CLINICAL STUDY PROTOCOL

Interventional Drug or Biologic

Mechanisms Underlying Hypotensive Response to ARB/NEP Inhibition – Aim 3

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Synopsis

Primary Objective The primary objective is to test the hypothesis that endogenous substance P contributes to effects of ARB/NEP inhibition on blood pressure, natriuresis, and diuresis at initiation.
Secondary Objective (if applicable) The secondary objective is to test the hypothesis endogenous substance P contributes to effects of ARB/NEP inhibition on blood pressure, natriuresis, and diuresis after up-titration.
Study Duration For an individual participant, the duration of the study from recruitment to completion is approximately 49 days. We expect to enroll 80 participants over five years.
Study Design This is a double-blind, randomized, two x two crossover (aprepitant vs placebo) during both initiation of Entresto, LCZ696, (50 mg dose) and at steady-state of Entresto (200 mg bid dose or the highest tolerated dose).
Number of Study Sites One – Yale
Study Population We will study patients with an EF ≤40% and a history of New York Heart Association (NYHA) class I through III HF at initiation and up-titration of Entresto. Subjects must have been treated with a stable dose of an ACEi or an ARB for at least four weeks before screening, and with a stable dose of a beta blocker (unless contraindicated or not tolerated). Patients with class II or III HF with an EF ≤ 35% should also be on a stable dose of an MR antagonist for at least four weeks unless precluded due to renal function or adverse reaction.
Number of Participants 80
Primary Outcome Variables Primary outcomes will be maximal change in mean arterial pressure (MAP) and urine sodium excretion following study drug.
Secondary and Exploratory Outcome Variables (if applicable) Secondary outcomes will include change in HR, urine volume excretion, RPF, GFR, and urine albumin/creatinine ratio. Biomarkers include plasma cGMP, urine cGMP, BNP, BNP/NT-proBNP ratio, ANP, NEP activity in neutrophils, bradykinin, endothelin, Ang II, and other peptides related to neprilysin inhibition, PRC, aldosterone, PAI-1, and t-PA.

Abbreviations

Abbreviation	Explanation
ACE	Angiotensin-converting enzyme
Ang	Angiotensin
ANP	Atrial natriuretic peptide
APP	Aminopeptidase P
ARB	Angiotensin receptor blocker
AT1R	Angiotensin receptor type 1
BNP	Brain natriuretic peptide
CAD	Coronary artery disease
cGMP	Cyclic guanine monophosphate
CNP	C-natriuretic peptide
DPP4	Dipeptidyl peptidase-4
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
eGFR	Estimated glomerular filtration rate
HF	Heart failure
HR	Heart rate
MAP	Mean arterial pressure

MR	Mineralocorticoid receptor
NEP	Neutral endopeptidase or neprilysin
NK	Neurokinin
PAH	Para-aminohippurate
PAI-1	Plasminogen activator inhibitor-1
PARADIGM	Prospective Comparison of ARNi with ACEi to Determine Impact on Global Mortality and Morbidity in Heart Failure
PRC	Plasma renin concentration
RAAS	Renin-angiotensin-aldosterone system
T-PA	Tissue-type plasminogen activator

Glossary of Terms

Glossary	Explanation

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1 Introduction

1.1 Introductory Statement

This document is a protocol for a human research study. The purpose of this protocol is to ensure that this study is to be conducted according to ICH GCP guidelines, and according to CFR 21 Part 312, other applicable government regulations and Institutional research policies and procedures.

2 Background

2.1.1 Preclinical Experience

In 2015, the FDA approved LCZ696, Entresto, a molecular complex of the ARB valsartan with an inhibitor of neprilysin (NEP, neutral endopeptidase-24.11) sacubitril, after the PARADIGM-HF (Prospective Comparison of ARNi with ACEi to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial demonstrated a mortality benefit over enalapril in patients with stable HF and reduced EF, and elevated circulating brain natriuretic peptide (BNP) or N-terminal pro-BNP (NT-proBNP) concentrations.(1, 2) The PIONEER-HF study further demonstrated the efficacy of Entresto in preventing rehospitalization in patients with acutely decompensated HF.(3) The combined ARB/NEPi targets at least two pathophysiological mechanisms underlying HF - activation of the renin-angiotensin-aldosterone system (RAAS) and decreased sensitivity to the natriuretic peptides.

Understanding mechanism(s) underlying hypotensive and renal effects of Entresto, particularly at initiation, could lead to strategies to decrease adverse effects. In addition to degrading natriuretic peptides, NEP degrades other peptides like angiotensins (Ang) I and II, endothelins, bradykinin, and substance P (**Figure 1**).(4, 5) Among these, bradykinin and substance P cause vasodilation, increase vascular permeability, and stimulate tissue plasminogen activator (t-PA) release. Bradykinin and substance P cause natriuresis and diuresis.(6-11) Bradykinin stimulates release of substance P from sensory fibers via its B_2 receptor.(12) Thus, bradykinin and substance P could contribute to hypotension as well as beneficial effects in those treated with Entresto. Also, substance P and BNP are substrates of dipeptidyl peptidase-4 (DPP4), target of the anti-diabetic DPP4i, creating the possibility that drug-drug interactions could promote hypotension in patients with HF and diabetes.

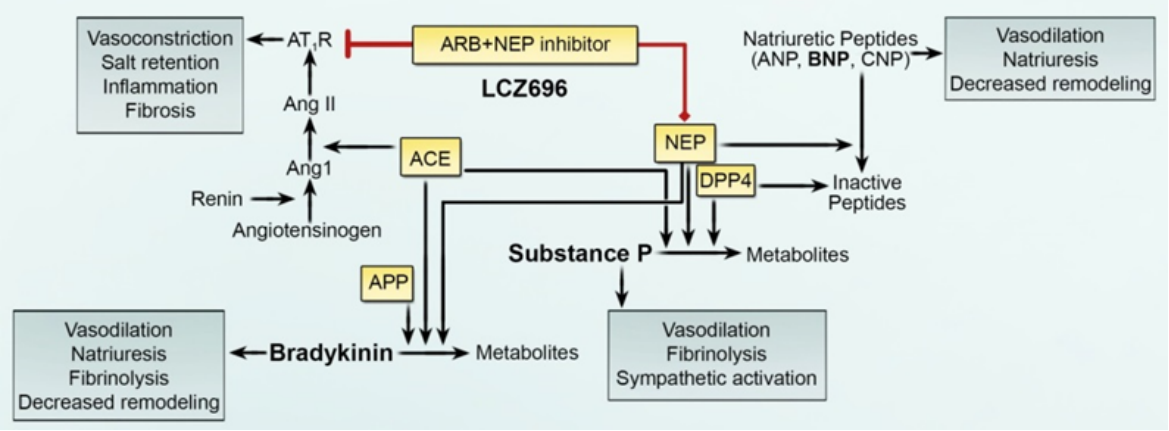


Figure 1: LCZ696 inhibits neprilysin (NEP) and blocks the angiotensin type 1 receptor (AT1R). Ang I indicates angiotensin I; Ang II, angiotensin II; ARB, angiotensin receptor blocker; ACE, angiotensin-converting enzyme; APP, aminopeptidase P; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DPP4, dipeptidyl peptidase-4

NEP cleaves other vasodilatory peptides such as bradykinin and substance P. In fact, the catalytic efficiency of NEP for bradykinin and substance P (Kcat/Km 69 and 158.7 min-1 μM-1, respectively)(13) is significantly higher than for ANP, BNP, and CNP (5.1, 0.53, and 7.58 min-1 μM-1).(14) In resistance arteries from patients with CAD, captopril, thiorphan, and omapatrilat all potentiated the vasodilator response to bradykinin.(15) The response to substance P was not potentiated, but NEP inhibition enhances effects of substance P in human vascular smooth muscle cells.(16) Substance P is dramatically increased in EDTA plasma from Entresto-treated HF patients.(17) (*Ex vivo* formation cannot be excluded without more robust inhibitors.) NEPis affect other peptides such as angiotensins, endothelin, vasoactive

intestinal peptide, adrenomedullin, and amyloid β peptide.(18) Patients given Entresto should not be taking an ACEi due the risk of angioedema, but could be taking a DPP4i for treatment of diabetes. There is potential for interactive effects of NEP and DPP4 inhibition on degradation of substance P and BNP (**Figure 2**).

