MINERALOCORTICOID RECEPTOR ANTAGONISM CLINICAL EVALUATION IN ATHEROSCLEROSIS

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SUMMARY

Atherosclerosis is the leading cause of morbidity and mortality in Type II diabetes. A cell type called the monocyte/macrophage is critical to development and complications of atherosclerosis. This project will evaluate the effectiveness of a medication called Spironolactone in preventing atherosclerosis in Type II diabetes through its effects on cells such as the monocyte. Spironolactone has been demonstrated to be effective for the treatment of patients after a heart attack and stroke. We will evaluate the impact of Spironolactone in reducing atherosclerosis plaque and additionally evaluate its potential in changing inflammation. We envision that our strategy of simultaneously probing effect of a drug combined with analysis of mechanisms of action and predictive response will likely provide key information with which to design hard event (heart attack, stroke etc.) based trials.

BACKGROUND

Pharmacological studies over > 20 years have defined the importance of the mineralocorticoid receptor (MR) in hypertension and heart failure. Large-scale clinical trials have validated the use of MR antagonists in the management of patients with heart failure (RALES, EMPHASIS) and post myocardial infarction (EPHESUS)1-4 . Recent studies suggest that MR may link metabolic dysregulation with susceptibility to type II diabetes (T2DM) and atherosclerosis5-12 . MR is an ancient member of the 51-member, steroid-thyroid-retinoid receptor family. In response to ligand binding, MR undergoes conformational changes and is translocated to the nucleus where it binds to response elements in promoters, increasing transcription of target genes 13,14 . Although effects of MR activation have been attributed to aldosterone, mechanisms independent of aldosterone have been suggested and play a role in promoting inflammation, fibrosis and target organ damage15-18 .

Cardiovascular Effects of MR Activation: MR is widely expressed in the cardiovascular system 19-22 and is a major determinant of endothelial function, smooth muscle tone, vascular remodeling, fibrosis and blood pressure (BP)23-36 . In vitro, animal, and human data support a role for MR activation in promoting vascular oxidative stress, inflammation, proliferation, migration, vasoconstriction, vascular remodeling and fibrosis (FIG. 2) 5,28,31,36-44 . Similarly, MR antagonism is anti-inflammatory and antifibrotic with evidence in humans indicating reversal of endothelial dysfunction, diastolic dysfunction and vascular stiffness45-49 .

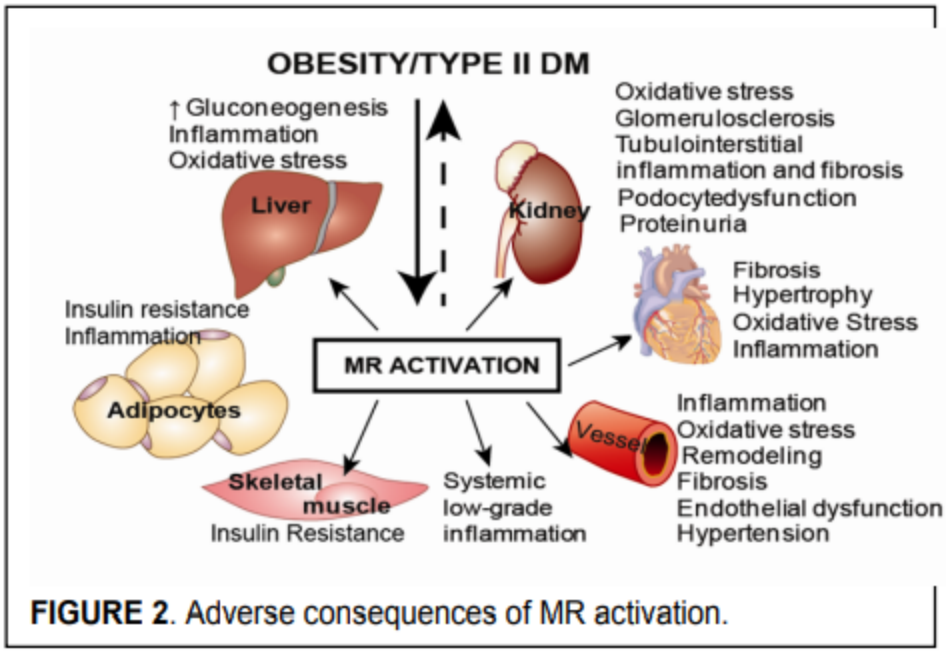

FIGURE 2. Adverse consequences of MR activation.

MR Activation Links Insulin Resistance (IR), Inflammation and Atherosclerosis: A number of reviews have already detailed in-vitro, experimental and human evidence linking aldosterone/MR activation with IR but is briefly summarized here.5-8,50-52 The visceral adipose renin-angiotensin-aldosterone (RAS) system synthesizes aldosterone, expresses MR, and predicts IR in both humans and animal models7,52,53 . MR expression on macrophages could potentially play a pro-inflammatory role in tissues such as adipose and vasculature.54-57 MR blockade/deletion has been shown in both experimental and human studies to ameliorate IR 58-60 . MR activation may affect IR through multiple mechanisms that include attenuation of insulin signaling in the heart, vasculature, and skeletal muscle. This may include impairment of expression of insulin receptor and substrate, decreased GLUT4 expression, abnormal phosphorylation of IRS, and activation of multiple stress kinases downstream of insulin receptor/IRS (leading to attenuation of insulin signaling)61-64 . MR activation also promotes hepatic oxidative stress, gluconeogenesis and enhances hyperglycemia 65 . Recently, MR activation has been shown to promote lipid storage in brown adipose and inhibit UCP1 expression 66,67 . Consistent with these roles, MR antagonism in humans improves IR 58-60,68 . Relevant to this proposal we have provided evidence for a role for MR in pro-inflammatory macrophage phenotype and reduction in atherosclerosis in animal models.

Role for the MR in Inflammation: Studies by Lumeng (co-investigator) and Chawla have provided the conceptual framework for understanding macrophage polarization state in the context of insulin resistance, obesity, and atherosclerosis 69-74 . Macrophages display heterogeneity of function depending on their micro-environment 75,76 . Th1 signals such as LPS/IFNγ result in "classical activation" leading to iNOS mediated NO production and synthesis of TNF-α and IL-6 77 . In contrast, an alternatively activated (AΦ) state of macrophage is generated upon exposure to IL-4/IL-13, which are generally implicated in resolution of inflammation and tissue repair 78-81 . Analysis of markers of AΦ have provided a remarkably conserved and stereotypic signature of genes corroborated by recent

genome-wide analysis of macrophage function.82 Although AΦ has been implicated in a range of physiologic and pathological processes, including inflammation, repair, and metabolic function, its role in atherosclerosis continues to evolve. PPARγ activation of macrophages by glitazones both in vitro and in vivo in patients with atherosclerosis, induces AΦ while deletion or antagonism (in mice) contributes to a classically activated phenotype 70,83,84 . In contrast to the function of PPARγ, MR activation potentiates classical activation while deletion of MR or antagonism results in AΦ 85 . The relative contribution and relevance of AΦ in atherosclerosis is not well studied. Studies in humans that provide insights on the role of AΦ and factors that may modulate macrophage phenotype may provide novel insights in atherosclerosis. Recently microRNAs (miRNAs) via their role as regulators of gene networks may represent mediators of functional networks and phenotypes such as AΦ and may provide insights into risk in atherosclerosis. We have provided important links between candidate miRs that play a role in macrophage AΦ through MR. We propose studies to investigate this in a clinical trial of MR antagonism in patients with T2DM.

