Mechanism(s) Underlying Cardiovascular Effects of ARB/NEP Inhibition – Aim 1

Nancy J. Brown, M.D.
D-3100 Medical Center North
Vanderbilt University Medical Center
Nashville, TN, 37232

Table of Contents:

1.0 Background

The lifetime risk of developing heart failure (HF) in the United States exceeds 20%.[1] Despite beneficial effects of angiotensin receptor blockers (ARB)s, angiotensin converting enzyme inhibitors (ACEi)s, beta blockers, and mineralocorticoid receptor (MR) antagonists on mortality, the five-year life expectancy of a patient with HF is 50%.[1] The combined ARB/neutral endopeptidase (NEP)i LCZ696 was approved by the FDA after the Prospective Comparison of ARNi with ACEi to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) showed a beneficial effect on mortality compared to enalapril in patients with HF and reduced ejection fraction (EF).[2, 3] Nevertheless, many questions regarding the treatment of patients with an ARB/NEPi remain to be answered.[4]

Beneficial effects of combined ARB/NEP inhibition have been attributed to potentiation of the vasodilatory, natriuretic, sympatholytic, antiproliferative, and antihypertrophic effects of natriuretic peptides (**Figure 1**).[5] In HF, decreased sensitivity to the natriuretic peptides results from altered processing of proBNP to BNP, as well as altered clearance.[6-8] LCZ696 decreases NT-proBNP, but increases BNP,[2, 9, 10] providing indirect evidence that BNP degradation is decreased; unfortunately commercially available assays cross-react with degradation fragments,[11] leaving interpretation of these data open.

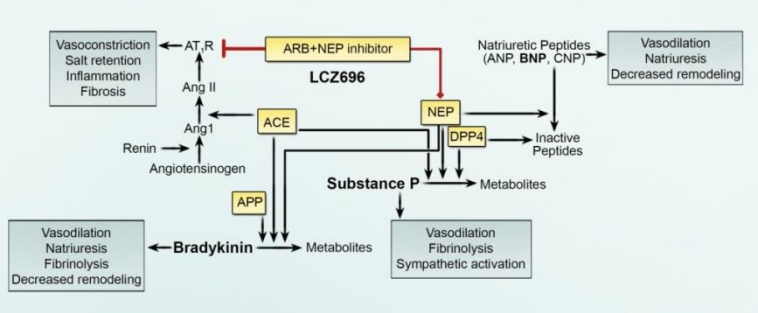


Figure 1: LCZ696 inhibits neprilysin (NEP) and blocks the angiotensin type 1 receptor (AT1R). Ang I indicates angiotensin I; Ang II, angiotensin II; ARB, angiotensin receptor blocker; ACE, angiotensin-converting enzyme; APP, aminopeptidase P; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide;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

Studies using exogenous natriuretic peptides in humans provide conflicting evidence regarding potentiation by NEP inhibition, however. For example, the NEPi thiorphan does not alter the vasodilator response to intra-arterial atrial natriuretic peptide (ANP) in HF patients taking an ACEi, but potentiates the response to bradykinin.[12] NEP inhibition enhances the effects of intravenous BNP on plasma cGMP and blood pressure, but does not alter urinary cGMP or sodium excretion in patients with left ventricular impairment.[13] In general, C-type natriuretic peptide (CNP) has minimal effect on vasodilation or natriuresis in humans.[14-18] Dalzell reported no effect of thiorphan, captopril, or omapatrilat on a weak vasodilator response to CNP in small resistance arteries from patients with CAD;[19] in one study, thiorphan potentiated the effect of intra-arterial CNP in healthy non-smokers.[20] In this study, we will investigate the effects of NEP inhibition on the vasodilator response to intra-arterial BNP based on 1) our prior data showing a significant vasodilator response to BNP,[21] 2) indirect evidence for decreased degradation of BNP in HF patients taking LCZ696 mentioned earlier,[9, 10] and 3) the potential for interaction between NEP and a dipeptidyl peptidase-4 (DPP4)i,[22] used in the treatment of diabetes, on the degradation of BNP (**Figure 2**).

In addition to cleaving the natriuretic peptides, NEP cleaves peptides such as bradykinin, substance P, vasoactive intestinal peptide, glucagon, neurotensin, adrenomedullin, and amyloid β peptide.[23] In fact, the Kcat/Km (a measure of catalytic efficiency) of NEP for bradykinin or substance P (69 and 158.7 min-1 μM-1, respectively)[24] is significantly higher than those for ANP, BNP, and CNP (5.1, 0.53, and 7.58 min-1 μM-1, respectively).[25] In small resistance arteries from patients with CAD and preserved EF, captopril, thiorphan, and omapatrilat all significantly potentiated the vasodilator response to bradykinin.[19] The response to substance P was not potentiated by ACE, NEP, or combined ACE/NEP inhibition in this study, but NEP inhibition potentiates effects of substance P in human vascular smooth muscle cells *in vitro*.[26] Patients given LCZ696 should not be taking an ACEi, but could be taking a DPP4i for the treatment of diabetes. As illustrated in Figure 2, there is potential for an interactive effect of NEP inhibition and DPP4 inhibition on the degradation of substance P and/or BNP.

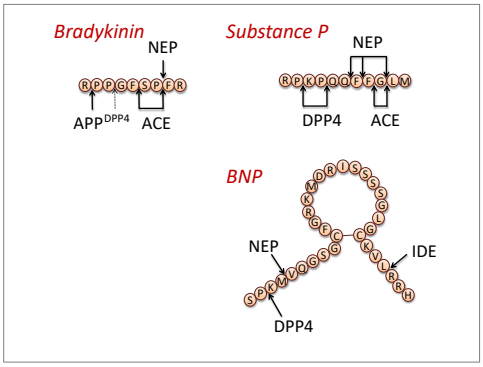


Figure 2: Cleavage of bradykinin, substance P, and brain natriuretic peptide (BNP) by neprilysin (NEP), angiotensin-converting enzyme (ACE), aminopeptidase P (APP), dipeptidyl peptidase-4 (DPP4), and insulin-degrading enzyme (IDE). The dotted arrow indicates that bradykinin must be cleaved by (and is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

Decreased degradation of bradykinin and substance P by NEP could lead to beneficial effects on vascular relaxation and fibrinolysis. Studies from our group and others using the pharmacological bradykinin B2 receptor blocker icatibant have shown that endogenous bradykinin contributes to the beneficial effects of ACEis on blood pressure (BP) and on endothelial fibrinolysis.[27-29] Substance P also causes vasodilation and tissue-plasminogen activator (t-PA) release from the vascular endothelium, and administration of a substance P (NK1) receptor antagonist blocks these effects in humans.[30, 31] Substance P also increases sympathetic activation,[30] an effect that may be detrimental in HF patients.[32]

ARB/NEPi use may be associated with angioedema.

Treatment with the combined ACE/NEPi omapatrilat results in a three-fold increase in angioedema compared to ACE inhibition.[33] The increased rate of angioedema has been attributed to simultaneous inhibition of two pathways involved in the degradation of bradykinin and substance P, major effectors of angioedema.[34] Bradykinin is inactivated primarily by ACE, but also by NEP and aminopeptidase P (APP), while substance P is inactivated by ACE, NEP, and DPP4 (**Figure 2**).[24, 35, 36] Consistent with a role for NEP in the pathogenesis of angioedema, a polymorphism in the gene encoding for NEP is associated with ACEi-associated angioedema.[37] Because LCZ696 inhibits NEP without inhibiting ACE, it has been suggested that the incidence of angioedema will be lower than that observed with omapatrilat.