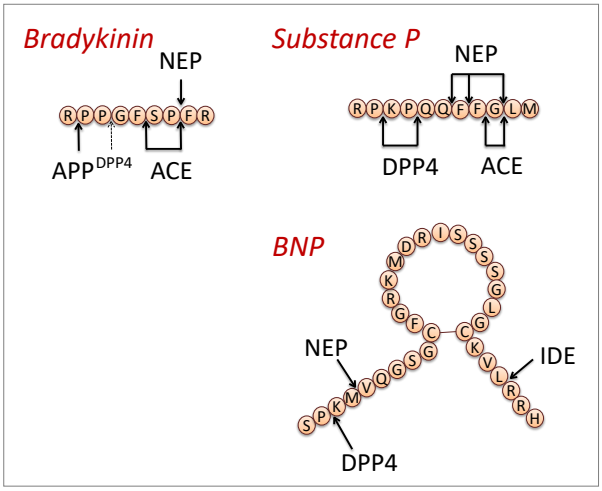


Figure 2: Cleavage of bradykinin, substance P, and brain natriuretic peptide (BNP) by neprilysin (NEP), angiotensin-converting enzyme (ACE), aminopeptidase P (APP), dipeptidyl peptidase-4 (DPP4), and insulin-degrading enzyme (IDE). The dotted arrow indicates that bradykinin must be cleaved by (and is inactivated by) APP before it is a DPP4 substrate

2.1.2 Clinical Experience

Heart failure (HF) affects approximately 5.7 million people in the United States, and the number is expected to grow to more than eight million by 2030.(19) Angiotensin-converting enzyme inhibitors (ACEi), angiotensin AT_1 receptor blockers (ARBs), beta blockers, and mineralocorticoid receptor (MR) antagonists reduce mortality and hospitalizations in patients with HF with reduced ejection fraction (EF).(20-24) Still mortality from HF remains high; HF causes over one million hospitalizations annually and contributes to two million more.(19)

In 2015, the FDA approved Entresto, a molecular complex of the ARB valsartan with an inhibitor of neprilysin (NEP, neutral endopeptidase-24.11) sacubitril, after the PARADIGM-HF (Prospective Comparison of ARNi with ACEi to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial demonstrated a mortality benefit over enalapril in patients with stable HF and reduced EF, and elevated circulating brain natriuretic peptide (BNP) or N-terminal pro-BNP (NT-proBNP) concentrations.(1, 2) The PIONEER-HF study further demonstrated the efficacy of Entresto in preventing rehospitalization in patients with acutely decompensated HF.(3)

One strategy to reduce hypotension following initiation of sacubitril/valsartan is to increase the dose gradually. The TITRATION study used such approach and serves as the basis for the dose of sacubitril/valsartan administered in the present study. In TITRATION, 92% of patients who entered the study and received the 50 mg dose of Entresto went on to the escalation phase.(25) In TITRATION, 84.3% were able to achieve and maintain the 200 mg bid regimen without dose interruption in the conservative arm.(25)

2.2 Background/prevalence of research topic

The lifetime risk of developing HF in the United States exceeds 20%.(19) Despite beneficial effects of ARBs, ACEis, beta blockers, and MR antagonists on mortality, five-year life expectancy of a patient with HF is 50%.(19) The combined ARB/NEPi Entresto was FDA-approved after PARADIGM-HF showed a beneficial effect on mortality versus enalapril in patients with HF and reduced EF.(2, 19) Entresto reduced a post hoc composite endpoint of coronary artery disease and improved glycemic control in diabetics.(26, 27) Entresto is in trials for HF with preserved EF. Nevertheless, Entresto has been underutilized in clinical practice given current guidelines.(28)

Effects of combined ARB/NEP inhibition may result from potentiation of vasodilatory, natriuretic, sympatholytic, antiproliferative, and antihypertrophic effects of natriuretic peptides (Figure 1).(29) In HF, decreased sensitivity to natriuretic peptides results from altered processing of proBNP to BNP, as well as altered clearance.(30-32) Entresto decreases NT-proBNP, but increases BNP,(1, 33, 34) providing indirect evidence that BNP degradation is decreased. Commercial, ***immunologic*** assays cross-react with degradation fragments, however, ***decreasing scientific rigor***.(35-37) We have developed a mass spectrometric assay specific for BNP1-32.

Decreased degradation of bradykinin and substance P by NEP could contribute to hypotension during initiation of ARB/NEPi therapy. Using the bradykinin B_2 receptor blocker icatibant, we and others have found that bradykinin contributes to favorable effects of ACEis on BP and fibrinolysis.(38-40) In Aim 2 being conducted at Vanderbilt, we are using icatibant to probe the contribution of endogenous bradykinin to the hemodynamic and renal effects of Entresto. Substance P also causes vasodilation and endothelial t-PA release, and a substance P (NK1) receptor antagonist blocks these effects.(41, 42) Substance P increases sympathetic activation,(41) an effect that may be harmful in HF patients.(43) We have used the FDA-approved NK1 receptor antagonist aprepitant to assess the contribution of substance P to effects of DPP4 inhibition and to explore the mechanism of pruritis in Sezary syndrome.(44) In Aim 3 we will use this strategy to investigate the contribution of substance P to effects of Entresto in patients with HF.

Bradykinin and substance P may contribute to angioedema during ARB/NEPi use. The risk of angioedema is three-fold higher during treatment with the ACE/NEPi omapatrilat than during ACEi alone,(45) due to simultaneous inhibition of two pathways degrading bradykinin and substance P.(46) Bradykinin is inactivated primarily by ACE, but also by NEP and aminopeptidase P (APP); substance P is inactivated by ACE, NEP, and DPP4 (**Figure 2**).(13, 47, 48) Supporting a role for NEP in the pathogenesis of angioedema, we have reported that a polymorphism in the gene encoding NEP is associated with ACEi-associated angioedema.(49) Entresto inhibits NEP without inhibiting ACE. In PARADIGM-HF, angioedema occurred in 0.45% of Entresto-treated patients compared to 0.23% (p=0.13) enalapril-treated patients.(1) Just 5% of patients in PARADIGM-HF were African American, a group at increased risk of ACEi- and NEPi-associated angioedema (and therefore a biologically relevant variable).(1, 45, 46, 50) In PIONEER-HF, 35.8% of patients were African American. 0.23% of Entresto- and 1.4% of enalapril-treated patients developed angioedema during only eight weeks of follow-up.(3) Concerns regarding angioedema may contribute to low adoption of Entresto, especially in African Americans.

3 Rationale/Significance

3.1 Problem Statement

Despite the beneficial effect of ARB/NEPi on mortality in patients with HF, the mechanism(s) underlying the beneficial or side effects of the ARB/NEPi are not understood. NEP degrades several vasodilator peptides including substance P. Here we test the hypothesis that endogenous substance P contributes to the effects of sacubitril/valsartan on blood pressure, natriuresis, and diuresis at initiation or after escalation to a steady-state dose.

3.2 Purpose of Study/Potential Impact

The prevalence of HF is increasing as our population ages, and mortality remains unacceptably high. ARB/NEPi reduces mortality compared to enalapril but concerns about side effects such as hypotension at initiation have limited use. Based on the prevalence of HF, labelled indications for Entresto, and the number needed to treat (NNT) to prevent one death, it is estimated that 2,287,296 HF patients are candidates for Entresto, with the potential to prevent 28,484 deaths per year.(51) Yet, a small fraction of eligible HF patients are treated with Entresto despite improved reimbursement.(52, 53) Concerns about hypotension, renal insufficiency, angioedema still limit use. Understanding mechanism(s) underlying hypotensive and renal effects of Entresto, particularly at initiation, could lead to strategies to decrease adverse effects.

3.2.1 Potential Risks

Potential Risks

1. Frequent blood draws can lead to anemia.
2. Collecting all of their urine in a jug for 24 hours and spending study days at the CSRU can be inconvenient for study subjects.
3. Entresto (0.45%) may cause angioedema, leading to swelling of the tongue, lips, or pharynx that can result in airway compromise. We will exclude patients with a history of angioedema.
4. Entresto can cause hypotension or orthostasis. The purpose of this study is to determine the mechanism of hypotension. We are using a conservative up-titration protocol used in the TITRATION study.(25) In the conservative arm, 5.2% developed a SBP <95 mmHg.
5. During the PARADIGM-HF trial, 2.2% of Entresto-treated patients had a decline in renal function, defined as end-stage renal disease or a decrease of 50% or more in the estimated glomerular filtration rate (eGFR) or a decrease of eGFR of more than 30 ml/min/1.73 m^2, to less than 60 ml/min/1.73 m^2 with a median follow-up of 27 months.
6. Withdrawal of a patient's angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) for 48 hours prior to study could lead to decompensation. However, the duration of withdrawal is compatible with label recommendations of at least 36 hours.
7. Entresto could cause hyperkalemia in patients with renal dysfunction. We are excluding patients with an eGFR less than 30 mL/min/1.73 m^2. We will monitor potassium during the study. If potassium is >5.2 mmol/L, we will repeat the blood sample. If potassium is persistently >5.2 mmol/L we will withdraw the subject from the study. In the conservative arm of TITRATION, the incidence of serum potassium >5.5mmol/L was 4%.
8. Para-aminohippurate (PAH) has been reported to cause flushing rarely. We have never observed this.
9. Iohexol can cause renal injury, particularly in patients with renal dysfunction and multiple myeloma. The dose used for measurement of GFR (5 mL) is significantly lower than that

used for CT scanning (80-180 mL) or for coronary procedures (e.g. 40 mL for a ventriculogram), and has proven safe for repeated measurements of GFR.(54) Iohexol rarely (1/2891 or 0.03%) causes hypersensitivity reactions when administered according to the protocol to be used in this study.(55)

10. The NK1 receptor antagonist aprepitant can rarely cause weakness, fatigue, nausea, constipation, hypotension, dizziness, dehydration, diarrhea, dyspepsia, hiccups, or impaired liver function. We have not observed this in our ongoing study in which we are giving aprepitant.
11. Aprepitant is a CYP3A4 inhibitor. In this study, we do not expect this to be an issue because participants receive a single dose of aprepitant on two separate occasions separated by approximately six weeks. No women participants will be taking oral contraceptives by the eligibility criteria.