Rationale for miRNA's as Regulator of AΦ and Marker(s) of Risk/Response: miRNAs hold the potential to modulate complex physiological/disease phenotypes and/or response to therapies by regulating entire functional networks. miRNAs are non-coding RNAs of 18–25 nucleotides that regulate multigene expression and modulate entire biologic pathways. It has been estimated that up to 1000 miRNAs are encoded by the human genome 86 . miRNAs are transcribed as precursors called primary miRNAs, which are processed to form mature miRNAs87 . Mature miRNAs recognize specific target mRNAs and induce gene silencing by mRNA degradation and/or inhibition of translation 86,87 . Assessment of miRNAs may provide insights into mechanisms of action/effect of therapies. In support of this, miRNAs are risk predictors in cancer and emerging studies suggest that miRNAs may predict risk in cardiovascular disease 88-93 .

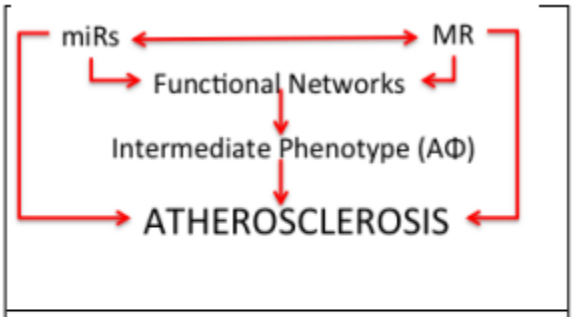

FIGURE 3. miRs and transcription factors such as MR regulate entire functional gene networks that through an intermediate phenotype (alternate macrophage activation) may affect atherosclerosis.

Preliminary Studies

MR antagonism is associated with Improvements in Vascular Function and Plaque Burden: Proof-ofconcept experiments from our group using two different MR antagonists (Eplerenone and

Spironolactone) in two distinct models of atherosclerosis provide experimental/mechanistic basis for the proposed investigation. In the first model, New Zealand white male rabbits were fed 1% cholesterol chow (HL) or normal chow (NC) over 8 weeks to induce atherosclerosis and then randomized to receive Eplerenone or placebo (Epl10 mg/kg) for 6 additional weeks. Eplerenone normalized peak endothelium dependent relaxation while nitroglycerin responses were unaltered (data for nitroglycerin not shown) (FIG. 4). Since we and others have established the importance of NADPH derived superoxide inactivation of •NO in response to angiotensin II (AII) and aldosterone, we investigated these sources 94-98 . Treatment with Eplerenone reduced •O2 - in aorta. We then studied the effects of Eplerenone in high-fat fed LDLR-/- model (Eplerenone 10 mg/kg for 12 weeks). Similar improvements in endothelial dilation in the LDLR-/- were seen (FIG 4). Epl reduced atherosclerosis and macrophage infiltration. These same data have been reproduced in the ApoE-/- by other groups 99,100 . In additional experiments, we have demonstrated that Eplerenone treatment prevents aldosterone induced thrombosis101 .

Role of the MR in Control of Macrophage Polarization: In collaboration with Dr. Lumeng, we and others have shown that various pro-inflammatory insults including highfat feeding and air-pollution can result in a shift to an M1 phenotype 72,102-104 . Cytokines from an M1 polarized macrophage, such as TNFα and CCL-2 play an important role in potentiation of inflammation and insulin resistance. Usher and Lumeng et al have recently shown that peritoneal macrophages in mice could be rendered pro-inflammatory via exposure to aldosterone, an effect prevented by Eplerenone but not the GR antagonist RU486 85 . Deletion of MR in myeloid cells recapitulated the effects of MR antagonism, by shifting phenotype to an alternately activated form concomitantly down regulating proinflammatory and pro-fibrotic genes (TGFβ and PAI-1). IL-4, an inducer of Aφ, synergized with macrophages lacking MR to display enhanced expression of Aφ markers. In vivo the effects of myeloid deletion of MR reduced, aortic and cardiac macrophage recruitment, cardiac hypertrophy, fibrosis and fetal gene re-programming in response to AII and L-NAME (model of MR activation) suggesting that myeloid MR was crucial to adverse cardiovascular remodeling 85 . Finally gene expression analysis revealed significant similarity between MR deletion and PPARγ activation.

Bioinformatic and in vitro assessment of miRNA regulation of NR3C2 (MR gene) in human monocytes: We first asked if NR3C2 is a target for miRNA regulation and investigated this using in-silico approaches. NR3C2 has one of the longest 3'-UTR's in the RAS cascade and contains the highest number of predicted miRNA targets based on Targetscan 5.2 analysis indicating that it may be under intricate miRNA control.

Fifteen miRNAs including 2 unique miRNA families (miR-30 and miR129) were identified that bound to 11 distinct sites on the 3'-UTR. These miRNAs were then analyzed in 10 miRNA prediction algorithms (Diana, MicroInspector, Miranda, mirtarget2, mitarget, nbmirtar, pictar, pita, rna22, rnahybrid). miRNAs that were common to at least 5 different prediction algorithms were identified. Based on this analysis, we investigated the expression of miRNAs initially identified in-silico, in human monocytes and then investigated their regulation in monocytes derived from a cohort of patients with atherosclerosis (derivation cohort, n=10; mean age=65±6 years) using an expression array with >600 miRNAs (Nanostring Technologies. All patients in the derivation cohort were receiving ACEI/ARB/statin therapy and were compared to control subjects without risk factors for atherosclerosis (n=6 per group). We used a miRNA expression array (Nanostring Technologies, CA) that provides quantitative assessment of >600 human miRs. miR's regulated in atherosclerosis were defined as those that were highly regulated ($p<10^{-10}$ vs. control subjects). The data set was filtered for outliers and analyzed. The putative miRNAs that target NR3C2, are expressed in monocytes AND are downregulated in atherosclerosis. We then analyzed

the relationship of miRNAs obtained in the derivation cohort, with aortic plaque measured by MRI. Correlation analysis with baseline atherosclerosis revealed that multiple miRs correlated with atherosclerosis plaque burden at baseline after adjustment of baseline covariates (age, gender, blood pressure and LDL cholesterol). This included 31 miRNAs involved in NR3C2 regulation (r2 =0.35) as well as several candidate miRs unrelated to MR expression (Down-regulated=miR-27, r 2=0.36; miR-125, r 2=0.25; miR-124, r 2=0.20; miR-7 r 2=0.19; Up-regulated: miR-223. r 2=0.25; mir-126. r 2=0.22; miR-1720, r 2=0.15.

Regulation of NR3C2-3UTR Luciferase Reporter: NR3C2 3-UTR was cloned downstream of a mammalian promoter-luciferase system and co-transfected in HeLa cells with plasmids expressing pmiR-19b and pmiR-124 have shown that 124 but not 19b reduced luciferase activity by more than 50%105 . Studies examining other miRNAs in HeLa cells are currently ongoing in the laboratory.

Assessment of Aortic Atherosclerosis at 3.0T MR using SPACE: We have described the implementation of a modified spin echo sequence T1W-SPACE and have successfully implemented this technique at both 1.5T and 3.0T 106,107 . We have developed a customized tool-set for assessment of plaque area over the aorta.