In PARADIGM-HF, there were 19 confirmed cases of angioedema in patients treated with LCZ696 compared to ten cases in patients treated enalapril (an incidence of 0.45% versus 0.23%, p=0.13).[2] The PARADIGM-HF trial likely underestimates the risk of angioedema in patients taking LCZ696 in the real world setting. Seventy-seven percent of patients enrolled in the trial were previously treated with an ACEi, and all were exposed to enalapril and LCZ696 during a single-blind, run-in period. Including a high proportion of patients who tolerated exposure to an

ACEi may have led to a decreased incidence of angioedema. Moreover, just 5% of patients in PARADIGM were African American, a group at increased risk of ACEi- and vasopeptidase inhibitor-associated angioedema.[2, 33, 38, 39]

Summary

The prevalence of HF is increasing with the aging of the population, and mortality remains unacceptably high. A newly approved ARB/NEPi reduced mortality in a single trial, but its mechanism of action has been inadequately studied. NEP cleaves many vasoactive peptides in addition to the natriuretic peptides, including bradykinin and substance P, two peptides that contribute to beneficial and adverse effects of ACEis. Understanding the mechanism of action of the ARB/NEPi will enable us to target its use appropriately and may lead to the development of new drugs for the treatment of HF.

2.0 Rationale and Specific Aims

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.[1] ACEis, ARBs, beta blockers, and MR antagonists reduce mortality in patients with HF with reduced EF.[40-44] Still mortality from HF remains high, and approximately 50% of individuals with HF die within five years of diagnosis.[1]

In 2015, the FDA approved LCZ696, a molecular complex of the ARB valsartan with an inhibitor of NEP (neutral endopeptidase-24.11) sacubitril,[45] after the PARADIGM-HF trial demonstrated a mortality benefit over enalapril in patients with HF, reduced EF, and increased circulating brain natriuretic peptide (BNP) or N-terminal pro-BNP (NT-proBNP) concentrations.[2, 3] This 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.

In addition to degrading natriuretic peptides, NEP degrades other vasoactive peptides including angiotensins (Ang) I and II, endothelins, bradykinin, and substance P (**Figure 1**). Indeed, observations that NEP inhibition increases endogenous Ang II and the pressor response to exogenous Ang II provided the rationale for combining NEPi with RAAS inhibitors.[46, 47] Increased angioedema during combined ACE and NEP inhibition (vasopeptidase inhibition),[33] presumably due to decreased degradation of bradykinin and substance P through two pathways,[48] led to development of the ARB/NEPi. Studies in isolated resistance vessels from patients with coronary artery disease (CAD) and preserved left ventricular function suggest that NEP inhibition alone (or with ACE inhibition) potentiates vasodilator effects of bradykinin, however.[19] NEP inhibition could potentiate bradykinin either by preventing its degradation or by enhancing effects at the B2 receptor.[49] Increased bradykinin can lead to decreased afterload, increased natriuresis, and enhanced fibrinolysis.[27, 28, 50, 51]

Bradykinin could also contribute to angioedema in patients taking an ARB/NEPi. We have reported the association of a polymorphism in the gene encoding NEP (MME) with ACEi-associated angioedema.[37] In addition to increasing vascular permeability directly, bradykinin induces the release of the NEP substrate substance P from nerve terminals.[52] Both bradykinin and substance P have been implicated in the pathogenesis of angioedema.[53, 54] In the PARADIGM-HF

trial the incidence of angioedema was 0.45% during combined ARB/NEPi versus 0.23% during ACEi (p=0.13).[2] While the incidence of angioedema was statistically comparable, the numerical differences raise concerns described below.

This proposal tests **the overarching hypothesis that bradykinin contributes to effects of combined ARB/NEP inhibition compared to ARB alone.** Specifically, we will:

Specific Aim 1: Test the hypothesis that ARB/NEP inhibition potentiates the effects of exogenous bradykinin, substance P, and BNP on forearm blood flow or endothelial t-PA release compared to ACE inhibition or ARB. We will compare LCZ696 to bioequivalent doses of valsartan to focus on the mechanistic effects of NEP inhibition in this aim.

3.0 Animal Studies and Previous Human Studies

Preliminary Studies

Our group has experience studying the effect of peptidase inhibitors on responses to vasoactive peptides such as bradykinin, substance P, and BNP in the human forearm vasculature. For instance, we and others have shown that bradykinin causes vasodilation and stimulates t-PA release from the forearm vasculature,[55] through the bradykinin B2 receptor,[56] that risk factors for CAD are associated with diminished bradykinin- and substance P-stimulated endothelial t-PA release,[57-59] and that forearm vascular t-PA release correlates with coronary t-PA release and predicts future coronary events.[60, 61] We have also found that 1) blocking the B2 receptor attenuates the BP response to ACE inhibition in patients with hypertension,[27] 2) that ACE inhibition increases endothelial t-PA release via the B2 receptor,[28] 3) that bradykinin contributes to hypotension following protamine in patients undergoing cardiopulmonary bypass,[62] and 4) that bradykinin contributes to inflammation during hemodialysis.[63] Relevant to the actions of LCZ696, bradykinin does not contribute to the BP response to valsartan alone.[64]

More recently, we have studied the effects of the DPP4i sitagliptin on responses to vasoactive peptides. We have reported that there is no effect of DPP4 inhibition on vasodilator responses to bradykinin or substance P (**Figure 3**), whereas DPP4 inhibition reduces substance P-stimulated endothelial t-PA release and increases norepinephrine release (from 212.5±60.7 pg/mL during placebo to 331.9±228.5 pg/mL during combined inhibition, p=0.02).[30] (Data are provided as mean ± SD unless indicated.) We found no effect of DPP4 inhibition alone on the vasodilator response to intra-arterial BNP.[21]

Figure 3: Effect of angiotensin-converting enzyme inhibition (Enalaprilat , enal) and dipeptidyl peptidase 4 inhibition (sitagliptin 200 mg) on forearm blood flow (FBF) and tissue-plasminogen activator (t-PA) release in response to exogenous intra-arterial bradykinin and substance P.[30] *P<0.05 versus placebo, †P<0.05 versus sitagliptin alone. Data are mean ± SEM.