Protections against Risk

1. Subjects with a hematocrit less than 35% will be excluded from study.

2. We will exclude patients with a history of angioedema. We have significant experience in studying patients with ACEis, dipeptidyl peptidase-4 inhibitors, and even an ACEi/neprilysin (NEP)i, as well as in treating patients with ACEi-associated angioedema. Should a patient exhibit angioedema, the offending medication will be promptly discontinued. Treatment of angioedema may include glucocorticoids and antihistamines with escalation to additional treatments (i.e. icatibant) as deemed necessary. (56)

3. Participants will receive their first and second doses of Entresto at the CSRU and we will monitor their blood pressure (BP) and heart rate for six hours after dosing. We will then monitor home BPs at each dose level. Prior to escalation of a dose, participants will report to the CSRU. We will take a history for adverse events, measure BP, potassium and creatinine.

6. We are determining eGFR at escalation visits. If a patient develops a decline in eGFR of 35% or a decrease in eGFR below 30 ml/min/1.73m^2, regardless of the magnitude of the decline, that patient's Entresto dose will be reduced to the highest tolerated dose. If the participant meets this eGFR criterion at the 50 mg bid dose, the participant will be discontinued. We will also measure GFR on each study day.

7. We will check serum potassium at screening, at initiation of study drug, and at each escalation visit. If potassium is >5.2 mmol/L, we will repeat the blood sample. If potassium is persistently >5.2 mmol/L, we will reduce Entresto to the highest tolerated dose. If the participant meets this criterion at the 50 mg bid dose, the participant will be discontinued. We will also measure potassium on each study day.

8. We will provide home BP cuffs and measure BP at each escalation visit. In patients who have a systolic BP (SBP) <95 mmHg or symptoms of hypotension or orthostasis, we will reduce their Entresto to the highest tolerated dose. If the participant meets this criterion at the 50 mg bid, he or she may be discontinued. The investigators may also make the clinical judgement to adjust diuretics since the study design allows for within-subject comparison of study day 3 and 4.

9. An investigator will be present during infusion of PAH and iohexol.

10. We are excluding patients with an eGFR less than 30 mL/min/1.73 m^2, as well as patients with hematological disorders, significantly reducing potential adverse effects of iohexol.

11. We will exclude any patients with a history of hypersensitivity reaction to contrast. Should any patient develop symptoms of hypersensitivity to iohexol we will treat them immediately with glucocorticoids and anti-histamines as appropriate and discontinue the study.

3.2.2 Potential Benefits

Individuals who participate in the studies will undergo much more detailed monitoring than might normally be done during initiation and up-titration of Entresto in a non-study setting. In addition, participants will receive study medication for eight weeks and laboratory examinations at no cost.

HF affects approximately 5.7 million people in the United States, and the number is projected to grow to more than eight million by 2030.(19) ACEis, ARBs, beta blockers, and MR antagonists reduce mortality in patients with HF and reduced ejection fraction (EF).(20-24) Despite the availability of these treatments, mortality from HF remains high.(19) In 2015, the first combined ARB/NEPi was approved for the treatment of HF with reduced EF. While this drug, Entresto, reduces mortality compared to enalapril,(1) a small fraction of eligible patients are treated with Entresto. Concerns about hypotension, renal insufficiency, and angioedema have limited use. Understanding whether endogenous substance P contributes to effects of Entresto on BP and renal function at initiation and up-titration can lead to new strategies to avoid excess vasodilation and hypotension or renal injury, while maximizing beneficial effects.

4 Study Objectives

4.1 Hypothesis

This study tests the hypothesis that endogenous substance P contributes to effects of ARB/NEP inhibition on blood pressure, catecholamines, natriuresis, and diuresis. We will use the specific neurokinin (NK)1 receptor antagonist aprepitant to examine the contribution of endogenous substance P to effects of Entresto.

4.2 Primary Objective

The primary objective is to test the hypothesis that endogenous substance P contributes to effects of ARB/NEP inhibition on blood pressure, natriuresis, and diuresis at initiation.

4.3 Secondary Objectives (if applicable)

The secondary objective is to test the hypothesis endogenous substance P contributes to effects of ARB/NEP inhibition on blood pressure, natriuresis, and diuresis after up-titration.

4.4 Exploratory Objectives (if applicable)

As an exploratory objective we will use the SomaScan (SomoLogic) platform to test the hypothesis that neprilysin inhibition alters levels of substrates such as BNP, ANP, endothelin, substance P, Ang II, and endorphins as well as unknown neprilysin substrates.

5 Study Design

5.1 General Design Description

Rationale

Substance P is one of the most efficient substrates for NEP.(13) Like bradykinin, substance P causes vasodilation and endothelial t-PA release.(41, 57) Data from our group and others indicate that substance P also increases HR and catecholamines.(41, 58, 59) Whereas responses to natriuretic peptides are impaired in HF, the vascular effects of substance P are preserved in HF.(60) In this Aim, we will use aprepitant to explore the contribution of endogenous substance P to the effects of ARB/NEPi at initiation and after up-titration in HF.

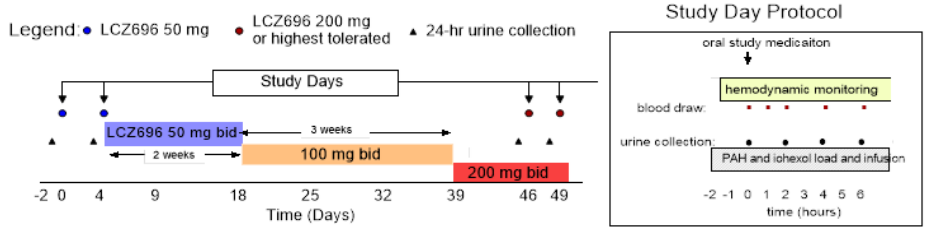
Subjects

We will study 80 patients (50% female, 50% Black) with an EF ≤40% and a history of New York Heart Association (NYHA) class I through III HF at initiation and up-titration of Entresto. Patients with a history of ACEi-associated angioedema will be excluded. Subjects must have been treated with a stable dose of an ACEi or an ARB for at least four weeks before screening, and with a stable dose of a beta blocker (unless contraindicated or not tolerated). Patients with class II or III HF should also be on a stable dose of an MR antagonist for at least four weeks unless precluded due to renal function or associated adverse reaction. Patients with an estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2 will be excluded. Women of child-bearing potential will be excluded. Detailed inclusion and exclusion criteria appear under **5.2.3.**

General Protocol

The Figure illustrates the randomized, double-blind (aprepitant versus placebo), cross-over design study protocol. After informed consent is obtained, subjects will undergo a screening history and physical examination. Upon enrollment, the subject's primary care physician or cardiologist will be notified via letter informing them of their enrollment into the study. Subjects will be asked to carry a wallet card noting their medications and risk of developing bradykinin-mediated angioedema. Subjects will collect their urine for 24 hours before each study day for measurement of volume, sodium and potassium. At the start of the study, subjects will discontinue their regular ACEi or ARB 48 hours prior to the first study day. Subjects will report to the CSRU for a study day, during which they will be given Entresto 50 mg and randomized to the NK1 receptor antagonist aprepitant or matching placebo. After a 96-hr washout period, subjects will again report to the CSRU for a second study day during which they will be given Entresto 50 mg and the opposite study drug [aprepitant or placebo]. Based on prior studies, we know that a 48-hr washout of prior ACE inhibition is sufficient to see an increase in ACE activity to baseline and to detect a decrease

Figure: Study protocol. Dose escalation follows the conservative protocol in TITRATION. Participants who do not meet criteria for dose escalation will be continued on and studied at the highest-tolerated dose.



in endogenous bradykinin to baseline.(61) Based on prior pharmacokinetic studies in HF, we expect 96 hrs between study day 1 and 2 to be sufficient for complete washout of valsartan ($t_{1/2}$ 13.7 hrs in HF during Entresto 200 mg bid), sacubitril ($t_{1/2}$ 3.9 hrs) and sacubitrilat ($t_{1/2}$ 18.4 hrs in HF during 200 mg bid).(62)

After completion of the two acute study days, subjects will take Entresto 50 mg bid for two weeks, followed by Entresto 100 mg bid for three weeks, and then Entresto 200 mg bid, following the conservative up-titration protocol from the TITRATION study.(25) During this period, subjects will be given a home BP cuff and log and instructed in the measurement of their BP. At the end of each dose titration, subjects will report to the CSRU for measurement of supine and standing BP, serum potassium and creatinine, and review of their home BP log. Subjects with a potassium ≤5.2 mmol/L, eGFR ≥30 ml/min/1.73M^2 and decrease in eGFR ≤35% compared to screening, SBP ≥95 mmHg without symptoms of hypotension or postural symptoms will continue with dose escalation. Subjects who fail to meet these criteria will be continued on the highest tolerated dose for the remainder of the study. On the 7th and 10th day of the 200 mg bid or highest tolerated dose, subjects will again undergo two more study days in which they are randomized to receive either aprepitant or placebo. Subjects will collect their urine every 24 hrs on days 6 and 9 of their highest tolerated dose for measurement of volume, sodium, potassium, and creatinine.