Plaque Progression over 9 months: In a recently concluded study in patients with established vascular disease on complete renin blockade with Aliskiren (ALPINE, n=37)108 . The results of this study demonstrated that aliskerin use on top of ACEI/ARB progresses atherosclerosis. These results are consistent with results of ALTITUDE published recently 109 . Interestingly one of the findings in the study was that aliskerin treatment resulted in decrease of miR-19a which we have shown to be important in upregulation of MR. This increase was particularly robust in those on ACEI/ARB therapy suggesting that this group could be potentially sensitive to the effects of MR blockade as they are overexpressing MR (product of NR3C2 gene).

STUDY RATIONALE

There are multiple innovative concepts in this trial. 1) There are currently no large trials of MR antagonism in patients with diabetic atherosclerosis and no trials that are investigating predictors of response. Our results will provide a basis for a definitive intervention trial. 2) Investigation of monocyte/macrophage polarization: The importance of monocyte/macrophage activation states in metabolic and cardiovascular disease is yet to be fully integrated into our understanding. Our preliminary studies and those by others support an important role for MR in AΦ, but whether or not pharmacologic blockade of MR modifies monocyte inflammation in humans is unknown. 3) Investigation of miRNAs: Prior studies have shown that miRNAs are involved in monocyte differentiation and inflammatory function 110,111 . Our approach to incorporate miRNA analysis to predict response is innovative and is based on preliminary data suggesting that several candidate miRNAs regulated MR expression and AΦ. 3) MRI Approaches in Aortic Imaging: MRI approaches can track serial changes in atherosclerosis 112-117 . Prior MRI approaches have assessed the carotid or a limited portion of the descending thoracic aorta (a relatively immobile structure) not susceptible to motion artifacts 118-121 . Current improvements in pulse sequence design have enabled motion-free high-resolution coverage of the entire aorta including the arch 122,123 . We have tested the ability of such an approach for wholebody plaque, carotid and lower extremity imaging 106,107,124 . Coverage of the entire aorta allows detection of smaller changes, allows inclusion of smaller numbers of patients and provides an

improvement over invasive surrogate measures acquired in limited portions of the arterial bed (83-86).125,126 4) Focus on monocyte miRNAs (versus plasma): While plasma miRNAs are under investigation as biomarkers, miRNA detection is limited by low amounts of RNA, lack of standardization, and lack of endogenous controls. In addition, since plasma miRNA expression is likely the result of several cell types, the biological and mechanistic relevance of changes are challenging. Peripheral blood mononuclear cells (PBMCs) provide unique advantages over plasma miRNAs in atherosclerosis (see below). Evidence of the importance of miRNAs in peripheral blood mononuclear cell (PBMC) is well established in diseases such as cancer and COPD 89,127- 130 . 5) miRNAs as Predictors of Response/Disease: Atherosclerosis is a complex disease and it is unlikely that expression of a single gene or marker may predict it. However understanding of candidate miRNAs and key factors such as MR through their role in regulating hundreds of genes and influencing phenotypes such as AΦ are far more likely to be informative. The figure illustrates this concept where miRNAs and the transcription factor MR may potentially influence functional networks of genes and thereby affect AΦ. Our investigations thus may shed light on the role of each of these "levels" of regulation in atherosclerosis.

Risk / Benefit Assessment

All potential patients will be informed of the potential side effect profile prior to enrollment in the study and full informed consent will be utilized.

Risks of an MRI Scan

There are no risks associated with the MRI scan. In our center approximately 10% of patients are genuinely claustrophobic.

Reproductive Risks

Spironolactone may cause risks to the unborn fetus and or the baby of a nursing mother. Therefore, pregnant women and women who are nursing their babies should not participate in the study. Woman must be either post-menopausal for one year, surgically sterile, or using effective contraception. A pregnancy test will be required at screening for all woman who may become pregnant. A negative test is required for the individual to enroll in the study. Participants who become pregnant will report to the study PI immediately, and stop taking the pills.

Spironolactone can decrease the effectiveness of birth control pills. Women who use oral birth control methods will be excluded from the study. Women of child bearing potential should use another reliable form of contraception. Acceptable methods of birth control include subcutaneous implants, intrauterine devices (IUD), barrier methods, surgical sterility, transdermal birth control path, and abstinence.

Side Effects Related to Study Drug

Common Side Effects

- Hyperkalemia: We anticipate that approximately 1% of patients may develop serious hyperkalemia that may warrant discontinuation. However this may be considerably higher in patients with GFR of 40-50 ml/minute who will constitute a minority of the patients in the trial.
- Increases in BUN and creatinine
- Gynecomastia or breast discomfort. Gynecomastia is a well-described adverse effect of spironolactone and is related to dose and duration of treatment; we anticipate that

approximately 10% of patients may develop gynecomastia that may warrant discontinuation. Generally, discontinuation of treatment results in resolution of gynecomastia.

Other Side Effects

- Cardiovascular: Vasculitis
- Central nervous system: Ataxia, confusion, drowsiness, headache, lethargy
- Dermatologic: Erythematous maculopapular rash, Stevens-Johnson syndrome, toxic epidermal necrolysis, urticaria
- Endocrine & metabolic: Amenorrhea
- Gastrointestinal: Abdominal cramps, diarrhea, gastritis, gastrointestinal hemorrhage, gastrointestinal ulcer, nausea, vomiting
- Genitourinary: Impotence, irregular menses, postmenopausal bleeding
- Hematologic & oncologic: Agranulocytosis, malignant neoplasm of breast
- Hepatic: Hepatotoxicity
- Hypersensitivity: Anaphylaxis
- Immunologic: DRESS syndrome
- Renal: Increased BUN, renal failure, renal insufficiency
- Miscellaneous: Fever

Potential Benefits of the Proposed Research to the Subjects and Others

It is possible that there may be a benefit with Spironolactone in terms of organ protection and patients may see benefits relating to reduction of atherosclerotic plaque including reduction in risk for events such as heart attacks, stroke, renal failure and need for hospitalization. The potential benefits must obviously be contrasted with the risk for renal failure and hyperkalemia.

Importance of the Knowledge to be Gained

There is an urgent need for clinical trials aimed at reducing the disproportionate cardiovascular disease burden and mortality in Type II diabetes. The multiple cardiovascular benefits of mineralocorticoid receptor (MR) blockade (using spironolactone or eplerenone) are well recognized in patients without Type II diabetes in the setting of congestive heart failure or myocardial infarction. However, randomized trials of MR Blockade have not been conducted in patients with atherosclerosis. A potential role for MR blockade for slowing atherosclerosis progression as well as influencing cardiovascular outcomes in diabetic patients is only beginning to attract the attention of cardiologists. Patients with atherosclerosis may benefit from MR blockade if, on one end of the spectrum, such intervention is made relatively early in the course of Type II diabetes to slow progression and of atherosclerosis and inflammation or plausibly at the other extreme when patients are already at very high risk patient population for cardiovascular events and mortality. The use of Spironolactone in patients with type II diabetes and atherosclerosis has not been well studied.

STUDY OBJECTIVES

Primary Objective

Evaluate the impact of Spironolactone in reducing atherosclerosis plaque as measured by change in percent total atheroma volume (PAV) in thoracic aorta with Spironolactone vs. placebo.

Secondary Objectives

Evaluate the functional significance of candidate miRNAs. Compare monocyte polarization and inflammatory gene expression at 6-weeks. Evaluate miRs expression to predict atherosclerosis and response.