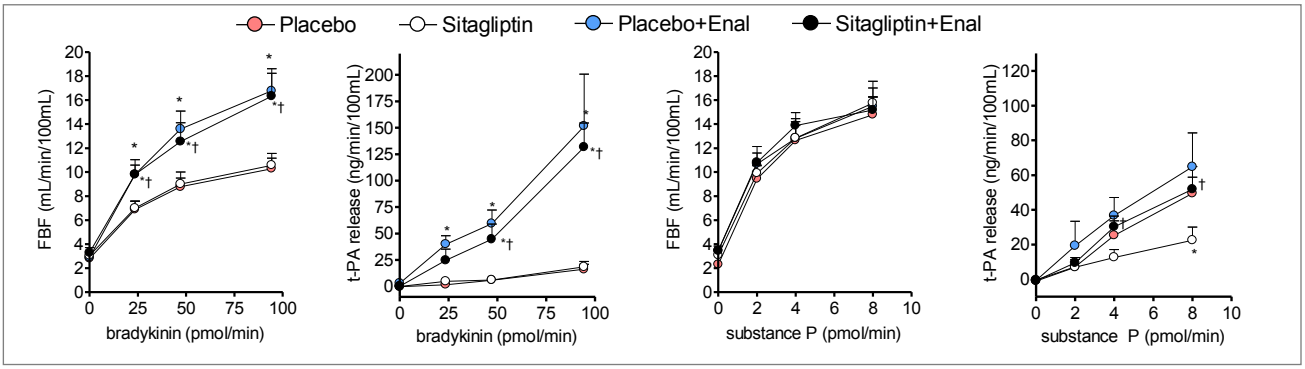
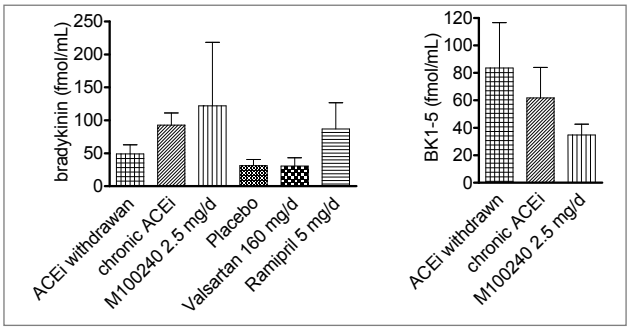


Figure 4: Bradykinin and BK1-5 concentrations measured prior to cardiac surgery in patients whose chronic ACE inhibitor was stopped 48 hours prior to surgery (ACEi withdrawn, N=12) or continued (chronic ACEi, N=19),[62] as well as in healthy volunteers treated daily with an ACE/NEPi for a week (M10024, N=8), and dialysis patients treated daily with placebo, valsartan, or ramipril for one week (N=13).[69] Mean ± SEM



4.0 Inclusion/Exclusion Criteria

Inclusion criteria

1) Patients with essential hypertension defined as having
    a) untreated, seated systolic BP (SBP) of 130 mmHg or greater on three separate occasions, or
    b) untreated, seated diastolic BP (DBP) of 80 or greater on three separate occasions, or
    c) taken anti-hypertensive agent(s) for a minimum of six months.

2) For female subjects, the following conditions must be met:
    a) postmenopausal status for at least one year, or
    b) status post-surgical sterilization, or
    c) if of childbearing potential, utilization of adequate birth control and willingness to undergo urine beta-hCG testing prior to drug treatment and on every study day.

3) Age 18 to 60 years of age

Exclusion criteria

1) Presence of secondary form of hypertension
2) Symptomatic hypertension and/or SBP>170 mmHg or DBP>110 mmHg, relevant to the washout period
3) History of hypersensitivity or allergy to any of the study drugs, drugs of similar chemical classes, ACEi, ARBs, or NEPi, as well as known or suspected contraindications to the study drugs
4) History of angioedema
5) History of pancreatitis or known pancreatic lesions
6) History of significant cardiovascular disease (other than essential hypertension and LV hypertrophy)
7) Symptomatic hypotension and/or a SBP<100 mmHg at screening or <95 mmHg during the study
8) Serum potassium >5.2 mmol/L at screening or >5.4 mmol/L during the study
9) Individuals using oral contraceptives and smokers in order to reduce the risk of thrombosis following arterial line placement
10) History of serious neurologic disease such as cerebral hemorrhage, stroke, seizure, or transient ischemic attack within six months
11) Presence of significant pulmonary disorders
12) Type 1 diabetes
13) Poorly controlled type 2 diabetes mellitus (T2DM), defined as a HgbA1c >9%
14) Hematocrit <35%
15) Impaired renal function (eGFR of <30mL/min/1.73 m2) 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: eGFR (mL/min/1.73m2)=175 • Scr-1.154 • age-0.203 • (1.212 if Black) • (0.742 if female)
16) Use of hormone-replacement therapy
17) Breast feeding and pregnancy
18) History or presence of immunological or hematological disorders
19) History of malignancy other than non-melanoma skin cancer
20) Diagnosis of asthma requiring use of inhaled beta agonist more than once a week
21) Clinically significant gastrointestinal impairment that could interfere with drug absorption
22) Impaired hepatic function [aspartate amino transaminase (AST) and/or alanine amino transaminase (ALT) >3.0 x upper limit of normal range]
23) 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
24) Treatment with chronic systemic glucocorticoid therapy within the last year
25) Treatment with lithium salts
26) History of alcohol or drug abuse
27) Treatment with any investigational drug in the one month preceding the study
28) Mental conditions rendering the subject unable to understand the nature, scope, and possible consequences of the study

29) Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

5.0 Enrollment/Randomization

Written advertisements which have been approved by the Vanderbilt Institutional Review Board (IRB) and which give the name and phone number of a contact research nurse will also be placed on Vanderbilt clinic bulletin boards. A description of the study will also be placed on the Vanderbilt patient portal. Potential participants who call for information will be given a brief description of the study protocol and, if interested, will be invited to the Vanderbilt CRC for more information. In addition, the research nurse will make himself available to talk with patients at the Vanderbilt Heart and Vascular Institute and at other clinics where a high number of potential subjects have been identified using Subject Locator. During meetings with potential subjects, the research nurse or investigator will describe the study protocol in detail. Interested participants will be invited to read and sign an IRB-approved consent form and will be given a copy of the consent form to take with them.

Assignment to treatment will be randomized and blinded to the investigators and subjects. Dr. Yu, study biostatistician, will provide an allocation schedule. The Vanderbilt Investigational Drug Service will be responsible for the storage, preparation, and labeling of all investigational agents and for maintaining accurate drug storage and dispensing logs. The Clinical Research Pharmacist will devise standard operating procedures for the pharmacy to follow with regard to preparing, labeling, blinding, and dispensing study drug.

After subjects have been consented and screened, the investigator or research nurse will fax a copy of the consent form and a prescription containing check boxes for the inclusion and exclusion criteria to the Vanderbilt Investigational Drug Service. The pharmacist will confirm that consent was obtained and the subject met entry criteria, assign the subject a randomization number, and will provide the investigator with labeled study drug as indicated in the protocol. An extra label containing the randomization number will be attached to each study drug. The investigator will affix this extra label to the subject's records. The Investigational Drug Service will retain a secure set of sealed envelopes containing the treatment assignment. These will be opened in the event of a clinical scenario which necessitates unblinding, as determined by the PI and the Data and Safety Monitoring Committee. Subjects randomized who do not complete the whole protocol for any reason will be replaced.

6.0 Study Procedures

Specific Aim 1: Test the hypothesis that ARB/NEP inhibition potentiates the effects of exogenous bradykinin, substance P, and BNP on forearm blood flow or endothelial t-PA release compared to ARB.