Study Day Protocol

On the morning of the study day, subjects will report to the CSRU at ~0700 in the fasting state. Subjects will be instructed to take any of their usual morning medications except Entresto prior to coming to the CSRU. At the CSRU, we will ask subjects to void, and we will weigh them. We will place two intravenous catheters, one for drug administration and one for sampling. Subjects will then remain in the supine position except as noted and will be provided a urinal or bed pan. We will measure BP and heart rate (HR) every fifteen min throughout the study using an oscillometric recording device (Dinamap, Critikon, Carlsbad, CA). Following a 30-min rest period, we will administer para-aminohippurate (PAH, 8 mg per kg body weight loading dose followed by a 12 mg/min steady-state infusion) and iohexol (5 mL Omnipaque 300, corresponding to 3.235 g of iohexol) to assess RPF and GFR,(54, 55) respectively. Ninety minutes later, immediately following the BP measurement, we will collect blood samples for measurement of BNP, ANP, ACE activity, t-PA, plasminogen activator inhibitor-1 (PAI-1), NEP activity, sodium, potassium, creatinine, PAH, iohexol, norepinephrine, cGMP, aldosterone, plasma renin concentration (PRC), endothelin, and NT-proBNP. We will also collect urine sample for β-HCG, sodium, potassium, creatinine, albumin, and cGMP.

We will then give Entresto (time 0) with 30 mL water. At the same time, we will give aprepitant or placebo (double-blind). We will measure BP and HR for six hours after study drug. We will measure sitting and standing BP and HR for six hours after study drug. Valsartan concentrations peak at 1.6-4.9 hours when given as Entresto, and concentrations of the active metabolite of sacubitril LBQ657 peak within three hrs.(63) At 1 and 2 hours after administration of study drug, we will collect blood for measurement of BNP, ANP, sodium, potassium, creatinine, PAH, and iohexol. We will also collect urine for sodium, potassium, creatinine, and cGMP. At 2 hours after administration, we will collect blood samples for measurement of NT-proBNP. At 4 hours after administration of study drug, we will collect blood for measurement of BNP, ANP, tPA, PAI-1, NEP activity, sodium, potassium, creatinine, PAH, iohexol, cGMP, aldosterone, PRC, endothelin, and NT-proBNP. We will also collect urine for sodium, potassium, creatinine, and cGMP. At 6 hours after administration of study drug, we will collect blood for measurement of BNP, ANP, sodium, potassium, creatinine, PAH, iohexol, and NT-proBNP. At times 0 and 6 hours, we will measure additional peptides that might be affected by neprilysin inhibition on the same blood samples. We will also collect urine for measurement of sodium, potassium, creatinine, and cGMP.

In multiple-dose studies of Entresto in healthy volunteers, increases in plasma cGMP and PRC were maximal at four hrs.(63) We will calculate the fractional excretion (FE) of sodium as FENa = [Na]urine × [Cr]plasma × [Na]plasma−1 × [Cr]urine−1. Following completion of the 4th

study day, subjects will continue on Entresto as standard of care.

5.1.1 Study Date Range and Duration

The duration of the study from recruitment to completion is approximately 49 days. We expect to enroll 80 participants over four years.

5.1.2 Number of Study Sites

This Aim will be conducted only at Yale.

5.2 Outcome Variables

5.2.1 Primary Outcome Variables

Primary outcomes will be maximal change in MAP and urine sodium excretion following study drug.

5.2.2 Secondary Outcome Variables (if applicable)

Secondary outcomes will include change in HR, urine volume excretion, RPF, GFR, and urine albumin/creatinine ratio. Biomarkers include plasma cGMP, urine cGMP, BNP, BNP/NT-proBNP ratio, ANP, NEP activity in neutrophils, bradykinin, endothelin, Ang II, PRC, aldosterone, PAI-1, and t-PA.

5.2.3 Exploratory Outcome Variables (if applicable)

Exploratory variables include levels of other peptides that may be affected by neprilysin inhibition.

5.3 Study Population

We will study patients with an EF ≤40% and a history of New York Heart Association (NYHA) class I through III HF at initiation and up-titration of Entresto. Subjects must have been treated with a stable dose of an ACEi or an ARB for at least four weeks before screening, and with a stable dose of a beta blocker (unless contraindicated or not tolerated). Patients with class II or III HF should also be on a stable dose of an MR antagonist for at least four weeks unless precluded due to renal function or related adverse reaction. Detailed inclusion and exclusion criteria appear **5.3.2**.

5.3.1 Number of Participants

We will enroll 80 participants. We anticipate screening approximately 320 potential participants to enroll 80.

5.3.2 Eligibility Criteria/Vulnerable Populations

Inclusion criteria

1. Black and white men and women
2. Stable patients with a reduced ejection fraction (EF)
    a. EF ≤55%, and

   b. history of symptoms of New York Heart Association (NYHA) class I, II, or III heart failure (HF)
   c. stable clinical symptoms including no hospitalizations for the last three months, or one month if hospitalized only once for initial diagnosis of HF
   d. who are not already taking Entresto
3. treatment with a stable dose of an angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) and with a beta blocker (unless contraindicated or not tolerated) for at least four weeks
4. for patients with NYHA Class II or III HF and EF ≤ 35%, treatment with a stable dose of a mineralocorticoid receptor (MR) antagonist for at least four weeks, unless not possible due to renal function or adverse reaction.
5. For female subjects, the following conditions must be met:
   a. postmenopausal status for at least one year
   b. status post-surgical sterilization
   c. or if childbearing potential, utilization of barrier methods of birth control or an oral contraceptive and willingness to undergo urine β-HCG testing on every study day
6. Age 18 years of age or older

Exclusion criteria

1. History of hypersensitivity or allergy to any of the study drugs, drugs of similar chemical classes, ACEi, ARBs, or neutral endopeptidase inhibitor (NEPi), as well as known or suspected contraindications to the study drugs
2. History of angioedema
3. History of decompensated HF within the last three months (exacerbation of chronic HF manifested by signs and symptoms that required intravenous therapy or hospitalization) or one month if hospitalized only once for initial diagnosis of HF
4. History of heart transplant or on a transplant list or with left ventricular assistance device
5. Symptomatic hypotension and/or a systolic blood pressure (SBP)<100 mmHg at screening or <95 mmHg during the study
6. Serum potassium >5.2 mmol/L at screening or during the study
7. Impaired renal function (eGFR of <30mL/min/1.73 m^2) as determined by the four-variable Modification of Diet in Renal Disease (MDRD) equation, where serum creatinine (Scr) is expressed in mg/dL and age in years:
   a. eGFR (mL/min/1.73 m^2)=175 • Scr-1.154 • age-0.203 • (1.212 if Black) • (0.742 if female)
8. Acute coronary syndrome, cardiac, carotid, or other major cardiovascular surgery, percutaneous coronary intervention, or carotid angioplasty within six months prior to screening
9. Coronary or carotid artery disease likely to require surgical or percutaneous intervention within six months of screening
10. History of serious neurologic disease such as cerebral hemorrhage, stroke, seizure, or transient ischemic attack with clinically significant residual deficits
11. History of ventricular arrhythmia with syncopal episodes
12. Symptomatic bradycardia or second- or third-degree atrioventricular block without a pacemaker
13. Presence of hemodynamically significant mitral and/or aortic valve disease, except mitral regurgitation secondary to LV dilatation

14. Presence of other hemodynamically significant obstructive lesions of the LV outflow tract, including aortic and subaortic stenosis
15. Type 1 diabetes
16. Poorly controlled type 2 diabetes mellitus (T2DM), defined as a HgbA1c >9%
17. In T2DM, dipeptidyl peptidase-4 inhibitor use for one month prior to enrollment will be excluded due to possible interaction with Entresto
18. Hematocrit <35%
19. Breast feeding and pregnancy
20. History or presence of immunological or hematological disorders
21. History of malignancy not felt to be cured, except non-melanoma skin cancer
22. Diagnosis of asthma requiring use of inhaled beta agonist more than once a week
23. History of hypersensitivity reaction to contrast
24. Clinically significant gastrointestinal impairment that could interfere with drug absorption
25. History of pancreatitis or known pancreatic lesions
26. Impaired hepatic function with evidence of advanced fibrosis or cirrhosis [stable aspartate amino transaminase (AST) and/or alanine amino transaminase (ALT) trend if >3.0 x upper limit of normal range as deemed of minimal clinical relevance by the investigators]
27. Any underlying or acute disease requiring regular medication which could possibly pose a threat to the subject or make implementation of the protocol or interpretation of the study results difficult, such as arthritis treated with non-steroidal anti-inflammatory drugs
28. Treatment with greater than 5 mg of prednisone or equivalent dose of chronic systemic glucocorticoid therapy within the last year or recent (within 6 weeks of first study day) treatment with burst dosed glucocorticoid therapy
29. Treatment with lithium salts
30. History of alcohol or drug abuse
31. Treatment with any investigational drug in the one month preceding the study
32. Mental conditions rendering the subject unable to understand the nature, scope, and possible consequences of the study
33. Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

6 Methods

6.1 Treatment

6.1.1 Identity of Investigational Product

FDA-approved drugs

Entresto (Valsartan/sacubitril) will be purchased from Novartis Pharmaceuticals (NDC# 0078-0696-20).