STUDY DESIGN

Vanguard Phase

Prior to the Main Study Phase, an open label vanguard (pilot) phase is planned with 10 subjects over 4 weeks with Spironolactone. This phase is designed to provide preliminary evidence and assess recruitment and methods.

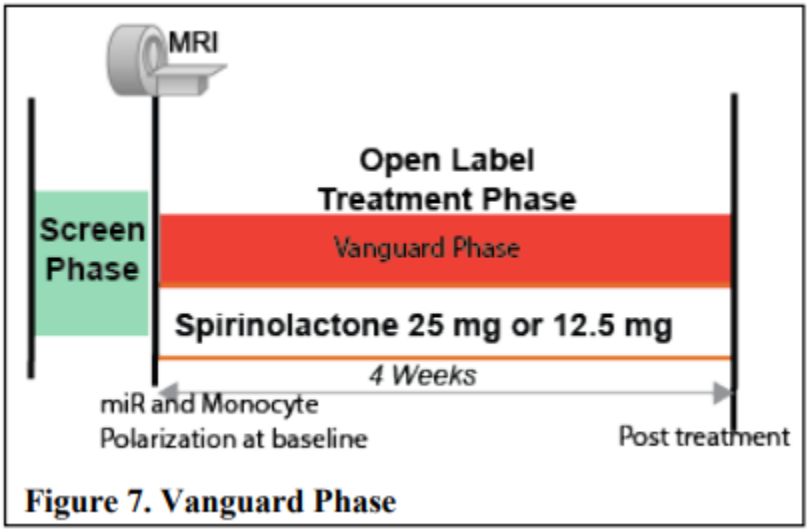

Figure 7. Vanguard Phase

Main Study Phase

The Main Study Phase is a muti-site, double-blind, randomized, placebo-controlled trial with spironolactone. 130 subjects are planned. There will be 4 phases (screening, placebo run-in, dose escalation and treatment phase (FIG. 8). All patients will be carefully evaluated for study eligibility during screening prior to randomization and will be recruited from clinics at the University Hospitals Cleveland Medical Center as well as nephrology clinics. The 2-week single-blind lead-in period will permit assessment of patient compliance as well as determination of baseline sitting blood pressures. Patients must have met all inclusion/exclusion criteria before being randomized 1:1 to Placebo or Spironolactone 12.5 mg on Week 0 (Visit 2). Patients will be escalated to 25 mg daily Spironolactone or maximal tolerated dose over a 4-week period with safety checks on K+ and creatinine (FIG. 8).

CRITERIA FOR EVALUATION

Co-Primary Efficacy Endpoints

The co-primary outcome measure is change in 24-hour SBP in Spironolactone treated patients versus placebo and percent change in total atheroma volume (PAV) in thoracic aorta of Spironolactone vs placebo.

Secondary Efficacy Endpoints

Change in central aortic SBP, Change in central aortic DBP, Change in central aortic Mean BP, 24-hour mean ambulatory BP, 24-hour ambulatory diastolic blood pressure, and HOMA-IR.

Evaluate miRs involved in regulating MR expression and differentially regulated miRs as predictors of disease progression and response.

Compare monocyte inflammatory activation in Spironolactone vs placebo at 6-weeks.

Investigate pre-miR/mimics and antagomirs to regulate NR3C2 expression and alternate macrophage phenotype in human macrophages.

Safety Evaluations

Change in clinical laboratory findings (e.g. BUN, Creatinine, electrolytes).

Incidence of adverse events

SUBJECT SELECTION

Study Population

Subjects with a diagnosis of Type II Diabetes who meet the inclusion and exclusion criteria will be eligible for participation in this study. Labs drawn within 3 months of the screening visit may be used to determine eligibility.

Inclusion Criteria

1. Male or female patients > 45 or > 40 years with known atherosclerotic events (examples include MI, Stroke) and able to provide informed consent (females must be either post-menopausal for one year, surgically sterile, or using effective contraception. Oral contraceptives are disallowed.
2. Patients with type II diabetes with HbA1c ≤ 9.0 on stable anti-glycemic regimen that may include oral and/or injectable therapy (GLP-1/Insulin etc.). Changes in dose of glycemic regimen is allowed during the course of the trial if felt to be clinically appropriate.

3. GFR <90 and evidence of proteinuria (Urine Albumin/Creatinine Ratio of >30 mg/g or equivalent) in a urine specimen within 12 months OR GFR <60 mg/g regardless of proteinuria.
4. Patients must be on ACE and/or ARB therapy with no planned dose adjustments.

Exclusion Criteria

1. Uncontrolled hypertension (SBP>160 and/or DBP>95 mmHg at visit 0 (screening) Protocol Number: 09-16-32 Confidential Version #: 1.5 Revised 01 April 2020 Page 14 of 39 and SBP >145 mm Hg at visit 2).
2. GFR (MDRD) of <15 at visit 0 (screening)
3. Hyperkalemia defined as serum K+≥ 5.1 meq/L at visit 0 (screening)
4. LDL cholesterol >150 mg/dL
5. Plasma triglycerides > 400 mg/dl
6. Contraindications to MRI (metallic implants, severe claustrophobia).
7. Acute coronary syndrome, Transient ischemic attack, CVA or critical limb ischemia during the last 6 months or coronary/peripheral revascularization within the last 3 months.
8. Evidence of a secondary form of hypertension
9. Initiation of new therapy with statins, ACEI/ARB, antioxidants, CCBs, diuretics, β blockers
10. Type I diabetes mellitus
11. Known contraindication, including history of allergy to Spironolactone
12. Any surgical or medical condition which might alter pharmacokinetics of drug (e.g. renal transplant, liver failure, liver transplant)
13. Concurrent potentially life threatening arrhythmia or symptomatic arrhythmia.
14. Significant hyponatremia defined as Na<130 meq/L
15. History of prior malignancy including leukemia and lymphoma (but not basal cell skin cancer, cured squamous cell cancer and cured prostate cancer).
16. History of any severe, life-threatening disease.
17. Any surgical or medical conditions which place the patient at higher risk derived from his/her participation into the study, or likely to prevent patient from complying with requirements
18. History of drug abuse within the last 2 years, noncompliance and unwillingness/inability to consent
19. Pregnant women and nursing mothers
20. Class III or IV Congestive Heart Failure
21. Primary Hyperaldosteronism

Recruitment Strategy

All patients will be carefully evaluated for study eligibility during screening prior to randomization and will be recruited from clinics at University Hospitals' healthcare systems. The medical records will be accessed to determine eligibility. PHI will be accessed during the pre-screening portion for medical review. A full waiver is requested. The 2 -week single -blind lead--in period will permit assessment of patient compliance as well as determination of baseline sitting blood pressures. Patients must have met all inclusion/exclusion criteria before being randomized 1:1 to Placebo or spironolactone on Week 0 (Visit 2).We may call patients who have expressed interest in the stud, using an approved phone script. No cold calling will be done. The phone script will be used for patients who would like to be contacted

regarding more information about the study after their physician has given them some initial background in person or by letter.

CONCURRENT MEDICATIONS

All subjects should be maintained on the same medications throughout the entire study period, as medically feasible. Changes in dose of glycemic regimen is allowed during the course of the trial if felt to be clinically appropriate. Patients must be on ACE and/or ARB therapy and statin therapy with no planned dose adjustments.