Rationale

NEP efficiently catalyzes the degradation of bradykinin and substance P (Kcat/Km 69 and 158.7 min-1 μM-1, respectively).[24] In small resistance arteries, NEP inhibition potentiates the vasodilator response to bradykinin.[19] NEP hydrolyzes substance P in human vascular smooth

muscle cells.[26] NEP inhibition has variable effects on the vasodilator response to exogenous natriuretic peptides in prior studies,[12, 13, 19] but LCZ696 increases the ratio of BNP to NT-proBNP in HF patients.[9, 10] Potentiation of bradykinin-, substance P-, or BNP-mediated vasodilation could contribute to beneficial effects of LCZ696 on BP and afterload reduction. Potentiation of t-PA release could reduce thrombotic events in patients taking LCZ696. Conversely potentiation of bradykinin or substance P could contribute to angioedema.[66]

Thirty-five percent of the patients with HF and reduced EF in PARADIGM-HF had known diabetes, and another 13% had undiagnosed diabetes.[2, 67] DPP4 inhibitors are often used to treat diabetes. Substance P and BNP are substrates common to NEP and DPP4 (**Figure 2**).[22, 36] For this reason, we will also test the novel hypothesis that the LCZ696 and the DPP4i sitagliptin interact to potentiate effects of substance P and/or BNP.

Subjects

We will study patients with essential hypertension. Thirty-four non-smoking hypertensive subjects, age 18 to 60 years of age (50% Black, 50% female) will participate in this randomized, double-blind, placebo-controlled, crossover study. Subjects will be defined as hypertensive if they have an untreated, seated systolic blood pressure (SBP) of 140 mmHg or greater on three separate occasions; have an untreated, seated diastolic blood pressure (DBP) of 90 mmHg or greater on three separate occasions; or have taken anti-hypertensive agent(s) for a minimum of six months. Subjects with secondary forms of hypertension, significant cardiovascular disease (other than essential hypertension and LV hypertrophy), pulmonary and neurological disorders, diabetes, anemia, and renal insufficiency will be excluded. Pregnancy will be excluded in women of child-bearing potential by urine beta-hCG, and women will be studied during the follicular phase of the menstrual cycle.

Protocol

Figure 5 illustrates the study protocol. After informed consent is obtained, subjects will undergo a screening history and physical exam, and anti-hypertensive medications will be withdrawn. If appropriate, as in the case of beta-blockers and clonidine or other medications for which there may be rebound hypertension if they are discontinued suddenly, medications will be tapered. During this period, BP will be measured every one to three days. If at any time the seated SBP is >170 mmHg, or seated DBP >110 mmHg, or if a subject develops symptoms of high BP regardless of BP, the subject will be discontinued, and his or her anti-hypertensives restarted. In our experience, about three percent of subjects may be excluded during washout. Excluded subjects will be replaced.

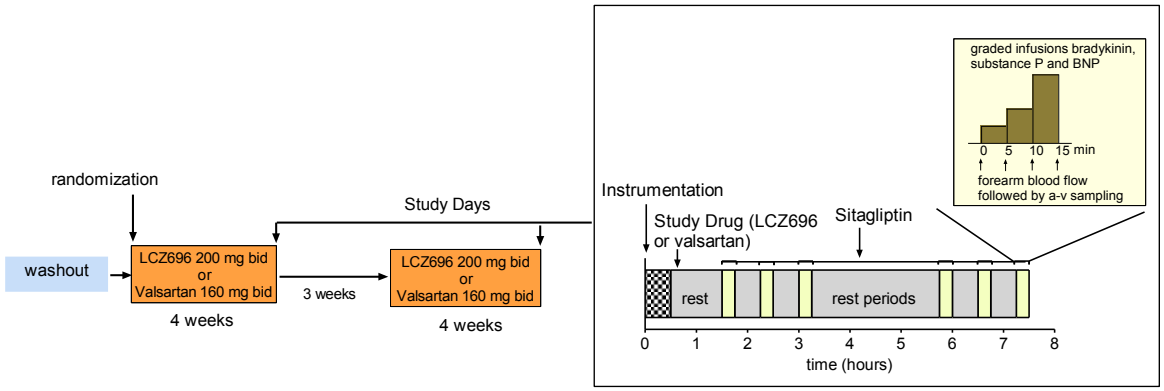


Figure 5: Study Protocol. Valsartan 160 mg and LCZ696 200 mg provide similar valsartan concentrations due to increased bioavailability of valsartan when administered as LCZ696.[64] The LCZ696 dose is that used in PARADIGM-HF.

After subjects have been off anti-hypertensive medications for three weeks (four for spironolactone), they will be randomized to four-week treatment with valsartan 160 mg bid (80 mg bid for one week, then 160 mg bid) or LCZ696 200 bid (100 mg bid for one week, then 200 mg bid) in a double-blind fashion. Valsartan bioavailability is enhanced when administered as LCZ696, and these doses of valsartan and LCZ696 give equivalent serum concentrations of valsartan.[68] The doses of LCZ696 is that used in PARADIGM. On the morning of the 28th day of study drug, subjects will report to the Vanderbilt Clinical Research Center (CRC) after an overnight fast. Subjects will be studied in the supine position in a temperature-controlled room. They will be instrumented for intra-arterial infusions, as described below. Subjects will be given their last dose of study drug. One hour after drug administration, we will measure forearm blood flow (FBF) and give bradykinin, substance P, or BNP. The order of bradykinin and substance P will be randomized. BNP will be given third because it has a half-life of 18 minutes (versus seconds for the other peptides) and requires a longer washout period to avoid carryover effects.[21] Each peptide will be infused in three graded doses for five minutes. After administration of all three peptides, subjects will be allowed to rest for an hour. Then they will be given a single oral dose of sitagliptin 200 mg and be allowed to rest for 90 minutes. This dose of sitagliptin achieves complete inhibition of DPP4 comparable to 100 mg but with an earlier (one hour) and more sustained maximal response.[69] We will repeat baseline measurements and the peptide infusions with an intervening rest period. The four-week study treatment and protocol will be repeated after a three-week washout, until subjects complete both arms.

Anticipated Results

We do not expect an effect of valsartan alone on the responses to bradykinin (or substance P or BNP) based on prior studies.[64] We anticipate that LCZ696 will increase vasodilator and t-PA responses to bradykinin and may increase vasodilator and t-PA responses to substance P, as well as the vasodilator response to BNP compared to during valsartan. We will compare the effect of valsartan and LCZ696 on the responses to exogenous bradykinin, substance P, and BNP. We hypothesize that effects of LCZ696 on responses to BNP or substance P will be enhanced by concurrent inhibition of DPP4. In the case of substance P, there may also be an effect on

norepinephrine release, as we observed in an earlier study of combined ACE and DPP4 inhibition.[30]

Sample Size Calculation and Statistical Analysis

The primary endpoints are FBF and net release of t-PA. Net norepinephrine release is a secondary endpoint. The primary analyses will focus on the LCZ696 versus valsartan comparisons on the primary and secondary endpoints. The secondary analysis will focus on the comparison of LCZ696 during sitagliptin versus LCZ696 alone on the primary and secondary endpoints.

