of 0.05. For matching purposes, we plan to study 26 subjects in each group. The subjects will be matched by age, race, gender, BMI, diabetic status.

- Specific Aim 2: The primary end-point is the half-time of PCr recovery (in minutes) measured by ^{31}P-MRS. Samples sizes were calculated using data from previous studies.[32, 35] A sample size of 11 will have 80% power to detect a difference in means (of the half time of PCr recovery) of 0.33 minutes (the placebo treatment mean of 1.1±0.35 minutes and the HOE-140 treatment mean of 0.77±0.35 minutes, a 30% reduction), assuming a correlation coefficient of 0.5 between the two measures, and that results to a standard deviation of differences of 0.35, using a paired t-test with a 0.05 two-sided significance level.

b Data analysis plan*.*

Standard graphing and screening techniques will be used to detect outliers and to ensure data accuracy. The distribution of continuous endpoints will be examined for normality. In the case of non-normally distributed data, proper data transformations will be performed or non-parametric tests will be used. ANOVA or Kruskal-Wallis tests will be used to compare the three groups and contrasts for specific two-group comparisons will be implemented. Two-group comparisons will also be made using two-sample T-tests or Wilcoxon rank sum tests. We will use individual matching; each triplet will be matched on race, gender, BMI and etiology of chronic kidney disease to ensure similar distribution among the 3 groups on these variables. We will also assess the comparability among the three groups. ANCOVA analysis adjusting for these factors along with two more additional factors will also be conducted. Specific Aim 2 is a 2x2 crossover study with repeated measurements during and after HD. For this aim we will use linear mixed effects models with a random subject effect and with the treatment (HOE-140 versus vehicle) and the time trend (before, during and after HD) as fixed effects. Carry over effect of HOE-140 treatment will be tested using the T-test approach proposed by Jones and Kenward.[68] Besides the evaluation of the treatment effect and the time trend using the mixed effect models, direct treatment effect will be estimated as within-subject mean difference along with their 95% confidence intervals. Based on our previous studies, we anticipate a drop-out rate of 10% or less. Subjects who drop out will be replaced. Specific inferences on effects of interest will be made by reporting a point estimate along with a 95% confidence interval and the p-value. Hypotheses will be tested at the level of α=0.05. SAS® release 9.3 (Cary, North Carolina) and the open source statistical package R (version 2.12)[69] will be used for the analysis with the assistance of Dr. Chang Yu from the Department of Biostatistics at Vanderbilt University Medical Center.

11.0 Privacy/Confidentiality Issues

There are many safeguards in place to prevent the release of information from this study. All research samples are bar coded with the subject's unique identifier. Data sets used for analysis only contain this identifier. The key to the code is protected. Only study team members have access to information that identifies subjects participating in the study. The results of tests run on research samples will not be recorded in any subject's medical record and neither the subject nor his or her doctor will be told of the results. Access to the Vanderbilt computer network is protected at the level of firewalls, TCP wrappers and university assigned user IDs. Data are secured with encryption algorithms and the network is maintained by the Medical Center's Network Computer Service.

12.0 Follow-up and Record Retention

We plan to enroll all subjects within the first 3.5 years of the grant. We will enroll 78 subjects in Aim 1 and 11 subjects in Aim 2. Aim 2 is a crossover study, so patients will come twice to the Vanderbilt CRC for each study day. We will utilize trained research nurses to assist with muscle biopsies, hemodialysis, and blood processing and sampling.

Research records will be maintained for at least seven (7) years from the date the research is closed with the Vanderbilt University IRB. All research records will be accessible for inspection and copying by authorized representatives of the IRB, federal regulatory agency representatives, and the department or agency supporting the research.

All Health Insurance Portability and Accountability Act (HIPAA) related documentation will be maintained for at least seven (7) years from the date of the last use or disclosure of the Protected Health Information (PHI).

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