Prohibited Medications and Treatments

The following medications are prohibited during the study:

Oral contraceptives

STUDY TREATMENTS

Method of Assigning Subjects to Treatment Groups

After the placebo run-in phase, eligibility with regards to blood pressure will be reassessed.

Up to 130 eligible patients will be randomly assigned to Spironolactone or placebo treatment groups in a 1:1 ratio using a computer-generated randomization scheme developed by the statistician.

All study participants will undergo a two week run-in period with placebo. Then study medication will be commenced at 12.5 mg Spironolactone or placebo and escalated to 25 mg Spironolactone or placebo over 4 weeks. Then the treatment phase will commence with tolerated dose of Spironolactone or placebo for 48 weeks.

Blinding

Due to the objectives of the study, the identity of test and control treatments will not be known to investigators, research staff, or patients. The following study procedures will be in place to ensure double-blind administration of study treatments.

Access to the randomization code will be strictly controlled.

A taste-matching agent.

Packaging and labeling of test and control treatments will be identical to maintain the blind.

Blinding of K+: All safety K+ values will be blinded. A protocol driven approach for management of K+ will be followed. These data will also be made available to the DSMB for review.

Blinded-end point assessment: Data sets will be de-identified and replaced with a random number. The newly generated data set will be saved and the data identity key will be stored in a secure location.

The study blind will be broken on completion of the clinical study and after the study database has been locked.

During the study, the blind may be broken only in emergencies when knowledge of the patient's treatment group is necessary for further patient management.

Formulation of Test and Control Products

Formulation of Test Product

Spironolactone is an antihypertensive potassium sparing diuretic and Mineralocorticoid (Aldosterone) Receptor Antagonist.

Formulation of Control Product

A matching placebo will be provided.

Dosage

During the dose escalation phase, Spironolactone dosing will start at 12.5 mg PO and escalate to 25 mg or maxim tolerated dose. The dose may need to be adjusted during the study due to safety lab results (e.g. K+) or if BP<100 mmHg.

Study Drug Accountability

An accurate and current accounting of the dispensing and return of study drug for each subject will be maintained on an ongoing basis by a member of the study site staff. The number of study drug dispensed and returned by the subject will be recorded.

Measures of Treatment Compliance

Subjects will be asked to keep a patient diary noting the day and date they take their study drug and any adverse events. They will be asked to bring their patient diary to each study visit along with all used and unused study drug containers.

HYPERKALEMIA MANAGEMENT

One of the most significant complications of the study medications is hyperkalemia. This risk is of particular importance in this study population as diabetic patients with chronic kidney disease are at increased risk for selective aldosterone deficiency, predisposing them to the development of hyperkalemia. Mild to moderate hyperkalemia is generally not associated with significant cardiac toxicity, especially when it is chronic (e.g., in dialysis patients) or slowly developing. In contrast, severe (> 6.5 mEq/L) or rapidly developing hyperkalemia is likely to be associated with the development of ECG abnormalities ranging from peaking of the T- wave; to atrial, AV nodal and ventricular conduction delays; and ultimately, with very severe hyperkalemia, to ventricular fibrillation.

Dietary intake of potassium is a major factor in producing hyperkalemia and transient dietary increases in potassium intake can precipitate hyperkalemia in our patients. For example, a single high potassium meal prior to blood draw could result in high potassium at the time of the draw but later on a repeat the potassium may be much lower, as much as 1 mEq/L lower.

Decreased cellular uptake of potassium occurs when insulin is relatively deficient. Even a patient who is not on insulin can have relative insulin deficiency (also known as insulin resistance). Generally decreased cellular uptake of potassium occurs when a patient has ingested a high potassium diet but does not have enough insulin to drive potassium into the cell. This can result in transient hyperkalemia.

Factors that impair renal excretion of potassium and can contribute to hyperkalemia in addition to the study medication include: 1) decreased GFR; 2) drugs including NSAIDS (e.g. ibuprofen), COX-2 inhibitors (e.g. celecoxib) and 3) any process which decreases distal tubular flow rate such as can occur in the setting of volume depletion.

One or all of the above factors can be operative in the same patient. For example, a volume depleted patient can have relative insulin deficiency, a high potassium intake and have taken NSAIDS for pain while ingesting the study medication.

Subjects will be counseled to avoid medications that may increase the risk of hyperkalemia, such as non-prescription non-steroidal anti- inflammatory medications, and potassium-containing salt substitutes. All subjects will be monitored for the development of hyperkalemia at each follow-up visit and an ECG will be obtained for any potassium value > 6.0 mEq/L.

The aggressiveness of treatment of hyperkalemia will depend on the degree of elevation and the presence or absence of ECG findings.

Treatment of hyperkalemia for Potassium > 5.0 meq/L: Dietary potassium

If during follow-up, a subject's potassium level increases to > 5.0 mEq/L, he/she will be prescribed a low potassium diet (0.7-0.8 meq/kg/day to a maximum daily intake of 60 meq/day). An initial step would be to restrict potassium in the diet. Input from a dietician can be helpful.

There is potassium in most foods, but certain foods are especially high in potassium and should be avoided. Some high potassium foods can be eaten after leaching the potassium by soaking. Many salt substitutes are potassium chloride and should be avoided. Fruits and vegetables should be limited to four servings per day. Dairy products should be limited to 1 cup/day.

<u>High Potassium Foods (avoid):</u>

Fruits and juice: apricots, avocado, banana, cantaloupe, honeydew, dried fruit (dates, raisins, prunes, figs), citrus fruits (orange, grapefruit, nectarines)

Vegetables: artichoke, butter beans, dried peas or beans, potatoes (unless leached), sweet potatoes, Swiss chard, winter squash.

Other: chocolate, molasses, nuts, peanuts

<u>Medium potassium foods</u>: Limit to one serving (1/2 cup)/day:

Fruits: blackberries, cherries, peach (1), pear (1), raspberries, strawberries, watermelon (1 cup), plums (2)

Vegetables: broccoli (cooked), carrot, mushroom, peas, corn, cabbage, eggplant, spinach (cooked), pumpkin, tomato, potato (leached), beets

<u>Low potassium foods</u> (note that portion size is 1/2 cup, eating a large amount of a lower potassium food will make it a higher potassium food)

Fruits (including juice): Apple, blueberries, cranberries, grapes, tangerine (1 small) Vegetables: green beans, wax beans, cucumber, green pepper, lettuce, yellow squash

Leaching vegetables: For potatoes, sweet potatoes, carrots, beets and rutabagas:

Peel and slice vegetables 1/8 inch thick

Rinse

Soak for a minimum of two hours in warm water, using 10 times the amount of water to the amount of vegetables. If soaking longer, change the water every few hours

Rinse under warm water

Cook with five times the amount of water to the amount of vegetable

Treatment of hyperkalemia for Potassium > 5.5 meq/L

In addition to a low potassium diet, if the potassium level increases to >5.5 mEq/L, conservative measures should be instituted.

Possible approaches include:

Increase in diuretics if volume depletion is not a concern. Volume depletion could worsen hyperkalemia

Administration of chronic alkali supplements, such as sodium bicarbonate 1300mg bid if acidosis is present

Liberalization of salt intake (if volume status and uncontrolled blood pressure are not concerns)

Chronic use of low-dose sodium polystyrene sulfonate (15-30 grams 3x/week)

Treatment of hyperkalemia for Potassium > 6.0 meq/L

If the potassium level is > 6.0 mEq/L, the study medication will be held until the level decreases to < 5.5 mEq/L.