Sample Size and Power Calculation:

In our study of the effect of ACE inhibition on responses to bradykinin, enalaprilat increased the FBF response to a 94.3 pmol/min dose of bradykinin from 10.57±3.24 (mean±SD) to 16.32±6.34 mL/min/100 mL, with a SD of the difference of 4.07 mL/min/100 mL and a within-subject correlation of 0.85. Substance P increased FBF from 2.29±0.51 to 14.79±4.86 mL/min/100 mL at the highest dose proposed, and the within-subject correlation was 0.76. BNP increased FBF from 2.54±0.84 mL/min/100 mL at baseline to *6.15±1.95* mL/min/100 mL at the *720* pmol/min dose, and the within-subject correlation was 0.71. With 16 subjects in this crossover study we would have *excellent (>90%)* power to detect an increase in FBF similar in magnitude during LCZ696 versus valsartan or LCZ696/sitagliptin versus LCZ696 alone (Table 2). We will study 32 subjects so we will have adequate power to detect this magnitude of difference within either racial or gender group alone. *We also have power to stratify women on menopause status. If half of the 16 women are pre-menopausal, we would have >90% power to detect the difference observed with bradykinin.* In our most recent study, enalaprilat increased the t-PA response to bradykinin (94.3 pmol/min) from 21.8±14.0 to 114.3±110.9 ng/mL/100mL, and the within-subject correlation was 0.80. An N of 13 would be required to provide 85% power to detect this magnitude of change between LCZ696 and valsartan.

Table 2: Expected FBF results. *For substance P and BNP, we conservatively projected LCZ696 would increase the FBF response 30 percent.*

Infusion	***valsartan***	**LCZ696, increase of the magnitude of *enalapril***				
		Mean ± SD	Correlation	Increase±SD	N for 85% power	Power for N=16
Bradykinin	10.57±3.24	16.32±6.34	0.85	5.75±3.97	7	>99%
Substance P	14.79±4.86	19.23±6.32	0.76	4.44±4.46	11	96
BNP	6.15±1.95	8.00±2.53	0.71	1.85±1.79	11	97

Data Analysis Plan:

We will use standard graphing and screening techniques to detect outliers and to ensure data accuracy. We will use non-parametric analysis methods due to the relatively small sample size. We will provide summary statistics for both numerical and categorical variables by study arm. We will assess comparability among randomization groups.

We are using a two-by-two crossover design in this study. Although we have designed the study with a prolonged three-week washout to avoid carryover of any ARB or NEPi effect, we will take additional baseline measurements of key study endpoints right before study subjects take study medication (valsartan or LCZ696) in the two treatment periods. These data will allow us to estimate directly any residual carryover effect using within-subject comparisons or the mixed effects models. In the mixed-effects models with a random subject effect, we will code the appropriate first-order carryover effect. For example, if a subject receives treatments valsartan and LCZ696 in the two periods, then the model has carryover effect null, CV, in the two periods. (CV denotes potential carryover effect due to valsartan in the prior period.) Since we are going to use the baseline measures for this evaluation, the main effect of the treatments will not be in the model. If they are not significant, the carryover effects will not be included in the model when we estimate the main treatment effects in the following. We will use an autoregressive model of order 1 (AR1) or other plausible covariance structures for the error covariance. We will use a similar approach to evaluate for potential carryover of bradykinin, substance P, or BNP effects.

We will use mixed-effects models with a random subject effect and with treatment factor one (valsartan or LCZ696) and treatment factor two (absence or presence of sitagliptin) as fixed effects. We will evaluate treatment effects and interactions using properly set up contrasts in the mixed-effects models. We will include sex and race as covariates and will conduct stratified analyses if appropriate. (We and others have observed racial differences in vasodilator responses to bradykinin.[70, 71] ACE inhibition increases t-PA response to bradykinin to a greater extent in women than in men.[72]) In addition to evaluating treatment effects and interactions using regression analyses, we will calculate within-subject mean differences and 95% confidence intervals for specific effects of interest and test for treatment effects using signed rank test. We will test all hypotheses at the level of α=0.05. We will use SPSS (version 23.0, SPSS, Chicago, IL) and the open-source statistical package R (version 3.1.0, R Core Team, 2014) for analyses.

Subjects who drop out 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.

Limitations and Future Directions

We will study people with hypertension rather than HF in this aim because the study is invasive and to permit safe washout of vasoactive drugs. Because LCZ696 increases urine cGMP similarly in patients with hypertension and with HF,[10, 73] we expect findings will be applicable across groups.

We are studying effects of four-week treatment with LCZ696 and valsartan. Prior studies indicate that hemodynamic effects of valsartan and LCZ696 are at steady state by four weeks.[73]

We have designed the study to avoid carryover effects. We know from many other studies that three weeks provides adequate washout for the effects of ARBs and ACEis,[74, 75] and studies using LCZ696 suggest that this duration is adequate for NEP inhibition.[2] As noted in Data

Analysis Plan, we will test for carryover and also use repeated baseline measurements to allow us to estimate any residual carryover effect.

Bradykinin, substance P, and BNP are just three substrates of NEP that may have cardiovascular effects. Adrenomedullin is another candidate NEP substrate that causes vasodilation and natriuresis.[76] NEP inhibition enhances the vasodilator response to intravenous adrenomedullin in sheep;[77] omapatrilat (but not thiorphan) potentiates the vasodilator response to adrenomedullin in resistance arteries from patients with CAD.[19] If we do not see an effect of NEP inhibition or NEP inhibition + DPP4 inhibition on the response to any one of the peptides, we will consider substituting adrenomedullin as one of the peptides. Likewise, it may be necessary to complete studies using ANP or CNP.

7.0 Risks of Investigational Agents/Devices (side effects)

1) Frequent blood draws can lead to anemia.
2) Withholding anti-hypertensive medications may result in elevation in BP. We will monitor BP every one to three days after stopping or decreasing these medications. If the BP is too high or if there are symptoms of high BP during the washout period, the study will be stopped and prior medications will be restarted.
3) LCZ696 may cause angioedema, leading to swelling of the tongue, lips, or pharynx that can result in airway compromise. DPP4i use has also been associated with angioedema and can increase the risk of ACEi-associated angioedema.[78, 79] We will exclude patients with a history of angioedema, and the duration of LCZ696 is short in each study. Subjects in Aim 1 will receive sitagliptin during LCZ696 only one time. We have not observed angioedema in studies of short-term sitagliptin and ACEi exposure.
4) Valsartan or LCZ696 can cause hypotension or orthostasis. We are giving the first dose in the CRC to monitor BP. We will start at a lower dose and escalate after one week. We will monitor BP after the first dose and throughout the study.
5) Valsartan or LCZ696 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 >5.4 mmol/L, we will repeat the blood sample. If potassium is persistently >5.4 mmol/L we will withdraw the subject from the study.
6) Insertion of brachial artery and venous catheters may cause bleeding, bruising, or infection. Insertion of a brachial artery catheter may cause damage to the artery or thrombosis. This is potentially serious and could result in loss of blood supply to the arm, requiring intra-arterial administration of fibrinolytic agents and/or surgery. Complications arising from arterial line insertion are usually seen in the setting of low flow and prolonged arterial line placement, neither of which occur in these protocols. We have conducted studies involving the insertion of brachial artery catheters safely in over 400 subjects.
7) We will use lidocaine to numb the skin prior to the insertion of the brachial artery catheter. Lidocaine can cause numbness and burning and/or local rash or irritation in subjects who are allergic to it.
8) Bradykinin (U-1080, Clinalfa, Bachem AG), substance P (U-1180, Clinalfa, Bachem AG, IND 110,721), and BNP are vasoactive substances that will be infused into the

brachial artery at doses that have no detectable effect on systemic BP or heart rate. Given at higher doses bradykinin, substance P, and BNP can lower BP and cause a compensatory tachycardia. We have not observed an effect on BP at the doses proposed in prior studies.