Aprepitant will be purchased from Merck and Co [National Drug Company (NDC) # 006-0461-06].

Iohexol 300 mg iodine/mL will be purchased from GE Healthcare Inc. (NDC# 0407-1413-95).

Placebo for aprepitant will contain microcrystalline cellulose [Professional Compounding Centers of America (PCCA) Part # 30-1130-500 GM]. Both aprepitant and the matching placebo will be encapsulated in #00 size green-opaque locking gelatin capsules (PCCA Part 30-3021-1000 EA).

Para-aminohippurate (PAH) 2 grams/10 mL will be purchased from Basic Pharma (NDC# 0006-3395-11).

6.1.2 Dosage, Administration, Schedule

Drugs will be prepared by Investigational Drug Services.

Sacubitril valsartan will be given as a single oral dose of 50 mg on each of the first two study days. Participants will then take 50 mg bid for two weeks, followed by 100 mg bid for three weeks, followed by 200 mg bid for ten days.

Aprepitant 120 mg or matching placebo will be given on study days 1 and 2 and again on study days 3 and 4.

Iohexol 3.235 g will be given once intravenously on each study day.

Para-aminohippurate will be given intravenously as a loading dose of 8 mg/kg followed by a steady-state infusion of 12 mg/min on each study day.

6.1.3 Method of Assignment/Randomization

Subjects will be randomized to one of the two treatment sequences (aprepitant placebo, or placebo aprepitant) for a two-by-two crossover design through a permuted-block randomization algorithm. Different randomization schedules will be generated for the initiation (study days 1 and 2) and the steady-state dose of Entresto (study days 3 and 4) studies.

6.1.4 Blinding and Procedures for Unblinding

A biostatistician not related to the study will provide the allocation schedules to the Investigational Drug Service (IDS).

6.1.5 Packaging/Labelling

IDS will devise standard operating procedures for the pharmacy to follow with regard to preparing, labeling, blinding, and dispensing study drug.

6.1.6 Storage Conditions

IDS will be responsible for the storage, preparation, and labeling of all investigational agents and for maintaining accurate drug storage and dispensing logs.

6.1.7 Concomitant therapy

The use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers other than the valsartan in sacubitril/valsartan is explicitly forbidden throughout this study.

Participants may take all other medications for heart failure as prescribed. It is not uncommon for diuretic doses to be reduced after initiation of sacubitril/valsartan.

6.1.8 Restrictions

There are no restrictions.

6.2 Assessments

6.2.1 Efficacy

We will measure BP and heart rate (HR) every fifteen min throughout the study using an oscillometric recording device (Intellivue MX400, Philips USA, Franklin, TN). Following a 30-min rest period, we will administer para-aminohippurate (PAH, 8 mg per kg body weight loading dose followed by a 12 mg/min steady-state infusion) and iohexol (5 mL Omnipaque 300, corresponding to 3.235 g of iohexol) to assess RPF and GFR,(54, 55) respectively.

Laboratory Analyses

cGMP will be measured by radioimmunoassay (RIA) (Perkin-Elmer, Waltham, MA). We will measure ACE (Olympus AU400/AU600, Alpco Diagnostics, Salem, NH) by kinetic analysis. We will measure NEP activity using a fluorometric substrate (Sensolyte® 520, AnaSpec, Freemont, CA) in blood polymorphonuclear granulocytes isolated using a Na diatrizoate/Dextran 500 density gradient (Lympholyte®-poly, Cedarlane, Burlington, NC).(64) BNP (Siemens, New York, NY), NT-proBNP (Roche Diagnostics, Indianapolis, IN), and ANP (RayBiotech, Norcross, GA) will be measured by immunoassay. We will also measure BNP by mass spectrometry (below). Iohexol will be measured by high-performance liquid chromatography (HPLC)(54) and PAH will be measured by colorimetric assay.(65) Creatinine will be measured by Jaffe method. Urine albumin will be measured by turbidimetric immunoassay with endpoint determination. We will measure PRC (DiaSorin, Stillwater, MN),(65) Ang II (drawn in protease inhibitors; Nichols Institute Diagnostic, San Clemente, CA),(66) and aldosterone [(MP Biomedicals, Irvine, CA) utilizing ^{125}I, with a primary antibody to aldosterone (NIDDK National Hormone and Peptide Program, Torrance, CA) and a secondary gamma globulin antibody (Linco Research, St. Charles, MO)](67) by chemiluminescent, immuno- or radioimmunoassay. We will measure endothelin by ELISA (R&D Systems, Inc., Minneapolis, MN). Blood for PAI-1 and t-PA will be collected in acidified 0.105M citrate (Becton Dickinson, Rutherford, NJ),(68, 69) and antigen assayed using a two-site ELISA (Diagnostica Stago, Inc. Parsippany, NJ).(69) Catecholamines will be quantified after batch alumina extraction by HPLC using electrochemical detection. Details of sample collection and assays appear in citations.

Peptide Measurement by Mass Spectrometry

Blood for bradykinin and substance P will be collected in plastic and added immediately to chilled ethanol (1:3 vol:vol) to denature peptidases.(70) After an hour at 4°C, samples will be centrifuged, and the supernatant stored at -80°C. The ethanolic supernatant will be prepared as described.(70) Bradykinin and substance P will be measured by ultraperformance LC-tandem MS (UPLC-ESI/MS/MS), using a modification of a prior assay.(70) For substance

P, we have achieved sensitivity by increasing the MS/MS working temperature to 325°C and using a 297°C heated ESI probe to reduce background noise, by utilizing a CHC_3N/H_2O/FA (5/94.9/0.1 to 30/69.9/0.1, v/v/v, over 7min) linear gradient to optimize separation, and by purifying ethanol-extracted blood with Nexus and C18 Sep-Pak cartridges before analysis. $^{13}C_6{}^{15}N_4$-substance P is internal standard (STD). Limit of detection is 0.05 pg. Assay is linear (r^2=0.995) from 0.1 pg to at least 50 pg. Coefficient of variation is 2.2%.

Blood (2 mL) for BNP will be collected into P-100 tubes (containing inhibitors, Becton Dickinson) mixed and immediately centrifuged at 3250 X g, 4°C for two min; 0.5 mL of supernatant (plasma) will be added to a low-protein-retention tube which contains 1.5 mL of 80% CH_3CN/H_20, 0.5% FA, 6 ng of internal STD ($^{13}C_{12}$,$^{15}N_8$-labeled BNP, CPC Scientific) in 20µL MeOH and 2 µL L-methionine (45mg/mL) solution. Pellets will be centrifuged at 16,000 X g, 4°C and removed. The supernatant will be flash frozen with liquid nitrogen and stored at -80°C until analysis.

For sample extraction and purification, 1.3 mL H_2O, 0.4 mL NH_4HCO_3 (1M), 2 µL L-methionine (45mg/mL) will be added and well mixed. The peptide will then be reduced and alkylated by adding 0.11 mL DTT (50 mM at 50°C for 20 min), and 0.12 mL Iodoacetamide (100 mM in dark at 37°C for 40 min). After the mixture is centrifuged, the supernatant will be passed through a 30,000 Da Amicon Ultra-4 filter (3250 X g at 4°C for 70 min), and purified (in 4°C cold room) using a 60 mg Nexus cartridge (Agilent), pre-conditioned with 3 mL MeOH, 5 mL H_2O and 3 mL KPi (10mM, pH 7.4). After the alkylated peptide is applied, the cartridge will be washed with 3 mL H_2O containing 45 µg/mL L-methionine, dried under a vacuum and the peptide eluded with 1.5 mL MeOH containing 0.1% HCl (1N) and 45 µg/mL L-methionine. The eluate will be kept at -80°C until analysis.

For LC/MS/MS analysis, the eluate will be evaporated in N_2 at room temperature and residue dissolved in 30 µL of 20% CH_2CN, 0.2% FA/H_2O. Samples will be centrifuged at 22,000 X g for two min to remove pellets before being resolved by UPLC (Waters) at 40°C with an Acquity BEH (C8) column (1.7µm, 1.0x50 mm) using a CH_3CN/H_2O/FA (5/94.8/0.2 to 30/69.8/0.2, v/v/v, over 5 min at flow 0.12 ml/min) linear gradient. The alkylated peptide will be quantified by positive ESI/MS/MS-SRM on a TSQ Vantage triple quadrupole mass spectrometer equipped with a heated ESI probe monitoring product ions: m/z 728.2 originating from m/z 597.7 (BNP) and m/z 734.0 originating from m/z 602.5 (internal STD). The ratio of BNP to internal STD is used for quantification. The limit of detection is 0.1 pg.

We will also use the SomaScan (SomaLogic) platform to measure peptides including BNP, NT-proBNP, endothelin, angiotensin II, endorphins, as well as other peptides that may be affected by neprilysin inhibition.

6.2.2 Safety and Pregnancy-related policy

We will provide home BP cuffs and measure BP at each escalation visit. Blood pressure (supine and standing), weight, potassium and creatinine will be assessed at each dose titration visit. Participants will also be questioned regarding symptoms such as dizziness, orthostatic dizziness, orthopnea, medication changes, symptoms since starting or increasing sacubitril/valsartan.