Acutely to treat the potassium:

An ECG should be checked. If there are no ECG changes and potassium level is < 6.5, then Kayexalate alone (60 grams) may be sufficient. If there are ECG changes, treatment would include insulin/glucose, beta-agonists and possibly calcium and bicarbonate. If there are ECG changes or if the potassium level is > 6.5 meq/L, the patient should be treated in the emergency room. If there are ECG changes, the patient should be placed on a cardiac monitor.

Reinstitution of Study Medication

If the potassium level was > 6.5, then the study medication will be permanently discontinued and the patient will be removed from the trial. If this is the second episode where potassium > 6.0, then the study medication will be permanently discontinued and the patient will be removed from the trial.

Recheck potassium level 7- 14 days after discontinuation. If K+ is > 5.5 mEq/L, do not restart study medications. Repeat potassium again in 7-14 days. If K+ is ≤ 5.5 mEq/L, the study medication will be reinstituted at 50% of prior dose.

If the study medication was restarted, check potassium level again in two weeks, if K+ is:

o > 6.0: stop study the study medication permanently

o 5.5-6.0: treat K+, maintain current dose of the study medication

o < 5.5: mEq/L and patient is on at least 12.5 mg spironolactone, increase spironolactone to 25mg daily.

STUDY PROCEDURES AND GUIDELINES

Prior to conducting any study-related activities, written informed consent and the Health Insurance Portability and Accountability Act (HIPAA) authorization must be signed and dated by the subject. If appropriate, assent must also be obtained prior to conducting any study-related activities. Study visits will be conducted on the scheduled day or within +/- 2 days for weekly study visits and +/- 7 days for monthly visits.

Schedule of Events

Vanguard Phase (update to reflect table below)

TABLE 3	SCREEN	Treatment Phase			
WEEK	W1	W2	W3	W4	W5
VISIT NUMBER	0	1	2	3	4
Screening Labs	X				
Safety Lab (BUN, Creat, Lytes)		X	X	X	X
History/Physical	X				
Clinic BP or 24-hour ABP*	X	X*		X	
Phone Call				X	
MRI		X			
MonocyteStudies		X			
Insulin/Glucose (HOMA-IR)		X			
HbA1C		X			
Central aortic BP		X			
PIP, PIIINP, CITP and chemokines CCL2, CX3 CL1, and CCL5		X			
Plasma Renin/Aldosterone		X			
Medication Log		X		X	
Pill counts		X			
Dietary survey/instructions	X	X			

Urine sample (Alb/Creat)	X	X			
Spot Na/K/Creat		X			
Interim Event log		X		X	
Phy activity, Smoking questionnaire	X				

Main Study Phase

TABLE 4	SCREEN		RUN-IN PHASE		RANDOMIZATION PHASE										
					DOSE ESCALATE				TREATMENT PHASE						
MONTH*	M1		M1		M2	M2	M 2		M 3	M 5	M 7	M 9	M 11	M 12	M13
WEEK	W 1	W2	W3	W 4	W5	W6	W8	W9	W11	W17	W25	W33	W41	W45	W53
VISIT NUMBER	0		1		2	3	4	5	6	TF1	7	TF2	TF3	TF4	8
Screening Labs (BMP, Lipid panel, HbA1C, Urine sample Alb/Creat)	X														
Safety Lab (BMP)					X	X	X	Optional	X		X				X
History/ Targeted assessment			X								X				X
EKG			X												
Vital Signs or 24-hour BP cuff *(includes central aortic BP)	X		X		X*		X		X*		X				X
Phone Call										X		X	X	X	
MRI					X										X
Research Labs/Monocyte Studies, including PIP, PIINP, CITP and chemokines CCL2, CX3, CL1, and CCL5					X				X						X
Insulin/Glucose (HOMA-IR)					X										X
HbA1C					X						X				X
Plasma Renin/Aldosterone					X										X
Medication Log			X				X		X	X	X	X	X	X	X
Pill counts					X				X		X				X
Dietary survey/instructions			X												X
Urine sample (Albumin/Creat)	X		X		X				X		X				X
Urine Na/K/Creat					X										
Interim Event log					X		X		X	X	X	X	X	X	X
Questionnaire & Instructions			X												

Clinical Assessments

A complete medical history and physical exam will be performed at baseline and will include a 12-lead electrocardiogram (EKG), cardiovascular risk factor assessment, intercurrent CV events, 7-day physical activity recall, active/passive smoking questionnaires and dietary recall questionnaire. Sitting systolic blood pressure, pulse rate, body weight, interim event logs, pill counts, medication logs will be recorded at most clinic visits. Central aortic blood pressure will be obtained during visit 2 (base), 6 (midpoint) and 8 (exit) while 24-hour BP will be measured at Visit 2 and 6. Dietary Na+ consumption: Since Na intake is an important determinant of MR responsiveness we will ensure that all patients receive dietary survey/instructions (Visit 0) on maintaining a personalized diabetic diet that is stable and is medium to low in sodium (<4g). We will obtain Na, K and Creatinine levels (Visit 2) to assess Na intake. The kawasaki formula will be used to estimate 24 hour urine.

Demographics

Demographic information (date of birth, gender, race) will be recorded at Screening.

Adverse Events

Information regarding occurrence of adverse events will be captured throughout the study.

Clinical Laboratory Measurements

Urinary Na levels, albumin and creatinine

Serum levels of carboxyl-terminal peptide of procollagen type I (PIP), carboxyl-terminal telopeptide of collagen type I (CITP), and amino-terminal peptide of procollagen type III (PIIINP) and chemokines

Safety labs (BUN, creatinine, electrolytes), HOMA-IR, Hem A1C and Plasma Renin Aldosterone

Urgent Study Alerts

An Investigator should be notified immediately if any of the following are noted at study visits:

<u>Blood Pressure Immediate Alert Values:</u>

- Systolic Blood Pressure < 80
- Systolic Blood Pressure >180
- Diastolic Blood Pressure >110

<u>Acute Distress: (signs or symptoms constituting an emergency):</u>

- Chest Pain
- Severe Respiratory Distress
- Acute Neurological Symptoms

<u>Urgent Lab Values:</u>

- Potassium ≤ 3.0 mEq/L
- Potassium ≥ 6.0 mEq/L
- Glucose < 50 mg/dL
- Glucose >350 mg/dL
- Creatinine doubling from last value

Coronavirus Outbreak (COVID-19) Management Plan under Public Health Mandates

The following guidelines were developed under polices set forth by UH Cleveland Medical Center, the University of Maryland, NYU Winthrop, and St. Michael's Hospital in Toronto. All participating sites will be taking the following preventative measures to reduce the spread of the COVID-19 virus to research patients and staff.

Recruitment and Enrollment

Under the new implemented COVID-19 policies, we will not enroll new participants in the United States and Canada. No new participants will be enrolled until deemed safe by the affiliated research institutions. Study procedures will revert to the original procedures described in Section 11 "Procedures and Guidelines" once the mandates are lifted. During this time, pre-screening may continue according to the discretion of each study site. Potentially eligible subjects will be placed on a waiting list to be screened and enrolled once mandates are lifted.

Active Participants and Follow up Visits

At the time COVID-19 policies were implemented, active participants fell into either the "run in phase" or the "treatment phase" of the study procedures timeline of events (See 11.1.2 Main Study Phase).

RUN-IN-PHASE Patients in the run-in-phase (visit 1) will postpone visit 2 until safe medication compliance and follow up labs can be performed.