9) DPP4 inhibitors can cause hypoglycemia. The risk of fasting hypoglycemia is lower than with non-incretin-based anti-diabetic agents due to the mechanism of action. We have not observed hypoglycemia in our prior studies using the dose of sitagliptin proposed in this study.

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

All protocols will be reviewed and approved by the Vanderbilt IRB before any subject is enrolled. The Principal Investigator will closely oversee the protocol in conjunction with the dedicated research nurse.

A DSMC will provide objective review of treatment results as they relate to human safety and data quality. The committee will be comprised of Marie Griffin, MD, Professor of Health Policy; Ashish S. Shah, MD, Professor of Cardiac Surgery and Surgical Director of Vanderbilt Heart Transplant and Mechanical Circulatory Support; and Alan B. Storrow, MD, Professor and Vice Chairman for Research and Academic Affairs for the Department of Emergency Medicine. Dr. Storrow will chair the committee. An important aspect of the membership of the DSMC is that all three members are senior faculty who hold a primary appointment outside the Department of Medicine.

The DSMC will meet at least three times, after the first one-third of the subjects have been enrolled, to receive reports of the progress of the study. These reports will provide information regarding recruiting, safety reporting, and data quality. No early stopping is planned. 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 and Yu. The randomization will be blinded and presented on a coded basis unless the Committee votes to receive unblinded data. The Committee will have the authority to modify the protocols or to terminate the study if it deems such actions to be warranted. The DSMC will provide summary reports to the investigators that will be submitted to the IRB.

The DSMC will also receive quarterly reports of enrollment, protocol adherence, data quality, and adverse events via e-mail. The DSMC will review all serious adverse events. Any serious adverse event will be reported to the DSMC, IRB, and NIH (and FDA if appropriate) as soon as possible, but not more than 7 business days from the PI's notification of the event. Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. 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. 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.

Non-serious, unexpected adverse events and incidences of noncompliance with the protocol will be reported to the IRB at the time of annual review. If warranted, appropriate changes will be made to the consent form. 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. Any instance of non-compliance with the protocol 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.

9.0 Study Withdrawal/Discontinuation

If at any time during the study, a subject develops any symptoms related to study participation that subject will be withdrawn from the study. If, in the opinion of the investigator, a subject is non-compliant, that subject will be withdrawn from the study. Subjects who are withdrawn will be followed until symptoms have resolved.

10.0 Statistical Considerations

See above under protocol.

11.0 Privacy/Confidentiality Issues

A unique identification case number will be used to protect the confidentiality of the study participants. Only case numbers will be included in spreadsheets used for the statistical analysis.

We will use the web-based Vanderbilt Research Electronic Data Capture (REDCap) system to design electronic data collection forms in all Aims. These forms will be pilot tested before use. Data will be 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. Results from the Vanderbilt clinical laboratory can also be directly imported to REDCap, which further reduces typographical data entry errors. 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 predoctoral student in the laboratory.

12.0 Follow-up and Record Retention

All records will be retained for 7 years following publication of the data. After that time, records may be archived for an additional 5 years and then shredded.

13.0 Standard Techniques

BP Measurements: During screening, washout, and active treatment outpatient BP, HR, and MAP will be measured with an aneroid sphygmomanometer (Welch Allyn, Skaneateles Falls, NY), using the appearance and complete disappearance of the Korotokoff sounds (K1 and K5) as SBP and DBP. The mean of three supine measurements will be used. During study days, BP will be measured before and using an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA).

Instrumentation for intra-arterial infusions: An intravenous catheter will be placed in the antecubital vein of the nondominant arm. After subdermal 1% lidocaine, an 18-gauge polyurethane catheter (Cook Inc., Bloomington, IN) will be inserted in the brachial artery of the same arm. Prior to the infusion of vasoactive peptides, arterial catheter patency will be maintained by infusion of 5% dextrose at rate of 1 mL/minute. After study drug, a one-hour rest period, and measurement of basal FBF and blood sampling, peptides will be infused. We will infuse bradykinin (U-1080, Clinalfa, Bachem AG) at 23.6, 47.2, and 94.3 pmol/min. The highest dose increases FBF to 10.57±3.24 mL/min/100 mL forearm volume in the absence of ACE inhibition. We will give substance P (U-1180, Clinalfa, Bachem AG), at 2, 4, and 8 pmol/min to achieve similar vasodilation (**Figure 3**). We will give BNP (Nesiritide, Scios Inc., Mountain View, CA) at 240, 480, and 720 pmol/min. These doses cause vasodilation comparable to the lower doses of the other agonists.[21] Each dose will be infused for five minutes, and FBF will be measured during the last two minutes. Drug concentrations in the infusate will be adjusted to maintain infusion volumes at one mL/minute. FBF will be measured using mercury-in-silastic strain-gauge plethysmography, as detailed elsewhere.[28]

Forearm blood flow measurements: FBF will be measured using mercury-in-silastic strain-gauge plethysmography. The wrist will be supported to raise the forearm above the level of the atrium, and a strain gauge placed on the widest part of the forearm. The strain gauge will be connected to a plethysmograph (Model EC-4, D.E. Hokanson, Issaquah, WA), calibrated to measure percent change in volume and connected to a chart recorder to record flow measurements. For each measurement, a cuff around the upper arm will be inflated to 40 mmHg with a rapid cuff inflator (Model E-10, Hokanson) to occlude venous outflow from the extremity. The hand will be occluded from measurement of FBF by inflation of a pediatric sphygmomanometer cuff to 200 mmHg around the wrist. Flow measurements will be recorded for approximately 7 sec out of 15 sec, and the slope will be derived from the first 3-4 pulses; 7 readings will be obtained for each mean.

Blood Sampling and Calculations: Following measurement of FBF, simultaneous arterial and venous samples will be obtained from the infused arm prior to and at the end of each dose of study drug for measurement of peptide concentrations, cGMP, t-PA (bradykinin and substance P), and norepinephrine. We will also measure venous NEP activity and DPP4 activity at baseline. Infusions will be interrupted during arterial sampling. All samples will be obtained after the first three mL of blood are discarded. Blood samples will be collected on ice and centrifuged immediately. Arterio-venous concentration gradients will be calculated by subtracting the plasma level measured in simultaneously collected venous (CV) and arterial (CA) blood. Forearm plasma flow (FPF) will be calculated from the FBF and arterial hematocrit corrected for 1% trapped plasma. Thus, individual net release or uptake rates at each time point will be calculated: Net release = (CV-CA) x [FBF x ((101-hematocrit)/100)].

References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW and Turner MB. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation*. 2015;131:e29-322.

2. McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K and Zile MR. Angiotensin-neprilysin inhibition versus enalapril in heart failure. *The New England journal of medicine*. 2014;371:993-1004.

3. Claggett B, Packer M, McMurray JJ, Swedberg K, Rouleau J, Zile MR, Jhund P, Lefkowitz M, Shi V and Solomon SD. Estimating the Long-Term Treatment Benefits of Sacubitril-Valsartan. *The New England journal of medicine*. 2015;373:2289-90.

4. Hubers SA and Brown NJ. Combined Angiotensin Receptor Antagonism and Neprilysin Inhibition. *Circulation*. 2016;133:1115-24.