In patients who have a systolic BP (SBP) <95 mmHg (or evidence of a home pressure < 90 mmHg more than once in a week), symptoms of hypotension or orthostasis, we will reduce their Entresto to the highest tolerated dose. If the participant meets this criterion at the 50 mg bid, he or she may be discontinued. The investigators may also make the clinical judgement to adjust diuretics since the study design allows for within-subject comparison of study day 3 and 4.

We are determining eGFR at escalation visits. If a patient develops a decline in eGFR of 35% or a decrease in eGFR below 30 ml/min/1.73m2, regardless of the magnitude of the decline, that patient's Entresto dose will be reduced to the highest tolerated dose. If

the participant meets this eGFR criterion at the 50 mg bid dose, the participant will be discontinued. We will also measure GFR on each study day.

We will check serum potassium at screening, at initiation of study drug, and at each escalation visit. If potassium is >5.2 mmol/L, we will repeat the blood sample. If potassium is persistently >5.2 mmol/L, we will reduce Entresto to the highest tolerated dose. If the participant meets this criterion at the 50 mg bid dose, the participant will be discontinued. We will also measure potassium on each study day.

Women of child-bearing age will be encouraged to utilize barrier methods of birth control or an oral contraceptive. They will undergo urine β-HCG testing during each study visit.

6.2.3 Adverse Events Definition and Reporting

Participants will be monitored for adverse events from consent until the end of the last study day. Participants will be monitored for specific adverse events such as anemia, hypotension, hyperkalemia, and decrease in renal function as specified in the protocol. In addition, participants will be questioned about adverse events using a standardized form at each study visit, including medication escalation visits.

Any serious adverse event will be reported to the DSMC, IRB, and NIH as soon as possible, but not more than seven business days from the PI's notification of the event. Any deviation from the IRB-approved protocol that increases risk or affects the participant's rights, safety, or welfare will also be reported to the IRB within seven days. If warranted, appropriate changes will be made to the consent form. The DSMC may choose to become unblinded; however, it is expected that such unblinding would not occur without reasonable concern related either to patient safety or to data validity. The Committee will have the authority to modify the protocols or to terminate the study if it deems such actions to be warranted. No early stopping is planned in any Aim.

Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. Adverse events will be graded as mild (no limitation of usual activities), moderate (some limitation), or severe (inability to carry out usual activities) and attributed according to the relationship to the study drug and/or procedures as Not related, Unlikely, Possible, Probable, or Definite. An adverse event will be classified as serious if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect.

Non-serious, unexpected adverse events will be reported to the IRB at the time of annual review. Any instance of non-compliance with the protocol that does not cause an adverse event or change the risk/benefit ratio or data integrity will be reported at the time of annual review. Summary reports will be submitted to the IRB at least annually and will contain a) the number of adverse events and an explanation of how each event was handled, b) the number of complaints and how each complaint was handled, c) the number of subject withdrawals and an explanation of why the subject withdrew or was withdrawn, and d) the number of instances of non-compliance with the protocol and how each was handled.

Relevant to the IND for PAH, we will submit Safety Reports to FDA:

- 7-Calendar-Day FDA Telephone or Fax Report: Via the sponsor-investigator we will directly notify the FDA, within 7 calendar days after initial receipt of the information, of any adverse event that is fatal or life-threatening, unexpected, and considered at least possibly related to the investigational product.
- 15-Calendar-Day FDA Written Report: Via the sponsor-investigator we will directly notify the FDA within 15 calendar days after initial receipt of the information, of any

serious adverse event (other than those that are fatal or life-threatening) that is unexpected and considered at least possibly related to the investigational product.

Serious Adverse Events which do not meet the criteria for expedited reporting will be reported to the FDA in the IND Annual Report.

6.2.4 Pharmacokinetics (if applicable)

N/A

6.2.5 Biomarkers (if applicable)

We will measure BP and HR for six hrs after study drug on each study day. We will measure sitting and standing BP and HR four hrs after study drug.

We will collect urine for sodium, potassium, cGMP, albumin, and creatinine every two hrs and blood for PAH, iohexol, creatinine, cGMP, NT-proBNP, BNP, ANP, endothelin, PRC, Ang II, aldosterone, PAI-1, and t-PA at baseline and 1, 2, 4, and 6 hrs after study drug on each study day. At times 0 and 6 hours, we will use the SomaScan (SomoLogic) platform to measure some of these biomarkers such as endothelin and the natriuretic peptides, and to detect other peptides that may be altered by neprilysin inhibition. (Valsartan concentrations peak at 1.6-4.9 hrs when given as Entresto, and concentrations of the active metabolite of sacubitril LBQ657 peak within three hrs.(63) In multiple-dose studies of Entresto in healthy volunteers, increases in plasma cGMP and PRC were maximal at four hrs.(63))

We will calculate the fractional excretion (FE) of sodium as FENa = [Na]urine × [Cr]plasma × [Na]plasma−1 × [Cr]urine−1.

6.3 Study Procedures

Screening Visit (see form attached)

Consent

Demographics

Concomitant Medications

Inclusion and Exclusion Criteria

Physical Assessment

Past Medical History

Review of Systems

Screening laboratory

CMP, CBC, urinalysis with microscopic, and EKG

Provision to participant: urine collection supplies, study day schedule, copy consent form, instructions form (see attached), BP diary log and cuff (if needed)

Study Day

See form attached

Dose Escalation visit

See form attached

6.3.1 Study Schedule

There are seven expected visits including consent and screening (one), study day visits (four), and dose escalation visits (two). A tabular Schedule of Events appears below.

CLINICAL STUDY PROTOCOL

Interventional Drug or Biologic

Schedule of Events

	Screening	Day -2	Study Day 1 (Day 0)	Study Day 2 (Day 4)	Days 4 to 18	Dose escalation visit (day 18±2 days)	Days 18 to 39	Dose escalation visit (Day Days 39 to 49	Study Day 3 (Day 46±1	Study Day 4 (Day 49±1 day but 3 days after Study Day 3)
Informed consent	X									
History and physical exam	X									
Screening laboratory & ECG	X									
24-hour urine collection end			X	X					X	X
Last dose of reg ACEi or ARB		X								
Entresto 50 mg x1			X	X						
Randomized treatment with aprepitant or placebo			X	X					X	X
Home BP monitoring					X		X	X		
BP, potassium, creatinine (eGFR) measurement			X	X		X		X	X	X
Pill count						X		X	X	X
Review adverse events			X	X		X		X	X	X
Concomitant meds			X	X		X		X	X	X
Entresto 50 mg bid					X					
Entresto 100 mg bid or highest tolerated dose							X			
Entresto 200 mg bid or highest tolerated dose								X	X	X
ACE activity			X	X						
Continuous BP and HR monitoring			X	X					X	X
Urine sodium and volume			X	X					X	X
PAH and iohexol clearance			X	X					X	X
UACR			X	X					X	X
NEP activity, BNP, ANP, BNP/NT-proBNP, cGMP			X	X					X	X
Bradykinin, Ang II, endothelin, and other neprilysin substrates, PRC, aldosterone, PAI-1, t-PA			X	X					X	X

Pregnancy test (urine β-HCG)	X	X	X	X

APPROVED BY THE YALE UNIVERSITY IRB 9/5/2024

CLINICAL STUDY PROTOCOL

Interventional Drug or Biologic

6.3.2 Informed Consent

A research nurse or investigator will be available to talk with patients at the at clinics where a high number of potential subjects have been identified using the electronic medical record. In addition, written advertisements which have been approved by the Institutional Review Board (IRB) and which give the name and phone number of a contact research nurse will be placed on clinic bulletin boards. Potential participants who call for information will be given a brief description of the study protocol and, if interested, will be invited to the CSRU for more information.

Subjects must consent in writing prior to participation. Each subject will be given an IRB-approved consent form describing the study in language that is understandable to the subject, and which meets all federal, local, ICH and HIPAA requirements.

Informed consent will be obtained using methods that comply with all federal and local regulations. The PI or her designee will carefully review the study and the consent form with any prospective participant in a way that is understandable to that subject. We will review the purpose and scope of the study, all procedures and timelines, and potential consequences to the study. We will discuss alternatives to participation. We will emphasize that participation is completely voluntary and that the subject can withdraw at any time without penalty or loss of benefits. We will use teach back methods to ensure that the potential subject understands. The PI or her designee and the potential study subject will sign and date the consent form before the subject participates in any aspect of the study including screening. We will retain the original and give a copy to the study participant.

Should the consent form be amended during the study, we will use the amended consent for all new participants and re-consent all current subjects using the amended consent form.

6.3.3 Screening

After informed consent is obtained, subjects will undergo a screening history and physical examination. Screening laboratory including a comprehensive metabolic panel, urinalysis, CBC, and ECG will be obtained. If a prospective participant has not had an echocardiogram within the last six months, an echocardiogram will be obtained.

6.3.4 Enrollment

Enrollment will occur after participants have been consented, screened, and meet eligibility criteria. Participants will be notified and scheduled for the first study day based on clinic availability at the CSRU. If the first study day is delayed greater than 8 weeks, the participant will be required to be rescreened.