TREATMENT-PHASE In order to simultaneously reduce transmission of the virus while maintaining the management of participant's follow up care to the best extent possible, pertinent study activities (i.e. safety labs & vital signs) will be attempted in conjunction with any provider scheduled clinical encounter deemed essential for clinical care. If this is not possible, patients will be given an additional prescription until the essential study activities can be performed safely (length of prescription may vary based on patients' recent bloodwork and BP/HR, specific site policies, and investigator's medical judgment).

Additional important details

Remote study visits are not appropriate for this study.

Participants will be compensated for their participation at the same amount specified in the original consent through site-specific means.

All enrolled participants at each site will be notified by phone call of these protocol changes enacted during the period of public health mandates.

ADVERSE EXPERIENCE REPORTING AND DOCUMENTATION

Adverse Events

An adverse event (AE) is any untoward medical occurrence in a clinical investigation of a patient administered a pharmaceutical product and that does not necessarily have a causal relationship with the treatment. An AE is therefore any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the administration of an investigational product, whether or not related to that investigational product. An unexpected AE is one of a type not identified in nature, severity, or frequency in the current Investigator's Brochure or of greater severity or frequency than expected based on the information in the Investigator's Brochure. The Investigator will probe, via discussion with the subject, for the occurrence of AEs during each subject visit and record the information in the site's source documents. Adverse events will be recorded in the patient CRF.

AE Severity

The National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) in Table 1 should be used to assess and grade AE severity, including laboratory abnormalities judged to be clinically significant. If the experience is not covered in the CTCAE, the guidelines shown in Table 2 below should be used to grade severity. It should be pointed out that the term "severe" is a measure of intensity and that a severe AE is not necessarily serious. K+ >6.0 will be considered an adverse event. K+5.0 will be treated according to the hyperkalemia protocol.

National Cancer Institute's Common Terminology Criteria for Adverse Events

Severity (Toxicity Grade)	Description
Grade 1, Mild	Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Grade 2, Moderate	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL*.
Grade 3, Severe	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL**.
Grade 4, Life-threatening	Life-threatening consequences; urgent intervention indicated.
Grade 5, Death	Death related to AE.

AE Severity Grading

Severity (Toxicity Grade)	Description
Mild (1)	Transient or mild discomfort; no limitation in activity; no medical intervention or therapy required. The subject may be aware of the sign or symptom but tolerates it reasonably well.
Moderate (2)	Mild to moderate limitation in activity, no or minimal medical intervention/therapy required.
Severe (3)	Marked limitation in activity, medical intervention/therapy required, hospitalizations possible.
Life-threatening (4)	The subject is at risk of death due to the adverse experience as it occurred. This does not refer to an experience that hypothetically might have caused death if it were more severe.

AE Relationship to Study Drug

The relationship of an AE to the study drug should be assessed using the following the guidelines in Table 3.

Table 3. AE Relationship to Study Drug

Relationship to Drug	Comment
Definitely	Previously known toxicity of agent; or an event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to the suspected drug; that is confirmed by stopping or reducing the dosage of the drug; and that is not explained by any other reasonable hypothesis.
Probably	An event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to the suspected drug; that is confirmed by stopping or reducing the dosage of the drug; and that is unlikely to be explained by the known characteristics of the subject's clinical state or by other interventions.
Possibly	An event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to that suspected drug; but that could readily have been produced by a number of other factors.
Unrelated	An event that can be determined with certainty to have no relationship to the study drug.

Serious Adverse Experiences (SAE)

An SAE is defined as any AE occurring at any dose that results in any of the following outcomes:

- death
- a life-threatening adverse experience
- inpatient hospitalization or prolongation of existing hospitalization
- a persistent or significant disability/incapacity
- a congenital anomaly/birth defect Other important medical events may also be considered an SAE when, based on appropriate medical judgment, they jeopardize the subject or require intervention to prevent one of the outcomes listed.

Serious Adverse Experience Reporting

Adverse events will be documented per the University Hospitals Cleveland Medical Center, Human Research Protections guidelines, reported to the IRB, DSMB and the sponsor. The collection period for all SAEs will begin after informed consent is obtained and end after procedures for the final study visit have been completed.

Medical Monitoring

Dr. Matthew Weir and/or Dr. Jeffrey Fink should be contacted directly to report medical concerns or questions regarding safety.

DISCONTINUATION OF SUBJECTS

Early Discontinuation of Study Drug

A subject may be discontinued from study treatment at any time if the subject, the investigator, or the Sponsor feels that it is not in the subject's best interest to continue. The following is a list of possible reasons for study treatment discontinuation:

Subject withdrawal of consent (or assent)

Subject is not compliant with study procedures

Adverse event that in the opinion of the investigator would be in the best interest of the subject to discontinue study treatment

Protocol violation requiring discontinuation of study treatment

Lost to follow-up

Sponsor request for early termination of study

Positive pregnancy test (females)

If a subject is withdrawn from treatment due to an adverse event, the subject will be followed and treated by the Investigator until the abnormal parameter or symptom has resolved or stabilized.

All subjects who discontinue study treatment should come in for an early discontinuation visit as soon as possible and then should be encouraged to complete all remaining scheduled visits and procedures.

All subjects are free to withdraw from participation at any time, for any reason, specified or unspecified, and without prejudice.

Reasonable attempts will be made by the investigator to provide a reason for subject withdrawals. The reason for the subject's withdrawal from the study will be specified in the subject's source documents.

DATA SAFETY MONITORING

A Data Safety Monitoring Board (DSMB) will be established to review data relating to safety and efficacy, to conduct and review interim analyses, and to ensure the continued scientific validity and merit of the study. Reviews will be conducted by the DSMB every 3 months for the purpose of monitoring study conduct and assessing patient safety.

STATISTICAL METHODS AND CONSIDERATIONS

Prior to the analysis of the final study data, a detailed Statistical Analysis Plan (SAP) will be written describing all analyses that will be performed. The SAP will contain any modifications to the analysis plan described below. General Statistical Considerations: All the tests are two-sided. We use log transformations for most continuous measures, and arc-sin square root transformations for percentages, both of which have excellent variance stabilizing properties for pooling variance.

Aim 1: To test change in percent total atheroma volume (PAV) in thoracic aorta with Spironolactone vs. placebo

N=52 per group is required in order to have 80% power to detect at least 1.5 fold difference in change in PAV between the two group (α=0.05; CV=83%). Assuming a 20% loss to follow up rate by 54 weeks, we need to recruit 130 patients. TABLE 7 lists the power calculations for the primary end-point in Aim 1.

TABLE 7. 1° End-point			Fold-Change		
Outcome	Power	CV	1.5	2	2.5
PAV	80% 85% 90%	0.83 0.83 0.83	52 59 68	19 21 24	11 13 15

TABLE 8 provides the power for mean differences on reproducibility of central aortic BP and PWV from our own data.