5. Jessup M. Neprilysin inhibition--a novel therapy for heart failure. *The New England journal of medicine*. 2014;371:1062-4.

6. Schmitt M, Gunaruwan P, Payne N, Taylor J, Lee L, Broadley AJ, Nightingale AK, Cockcroft JR, Struthers AD, Tyberg JV and Frenneaux MP. Effects of exogenous and endogenous natriuretic peptides on forearm vascular function in chronic heart failure. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:911-7.

7. Forfia PR, Lee M, Tunin RS, Mahmud M, Champion HC and Kass DA. Acute phosphodiesterase 5 inhibition mimics hemodynamic effects of B-type natriuretic peptide and potentiates B-type natriuretic peptide effects in failing but not normal canine heart. *Journal of the American College of Cardiology*. 2007;49:1079-88.

8. Chen HH. Heart failure: a state of brain natriuretic peptide deficiency or resistance or both! *Journal of the American College of Cardiology*. 2007;49:1089-91.

9. Solomon SD, Zile M, Pieske B, Voors A, Shah A, Kraigher-Krainer E, Shi V, Bransford T, Takeuchi M, Gong J, Lefkowitz M, Packer M and McMurray JJ. The angiotensin receptor neprilysin inhibitor LCZ696 in heart failure with preserved ejection fraction: a phase 2 double-blind randomised controlled trial. *Lancet (London, England)*. 2012;380:1387-95.

10. Packer M, McMurray JJ, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile M, Andersen K, Arango JL, Arnold JM, Belohlavek J,

Bohm M, Boytsov S, Burgess LJ, Cabrera W, Calvo C, Chen CH, Dukat A, Duarte YC, Erglis A, Fu M, Gomez E, Gonzalez-Medina A, Hagege AA, Huang J, Katova T, Kiatchoosakun S, Kim KS, Kozan O, Llamas EB, Martinez F, Merkely B, Mendoza I, Mosterd A, Negrusz-Kawecka M, Peuhkurinen K, Ramires FJ, Refsgaard J, Rosenthal A, Senni M, Sibulo AS, Jr., Silva-Cardoso J, Squire IB, Starling RC, Teerlink JR, Vanhaecke J, Vinereanu D and Wong RC. Angiotensin receptor neprilysin inhibition compared with enalapril on the risk of clinical progression in surviving patients with heart failure. *Circulation*. 2015;131:54-61.

11. Miller WL, Phelps MA, Wood CM, Schellenberger U, Van Le A, Perichon R and Jaffe AS. Comparison of mass spectrometry and clinical assay measurements of circulating fragments of B-type natriuretic peptide in patients with chronic heart failure. *Circulation Heart failure*. 2011;4:355-60.

12. Cruden NL, Fox KA, Ludlam CA, Johnston NR and Newby DE. Neutral endopeptidase inhibition augments vascular actions of bradykinin in patients treated with angiotensin-converting enzyme inhibition. *Hypertension*. 2004;44:913-8.

13. Lainchbury JG, Richards AM, Nicholls MG, Espiner EA and Yandle TG. Brain natriuretic peptide and neutral endopeptidase inhibition in left ventricular impairment. *The Journal of clinical endocrinology and metabolism*. 1999;84:723-9.

14. Barletta G, Lazzeri C, Vecchiarino S, Del Bene R, Messeri G, Dello Sbarba A, Mannelli M and La Villa G. Low-dose C-type natriuretic peptide does not affect cardiac and renal function in humans. *Hypertension*. 1998;31:802-8.

15. Pham I, Sediame S, Maistre G, Roudot-Thoraval F, Chabrier PE, Carayon A and Adnot S. Renal and vascular effects of C-type and atrial natriuretic peptides in humans. *The American journal of physiology*. 1997;273:R1457-64.

16. Nakamura M, Arakawa N, Yoshida H, Makita S and Hiramori K. Vasodilatory effects of C-type natriuretic peptide on forearm resistance vessels are distinct from those of atrial natriuretic peptide in chronic heart failure. *Circulation*. 1994;90:1210-4.

17. Hunt PJ, Richards AM, Espiner EA, Nicholls MG and Yandle TG. Bioactivity and metabolism of C-type natriuretic peptide in normal man. *The Journal of clinical endocrinology and metabolism*. 1994;78:1428-35.

18. Ichiki T, Huntley BK, Sangaralingham SJ and Burnett JC, Jr. Pro-Atrial Natriuretic Peptide: A Novel Guanylyl Cyclase-A Receptor Activator That Goes Beyond Atrial and B-Type Natriuretic Peptides. *JACC Heart failure*. 2015;3:715-23.

19. Dalzell JR, Seed A, Berry C, Whelan CJ, Petrie MC, Padmanabhan N, Clarke A, Biggerstaff F, Hillier C and McMurray JJ. Effects of neutral endopeptidase (neprilysin) inhibition on the response to other vasoactive peptides in small human resistance arteries: studies with thiorphan and omapatrilat. *Cardiovascular therapeutics*. 2014;32:13-8.

20. Honing ML, Smits P, Morrison PJ, Burnett JC, Jr. and Rabelink TJ. C-type natriuretic peptide-induced vasodilation is dependent on hyperpolarization in human forearm resistance vessels. *Hypertension*. 2001;37:1179-83.

21. Devin JK, Pretorius M, Nian H, Yu C, Billings FTt and Brown NJ. Dipeptidyl-peptidase 4 inhibition and the vascular effects of glucagon-like peptide-1 and brain natriuretic peptide in the human forearm. *Journal of the American Heart Association*. 2014;3.

22. Brandt I, Lambeir AM, Ketelslegers JM, Vanderheyden M, Scharpe S and De Meester I. Dipeptidyl-peptidase IV converts intact B-type natriuretic peptide into its des-SerPro form. *Clinical chemistry*. 2006;52:82-7.

23. Rawlings ND, Barrett AJ and Bateman A. Using the MEROPS Database for Proteolytic Enzymes and Their Inhibitors and Substrates. *Current protocols in bioinformatics*. 2014;48:1.25.1-33.

24. Matsas R, Kenny AJ and Turner AJ. The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. *The Biochemical journal*. 1984;223:433-40.

25. Watanabe Y, Nakajima K, Shimamori Y and Fujimoto Y. Comparison of the hydrolysis of the three types of natriuretic peptides by human kidney neutral endopeptidase 24.11. *Biochemical and molecular medicine*. 1997;61:47-51.

26. Wang L, Sadoun E, Stephens RE and Ward PE. Metabolism of substance P and neurokinin A by human vascular endothelium and smooth muscle. *Peptides*. 1994;15:497-503.

27. Gainer JV, Morrow JD, Loveland A, King DJ and Brown NJ. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. *The New England journal of medicine*. 1998;339:1285-92.

28. Pretorius M, Rosenbaum D, Vaughan DE and Brown NJ. Angiotensin-converting enzyme inhibition increases human vascular tissue-type plasminogen activator release through endogenous bradykinin. *Circulation*. 2003;107:579-85.

29. Witherow FN, Helmy A, Webb DJ, Fox KA and Newby DE. Bradykinin contributes to the vasodilator effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Circulation*. 2001;104:2177-81.

30. Devin JK, Pretorius M, Nian H, Yu C, Billings FTt and Brown NJ. Substance P increases sympathetic activity during combined angiotensin-converting enzyme and dipeptidyl peptidase-4 inhibition. *Hypertension*. 2014;63:951-7.