6.3.5 On Study Visits

See attached schedule.

6.3.6 End of Study and Follow-up

Following completion of the last study day, participants will be provided a prescription for the final dose of valsartan/sacubitril they tolerated. We will send a letter to the participant's HF

and primary care physicians documenting the final dose and any side effects that the participant may have had at higher doses. Should a participant withdraw prior to completion due to adverse effects we will share information about the adverse effects with the HF and primary care physicians. There are no plans to provide any additional information to participants. Should we discover an incidental health finding during screening or at any point during the study, however, we will ask permission to share that information with the participant's physician.

6.3.7 Removal of subjects

Participants may be withdrawn due to adverse events related to sacubitril/valsartan. We are specifically monitoring blood pressure, potassium and creatinine and will adhere to criteria for reducing the dose or if adverse events happen at the lowest dose, discontinuing sacubitril/valsartan. (See titration visit form.) Should subjects withdraw, we will continue them at the lowest tolerated dose of sacubitril/valsartan. If they do not tolerate sacubitril/valsartan, we will recommend that they reinstate their ACE inhibitor or angiotensin receptor blocker after 48 hours.

6.4 Statistical Method

6.4.1 Statistical Design

We have designed the study with a two x two crossover (aprepitant vs placebo) during both initiation of Entresto (50 mg dose) and at steady state of Entresto (200 mg bid dose or the highest tolerated dose). The primary analysis will focus on the maximal change in MAP and urine sodium excretion during aprepitant vs placebo comparison at the initiation dose. The same comparisons at the steady-state dose will be secondary.

We will use mixed-effects models with a random subject effect and with treatment (aprepitant vs placebo) as fixed effects. We will include gender, race, and baseline measures as covariates. In addition to evaluating the treatment effect using regression analyses, we will calculate within-subject mean differences and their 95% confidence intervals for the aprepitant vs placebo comparison and test for the treatment effect using paired t-test or sign rank test as appropriate. Although we have designed the study to avoid carryover effect based on our prior experience and other published studies, we will test for carryover effect using the T-test approach described in Section 2.3 (page 21) of Jones and Kenward.

If a carryover effect appears to exist, we will combine the four study days over the two doses of Entresto into one crossover study with four periods to model explicitly the potential carryover effect. We will then use a mixed-effects model to analyze the data with four treatment combinations (initiation Entresto+aprepitant, initiation Entresto+placebo, steady-state Entresto+aprepitant, and steady-state Entresto+placebo) over the four periods. We can then include the appropriate first-order carryover effect in the mixed-effects model. For example, if a subject receives treatments initiation Entresto+aprepitant (II) and initiation Entresto+placebo (IP) in periods 1 and 2, then the model has main effect II in period 1 and main effect IP and carryover effect CII in period 2. (CII denotes potential carryover effect due to II in the prior period.) Both the main effects and carryover effects can be estimated in the model (Chapter 5 of *Jones and Kenward*, 2003). The mixed-effects model will have a random subject effect and the four treatment combinations and carryovers as a fixed effect. We will use an autoregressive model of order 1 (AR1) or other plausible covariance structures for the error covariance. Mixed-effects models provide flexibility of adjusting for and of evaluating covariates, including sex, race, BNP, ANP, prior ACEi or ARB use, and their potential interactions with treatment. As noted earlier, dropouts prior to completion of both 50 mg Entresto study days will be replaced. Other missing data will be handled as in Aim 1.

6.4.2 Sample Size Considerations

MAP: In our prior study of the effect of icatibant (a bradykinin receptor antagonist) on the acute BP response to ACE inhibition, there was a 3.5±5.2 mmHg difference in the decrease in MAP in response to ACEi with (10.5±5.2) and without (14.0±5.2) icatibant.(38) We anticipate the effect of a substance P receptor antagonist on the response to sacubitril/valsartan would be similar. With 80 subjects we have 84% power to detect half this difference (1.75 mmHg) with an SD of the difference of 5.2. With 67 subjects we have 80% power to detect a difference of 1.81±5.2 mmHg and excellent (>95%) power to detect a difference of 3.5±5.2 mmHg.

Urine Na excretion: Little is published about the acute natriuretic or diuretic response to Entresto in HF patients. In a study of salt-sensitive hypertensive subjects, acute administration of Entresto 400 mg caused a 24.5±33 (mean±SD) mmol greater six-hr sodium excretion compared to 320 mg valsartan.(71) With 80 subjects we have 80% power to detect a difference of 10.5 mmol. With 67 subjects we have 80% power to detect a difference of 11.5 mmol with an SD of the difference of 33.

6.5 Planned Analyses

6.5.1 Primary Objective Analysis

We will measure blood pressure every 15 minutes throughout the study. We will determine the change in MAP as the change in BP from the time of sacubitril/valsartan administration to the nadir of MAP after drug administration. We will compare the change in MAP following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

We will collect urine for six hours after administration of sacubitril/valsartan. We will compare urine sodium excretion following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

6.5.2 Secondary Objectives Analyses

We will measure HR every 15 minutes throughout the study. We will determine the change in HR as the change in HR from the time of sacubitril/valsartan administration to the maximal HR after drug administration. We will compare the change in HR following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

We will collect urine for six hours after administration of sacubitril/valsartan. We will compare urine volume excretion following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

We will calculate RPF based on the steady-state concentration of PAH. We will calculate the change in RPF as the change from RPF at the time of sacubitril/valsartan to maximal RPF following drug administration. We will compare the change in RPF following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

We will measure GFR as the clearance of iohexol. We will compare GFR following acute sacubitril/valsartan during administration of aprepitant versus during administration of placebo (study days 1 and 2). We will make the same comparison after chronic treatment with sacubitril/valsartan (study days 3 and 4).

For each of the biomarkers, we do not expect to detect an effect of aprepitant, but measurement will either confirm the effect of drug administration (e.g. demonstrate an increase in PRC and a decrease in aldosterone due to valsartan) or provide new descriptive information on the time-course of the hormonal response to sacubitril/valsartan.

6.5.3 Exploratory Objectives Analyses (if applicable)

As an exploratory objective we will use the SomaScan (SomoLogic) platform to measure neprilysin substrates such as BNP, ANP, endothelin, angiotensin II and other peptides that may be affected by neprilysin inhibition. As noted above, we do not expect to see an effect of aprepitant on the biomarkers. The primary analyses will focus on peptide concentrations prior to and six hours after acute administration of sacubitril/valsartan and on baseline concentrations on days 1 and 2 versus after 12-week treatment with sacubitril valsartan on days 3 and 4 in samples collected in Aims 2 and 3.

6.5.4 Safety

As noted, we will assess BP, hematocrit, potassium, and creatinine at each titration visit. We will also assess symptoms, changes in diuretic use. Since this is a mechanistic study of an FDA-approved drug and all participants receive sacubitril/valsartan we are not assessing safety as a primary or secondary endpoint.

6.5.5 Analysis of Subject Characteristics

We will define the baseline characteristics of those studied to include age, self-defined race, gender, BMI, BP, HR ejection fraction, underlying conditions such as coronary artery disease, baseline medications, baseline laboratory.

6.5.6 Interim Analysis (if applicable)

No interim analysis is planned.

6.5.7 Health economic evaluation

Not applicable

6.5.8 Other

6.5.9 Subsets and Covariates

The detailed phenotyping in Aims 2 (being conducted at Vanderbilt) and Aim 3 (this protocol) provides a unique opportunity to assess patient factors that predict the hypotensive, natriuretic and diuretic responses to Entresto. Because the two Aims use the same protocol, we can combine data from the Entresto + placebo study days. Beneficial effects of Entresto were similar in men and women in PARADIGM but acute BP effects could differ.(1) Five percent of patients in PARADIGM and 35% in PIONEER-HF were Black.(1, 72) Blacks and women may be more sensitive to potentiation of bradykinin and downstream substance P.(73, 74) The complex neurohormonal adaptions to HF vary considerably among individuals. We hypothesize that baseline or change in BNP, ANP or NEP activity will predict hypotension or diuresis following Entresto.

6.5.10 Handling of Missing Data

Subjects who drop out prior to study day 2 will be replaced. For this reason and based on prior experience, missing data will be unusual. Nevertheless, if data are missing for a particular time point, mixed-effects models are robust in that subjects with missing data at some time points can be included to estimate effects of interest. In addition, we will conservatively impute missing data to perform corroborative analyses with and without missing data.

7 Trial Administration

7.1 Ethical Considerations: Informed Consent/Assent and HIPAA Authorization

This study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki.

This is a double-blind study. There is a placebo for the study drug that is used to probe mechanism (aprepitant) but not for the study drug (sacubitril/valsartan) that is given to heart failure patients. Participants will be advised of the use of placebo in the informed consent document.

Reimbursement will be provided to each participant for his or her time.

Sensitive data are not being collected as part of this study.

It is highly unlikely that any previously unknown condition will be discovered as part of this study. If a previously unknown condition is discovered, we will inform the participant and ask him or her permission to inform the participant's regular physician.

7.2 Institutional Review Board (IRB) Review

The protocol will be submitted to the Yale IRB for review and approval. Approval of the protocol must be obtained before initiating any research activity. Any change to the protocol or study team will require an approved IRB amendment before implementation. The IRB will determine whether informed consent and HIPAA authorization are required.