TABLE 8. 2° Endpoints	SD	Change (Δ)		
Central Systolic BP (mm Hg)	5.4	5	7.5	10
Power		>90%	>90%	>90%
PWV (m/s)	1.4	0.8	1.0	1.2
Power		8782 %	>90%	>90%
HOMA-IR (units)	0.9	0.5	1.0	2.0
Power		8580 %	>90%	>90%

Analysis Plan: After distributions are checked and outliers are investigated for veracity, descriptive statistics by treatment arm will be reported for patient demographics and important potential confounding variables at baseline. These will include age, gender, BMI, baseline blood pressure, HbA1c, 24-hour urine Na, plasma and renin/aldo activity at baseline, HOMA-IR, HDL and non-HDL cholesterol. To analyze these data (primary end points and secondary outcome measures), linear mixed models with group (drug/placebo), baseline plaque volume, time points (before/after), stratification variables (age, high-risk feature) and other potential confounders (such as demographics and other comorbidities) as well as a patient random effects (to account for the correlation of measures within same patient) will be used. Different covariance structures will be used and best model will be selected based on AIC criteria by first fitting most complicated mean structure model, selecting the best covariance structure, then selecting the best mean structure, using SAS procedure GLIMMIX or MIXED) and convergence and model performance will be evaluated using SAS fit statistics (e.g. AIC criteria). Clinically relevant interactions will also be included in the models. Forward stepwise selection followed by backward elimination will be used to retain features with the greatest combined prediction power while maintaining parsimony using a training data set. Traditional methods, such as AIC and BIC involve a combinatorial optimization problem, which is NP-hard, with computational time increasing exponentially with the dimensionality. The expensive computational cost makes traditional procedures infeasible for high-dimensional data analysis. To overcome this, penalized likelihood maximization and penalized risk minimization have been intensively used for classification. Penalized methods with Lp-penalty, especially when $p \leq 1$, automatically perform variable selection by removing predictors with very small estimated coefficients. Fan et al. proposed SCAD-penalty with better oracle properties 131 . It automatically selects features and avoids excessive estimation bias compared to L1- penalty. We will use penalized methods for classification in order to avoid over-fitting problems by producing sparse solutions via feature selection.

Aim 2a: To evaluate the functional significance of candidate miRNAs.

Analysis Plan: An interaction contrast in an ANOVA model will be used to test the effects of overexpression of miRNAs (miR mimic) or antagomiRs in various experiments where we will test the effect of miRNAs alone or in the presence of LPS or IL4/13. The effect of various interventions on NR3C2 gene expression and MR protein expression as well as markers of alternate activation will be tested.

Aim 2b: To compare monocyte polarization and inflammatory gene expression at 6-weeks

Analysis Plan: Comparison of expression of these markers between groups will be done with ANOVA. Alterations in the percent change in monocyte subsets will be correlated with both the primary end-point (Δ PAV) and secondary end-points including change in HOMA-IR, SBP (central aortic) and PWV.

Aim 2c: To evaluate miRs expression to predict atherosclerosis and response

In an exploratory analysis, similar model selection strategy as in aim 1 will be applied which will include traditional risk factors. Based on the model we generated in aim 1, we will add miRNAs as additional factors (singly and in combination). Using this model, we will be able to test the effect of miRNA(s) at baseline on the rate of atherosclerosis progression and to identify patients who demonstrate greater response (e.g. decreases in PAV). The first half of patients (n=30 per group) will be used to build our model (training stage). The rest of the patients enrolled will be used to validate this model (validation stage). Features that are dropped from the model because of redundancies will be retained for further univariate testing in the validation data. Combining biological and clinical deliberations with statistical

results will likely generate a few alternative models with similar prediction accuracy. Cross-validation will be used to evaluate a classifier's prediction accuracy and for choosing the best tuning parameters. The classifier's prediction accuracy will be the main objective of this aim. For sample size calculations, we use the method described by Dobbin et al. 183, 184, which is based on the assumption that the expected accuracy of the classifier is controlled within a percentage (tolerance) of the best possible classifier. Assuming a maximum tolerance of 5% (and at most a 2-fold difference in the differentially expressed miRNAs (assuming a median SD of 0.71), a sample size of n=60 total (training and validation sets) is required. Tolerance represents the difference in the probability of correct classification in the training sample with respect to the same probability from an optimal signature. This sample size does not guarantee that there will be differentially expressed miRNAs. Instead, this sample size will be enough to validate a differentially expressed miRNAs, if such miRNAs is found in the data. Baseline miRNAs will also be correlated with markers of alternate activation (CD206+ and CD163+ monocytes) and MR gene/protein expression.

DATA COLLECTION, RETENTION AND MONITORING

Data Collection Instruments

The Investigator will prepare and maintain adequate and accurate source documents designed to record all observations and other pertinent data for each subject treated with the study drug. Study personnel at each site will enter data from source documents corresponding to a subject's visit into the protocol-specific electronic Case Report Form (eCRF) OR paper CRF when the information corresponding to that visit is available. Subjects will not be identified by name in the study database or on any study documents but will be identified by a subject number.

Data Management Procedures

The data will be entered into a validated database. The Data Management group will be responsible for data processing, in accordance with procedural documentation. Database lock will occur once quality assurance procedures have been completed. All procedures for the handling and analysis of data will be conducted using good computing practices meeting FDA guidelines for the handling and analysis of data for clinical trials.

Archival of Data

The database is safeguarded against unauthorized access by established security procedures; appropriate backup copies of the database and related software files will be maintained. Databases are backed up by the database administrator in conjunction with any updates or changes to the database. At critical junctures of the protocol (e.g., production of interim reports and final reports), data for analysis is locked and cleaned per established procedures.

Availability and Retention of Investigational Records

The Investigator must make study data accessible to the monitor, other authorized representatives of the Sponsor (or designee), IRB/IEC, and Regulatory Agency (e.g., FDA) inspectors upon request. A file for each subject must be maintained that includes the signed Informed Consent, HIPAA Authorization and copies of all source documentation related to that subject. The Investigator must ensure the reliability and availability of source documents from which the information on the CRF was derived.

Subject Confidentiality

In order to maintain subject confidentiality, only a subject number will identify all study subjects on CRFs and other documentation submitted to the Sponsor.

ADMINISTRATIVE, ETHICAL, REGULATORY CONSIDERATIONS

The study will be conducted according to the Declaration of Helsinki, Protection of Human Volunteers (21 CFR 50), Institutional Review Boards (21 CFR 56), and Obligations of Clinical Investigators (21 CFR 312). To maintain confidentiality, all laboratory specimens, evaluation forms, reports and other records will be identified by a coded number only. All study records will be kept in a locked file cabinet and code sheets linking a patient's name to a patient identification number will be stored separately in another locked file cabinet. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by the FDA. The Investigator must also comply with all applicable privacy regulations (e.g., Health Insurance Portability and Accountability Act of 1996, EU Data Protection Directive 95/46/EC).

Informed Consent Form

Informed consent will be obtained in accordance with the Declaration of Helsinki, ICH GCP, US Code of Federal Regulations for Protection of Human Subjects (21 CFR 50.25[a,b], CFR 50.27, and CFR Part 56, Subpart A), the Health Insurance Portability and Accountability Act (HIPAA, if applicable), and local regulations. A properly executed, written, informed consent will be obtained from each subject prior to entering the subject into the trial. Subjects will be given ample opportunity to inquire about details of the study. If a subject is unable to sign the informed consent form and the HIPAA authorization, a legal representative may sign for the subject. A copy of the signed consent form will be given to the subject or legal representative of the subject and the original will be maintained with the subject's records.

Publications

The preparation and submittal for publication of manuscripts containing the study results shall be in accordance with a process determined by mutual written agreement with the study Sponsor. The publication or presentation of any study results shall comply with all applicable privacy laws, including, but not limited to, the Health Insurance Portability and Accountability Act of 1996.

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