The IRB will conduct continuing review at intervals appropriate to the degree of risk, but not less than once per year.

A study closure report will be submitted to the IRB after all research activities have been completed.

Other study events (e.g. data breaches, protocol deviations) will be submitted per Yale's IRB's policies.

7.3 Subject Confidentiality

A unique identification case number will be used to protect the confidentiality of the study participants. Only case numbers will be included on paper case report forms or in spreadsheets used for the statistical analysis. The link between the unique identification and participant identifiers will be maintained in REDCap.

7.4 Deviations/Unanticipated Problems

Any departure from the IRB-approved protocol procedures (e.g., the protocol, the informed consent document, the recruitment process or the study materials) that was made without prior IRB-approval will be considered a protocol deviation. Protocol deviations that alter the risk/benefit ratio, that are associated with serious adverse events, or that affects the subject's rights, safety or welfare and/or the integrity of the research data will be reported to the IRB and the DSMC as soon as possible but no later than ten days after the event.

7.5 Data Collection

We will use the web-based Research Electronic Data Capture (REDCap) system to design electronic data-collection forms. These forms have been pilot tested in Aim 2. Data will be collected initially on a paper form and then input into a protected, web-based case report form (which can be readily downloaded into SAS, STATA, R, or SPSS). The form allows for direct data entry by investigators and is designed to minimize errors and erroneous values. Expected ranges are pre-specified to prevent errors such as the shifting of decimal points. The program includes a computerized audit trail so that the identity of individuals entering or changing data and, in the case of changes, both original and revised data are saved. Data are backed up daily.

Clinical data, including clinical laboratory, will be entered by the research nurse. Research laboratory data will be entered by a fellow or research technician in the laboratory. One of the PIs will review each subject's CRF in REDCap at the completion of the study to assess for completeness.

7.6 Data Quality Assurance

Prior to initiating the study, we will convene a meeting of the investigators, research nurse, statisticians, and investigational pharmacists. During this meeting we will review all standard operating procedures and case report forms.

We use standardized protocols to calibrate and to use an oscillometric recording device (Dinamap, Critikon, Carlsbad, CA) to measure blood pressure and heart rate.

Clinical laboratory assays will be run in the CLIA-approved laboratory of Yale New Haven Hospital. All blood samples drawn for research assays will be centrifuged for 20 min immediately after blood drawing, and plasma or serum will be divided into aliquots, labeled with the case identification number and stored separately at -80^{o}C until shipping to Vanderbilt University for analysis. The laboratory at Vanderbilt will log and track all samples using its inventory management system (CryoTrack^{TM}, Los Altos, CA). Standard quality assurance measures will be applied to all laboratory analyses including but not limited to, tracking of inter-assay variability and the reanalysis of blinded internal controls at different times.

7.7 Study Records

Study records include regulatory documents, the protocol, consent form, case report forms, and participant medical records.

7.8 Access to Source Documents

Source documents include blood pressure printouts from the automated blood pressure cuff and laboratory data generated in the electronic medical record (clinical data) or in spreadsheet form (research data). These data will be downloaded into REDCap. Only HIPPA-trained and GCP-trained key study personnel will have access to the database.

7.9 Data or Specimen Storage/Security

Original data such as blood pressure printouts or laboratory spreadsheets will be kept on paper in a locked drawer or electronically on an encrypted computer. Data will be uploaded into the secure platform, REDCap. Specimens will be batch-shipped to Vanderbilt and stored

in the laboratory of Drs. Brown and Stevenson (co-PI on the NIH grant) funding this study. All specimens will be labeled with the unique identifier.

7.10 Retention of Records

All records will be retained for seven years following publication of the data. After that time, records may be archived for an additional five years and then shredded.

7.11 Study Monitoring

Clinical site monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, that the facilities and staffing are adequate for continued study conduct, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with International Council for Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s) including federal, state and local regulations and institutional policies and procedures.

Clinical site monitoring will be performed by the Yale Center for Clinical Investigation (YCCI). Monitoring visits will include regularly scheduled on-site interim monitoring visits and remote interim monitoring visits while subjects are on study, and a site close-out visit at the site. Following each monitoring visit, a visit report will be generated containing information on site activities and a summary of pertinent points and action items. The report will be provided with a follow-up letter. Site-specific data status reports will be distributed to the site regularly to outline planned, missing or incomplete case report forms and any outstanding data queries.

During monitoring visits, the following may be reviewed:

- Protection of the rights, safety and welfare of subjects through review of informed consent process and documentation, adverse events (AEs) and serious adverse events (SAEs) and safety procedures
- Subject eligibility
- Source verification
- Protocol compliance
- Deviations and Non-compliance
- Investigator Site File
- ICH GCP compliance
- Investigational Drug Storage and Accountability (including quantity and disposal procedures)
- Laboratory documentation related to biospecimen storage, shipment records, and temperature requirements
- Additional study supplies inventory and assessment
- Study progress and/or follow-up on issues with Site Principal Investigator (PI) and relevant members of the study team

The Sponsor-Investigator and YCCI will define the required study monitoring activities in a Clinical Monitoring Plan (CMP). The CMP describes in detail who will conduct the monitoring, at what frequency monitoring will be done, at what level of detail monitoring will be performed, and the distribution of monitoring reports.

7.12 Data Safety Monitoring Plan

A Data and Safety Monitoring Committee (DSMC) was established to provide objective review of treatment results as they relate to human safety and data quality for all Aims of HL145293. The committee is comprised of Thomas Wang, Chair of Medicine, University of Texas Southwestern; Ashish S. Shah, MD, Alfred Blalock Endowed Director and Chairman Department of Cardiac Surgery, Vanderbilt University Medical Center; and Alan B. Storrow, MD, Professor and Vice Chairman for Research and Academic Affairs for the Department of Emergency Medicine, Vanderbilt University Medical Center. Dr. Storrow chairs the committee. An important aspect of the membership of the DSMC is that all three members are senior faculty who are at another institution and therefore do not report to Dean Brown.

The DSMC will meet once after the first one-third of the subjects has been enrolled in this Aim, to receive reports of the progress of the Aim. This report will provide information regarding recruiting, safety reporting, and data quality. The committee will assess safety data including hypotension or hypertension, angioedema, hyperkalemia, common adverse events, hospitalizations, and other serious adverse events. Interim data will be provided to the committee by Drs. Brown, Stevenson (Dr. Brown's co-PI at Vanderbilt, who is leading Aim 2), and Yu (biostatistician for R01 grant HL145293). The DSMC will also receive quarterly reports of enrollment, protocol adherence, data quality, and adverse events via e-mail. Serious adverse events and unexpected events will be provided to the DSMC when they occur. Data will be blinded and presented on a coded basis unless the Committee votes to receive unblinded data. The DSMC will provide summary reports to the investigators that will be submitted to the Yale IRB.

7.13 Study Modification

Should any study modification be required, an amendment will be submitted to the IRB for approval. Changes would be implemented after approval. Any enrolled participant would be advised of the change and asked to sign the updated IRB-approved consent form.

7.14 Study Discontinuation

No early stopping is planned in any Aim. The Data Safety and Monitoring Committee (DSMC) will receive reports of any serious adverse events. The committee will assess safety data after the first third of participants have been enrolled and quarterly. The Committee will have the authority to modify the protocols or to terminate the study if it deems such actions to be warranted.

7.15 Study Completion

The study will be completed after 80 participants have completed study day 1 and 2 and any of these who have not withdrawn have completed study days 3 and 4 at the highest tolerated dose. After all data have been entered into the study Research Electronic Data Capture (REDCap) data base (see below), masters level biostatistician Nian Hui will use standard graphing and screening techniques to detect outliers and to ensure data accuracy of the blinded data in collaboration with the PIs. Any errors will be corrected. Only after data cleaning is complete, the database will be locked, and data analysis will be completed according to the Data Analysis Plan (see **6.4.**) We will write a manuscript describing the study findings. Results will be posted at ClinicalTrials.gov and the study will be closed.

7.16 Conflict of Interest Policy

The investigators will abide by the Conflict of Interest and Commitment policies and standards of Yale University.

7.17 Funding Source

The study will be funded by NIH grant HL14293 and Dr. Brown's C.N.H. Long professorship.

7.18 Publication Plan

The investigators will adhere to all federal policies regarding the submission of results and adverse events information at ClinicalTrials.gov. The PIs will also publish the results of this study in the appropriate peer-reviewed journal. The PIs will adhere to guidelines for authorship such as those promulgated by the International Committee of Medical Journal Editors (ICMJE). The investigators will submit any final peer-reviewed manuscripts that arise to the digital archive PubMed Central upon acceptance of the publication. The investigators will adhere to Yale and Vanderbilt University policies regarding intellectual property.

8 Appendices

Appendix #	Title	Section	Topic
1	Demographic and Screening CRF	**6.3.3**	Screening
2	Study Day CRF Aim 3	**6.3.5**	Study Day Visits
3	MD_Brown Lab Study Day Checklist	**6.3.5**	Study Day Visits
4	Medication Titration Questionnaire Aim 3	**6.3.5**	Dose Escalation Visits

9 List of Tables

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