31. Newby DE, Sciberras DG, Ferro CJ, Gertz BJ, Sommerville D, Majumdar A, Lowry RC and Webb DJ. Substance P-induced vasodilatation is mediated by the neurokinin type 1 receptor

but does not contribute to basal vascular tone in man. *British journal of clinical pharmacology*. 1999;48:336-44.

32. Latini R, Masson S, Anand I, Salio M, Hester A, Judd D, Barlera S, Maggioni AP, Tognoni G and Cohn JN. The comparative prognostic value of plasma neurohormones at baseline in patients with heart failure enrolled in Val-HeFT. *European heart journal*. 2004;25:292-9.

33. Kostis JB, Packer M, Black HR, Schmieder R, Henry D and Levy E. Omapatrilat and enalapril in patients with hypertension: the Omapatrilat Cardiovascular Treatment vs. Enalapril (OCTAVE) trial. *American journal of hypertension*. 2004;17:103-11.

34. Byrd JB, Adam A and Brown NJ. Angiotensin-converting enzyme inhibitor-associated angioedema. *Immunology and allergy clinics of North America*. 2006;26:725-37.

35. Ward PE, Chow A and Drapeau G. Metabolism of bradykinin agonists and antagonists by plasma aminopeptidase P. *Biochemical pharmacology*. 1991;42:721-7.

36. Wang LH, Ahmad S, Benter IF, Chow A, Mizutani S and Ward PE. Differential processing of substance P and neurokinin A by plasma dipeptidyl(amino)peptidase IV, aminopeptidase M and angiotensin converting enzyme. *Peptides*. 1991;12:1357-64.

37. Pare G, Kubo M, Byrd JB, McCarty CA, Woodard-Grice A, Teo KK, Anand SS, Zuvich RL, Bradford Y, Ross S, Nakamura Y, Ritchie M and Brown NJ. Genetic variants associated with angiotensin-converting enzyme inhibitor-associated angioedema. *Pharmacogenetics and genomics*. 2013;23:470-8.

38. Brown NJ, Ray WA, Snowden M and Griffin MR. Black Americans have an increased rate of angiotensin converting enzyme inhibitor-associated angioedema. *Clinical pharmacology and therapeutics*. 1996;60:8-13.

39. Miller DR, Oliveria SA, Berlowitz DR, Fincke BG, Stang P and Lillienfeld DE. Angioedema incidence in US veterans initiating angiotensin-converting enzyme inhibitors. *Hypertension*. 2008;51:1624-30.

40. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *The New England journal of medicine*. 1987;316:1429-35.

41. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. The SOLVD Investigators. *The New England journal of medicine*. 1991;325:293-302.

42. Cohn JN and Tognoni G. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. *The New England journal of medicine*. 2001;345:1667-75.

43. Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). *Lancet (London, England)*. 1999;353:2001-7.

44. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J and Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *The New England journal of medicine*. 1999;341:709-17.

45. Feng L, Karpinski PH, Sutton P, Liu Y, Hook DF, Hu B, Blacklock TJ, Fanwick PE, Prashad M, Godtfredsen S and Ziltener C. LCZ696: a dual-acting sodium supramolecular complex. *Tetrahedron Letters*. 2012;53:275-276.

46. Richards AM, Wittert GA, Espiner EA, Yandle TG, Ikram H and Frampton C. Effect of inhibition of endopeptidase 24.11 on responses to angiotensin II in human volunteers. *Circulation research*. 1992;71:1501-7.

47. Richards AM, Wittert GA, Crozier IG, Espiner EA, Yandle TG, Ikram H and Frampton C. Chronic inhibition of endopeptidase 24.11 in essential hypertension: evidence for enhanced atrial natriuretic peptide and angiotensin II. *Journal of hypertension*. 1993;11:407-16.

48. Lu B, Figini M, Emanueli C, Geppetti P, Grady EF, Gerard NP, Ansell J, Payan DG, Gerard C and Bunnett N. The control of microvascular permeability and blood pressure by neutral endopeptidase. *Nature medicine*. 1997;3:904-7.

49. Deddish PA, Marcic BM, Tan F, Jackman HL, Chen Z and Erdos EG. Neprilysin inhibitors potentiate effects of bradykinin on b2 receptor. *Hypertension*. 2002;39:619-23.

50. Witherow FN, Dawson P, Ludlam CA, Fox KA and Newby DE. Marked bradykinin-induced tissue plasminogen activator release in patients with heart failure maintained on long-term angiotensin-converting enzyme inhibitor therapy. *Journal of the American College of Cardiology*. 2002;40:961-6.

51. Cruden NL, Witherow FN, Webb DJ, Fox KA and Newby DE. Bradykinin contributes to the systemic hemodynamic effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:1043-8.

52. Seyedi N, Maruyama R and Levi R. Bradykinin activates a cross-signaling pathway between sensory and adrenergic nerve endings in the heart: a novel mechanism of ischemic norepinephrine release? *The Journal of pharmacology and experimental therapeutics*. 1999;290:656-63.

53. Emanueli C, Grady EF, Madeddu P, Figini M, Bunnett NW, Parisi D, Regoli D and Geppetti P. Acute ACE inhibition causes plasma extravasation in mice that is mediated by bradykinin and substance P. *Hypertension*. 1998;31:1299-304.

54. Sulpizio AC, Pullen MA, Edwards RM and Brooks DP. The effect of acute angiotensin-converting enzyme and neutral endopeptidase 24.11 inhibition on plasma extravasation in the rat. *The Journal of pharmacology and experimental therapeutics*. 2004;309:1141-7.

55. Brown NJ, Gainer JV, Stein CM and Vaughan DE. Bradykinin stimulates tissue plasminogen activator release in human vasculature. *Hypertension*. 1999;33:1431-5.

56. Brown NJ, Gainer JV, Murphey LJ and Vaughan DE. Bradykinin stimulates tissue plasminogen activator release from human forearm vasculature through B(2) receptor-dependent, NO synthase-independent, and cyclooxygenase-independent pathway. *Circulation*. 2000;102:2190-6.

57. Pretorius M, Rosenbaum DA, Lefebvre J, Vaughan DE and Brown NJ. Smoking impairs bradykinin-stimulated t-PA release. *Hypertension*. 2002;39:767-71.

58. Newby DE, Wright RA, Labinjoh C, Ludlam CA, Fox KA, Boon NA and Webb DJ. Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation*. 1999;99:1411-5.

59. Ridderstrale W, Ulfhammer E, Jern S and Hrafnkelsdottir T. Impaired capacity for stimulated fibrinolysis in primary hypertension is restored by antihypertensive therapy. *Hypertension*. 2006;47:686-91.

60. Minai K, Matsumoto T, Horie H, Ohira N, Takashima H, Yokohama H and Kinoshita M. Bradykinin stimulates the release of tissue plasminogen activator in human coronary circulation: effects of angiotensin-converting enzyme inhibitors. *Journal of the American College of Cardiology*. 2001;37:1565-70.

61. Robinson SD, Ludlam CA, Boon NA and Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1651-6.

62. Pretorius M, Scholl FG, McFarlane JA, Murphey LJ and Brown NJ. A pilot study indicating that bradykinin B2 receptor antagonism attenuates protamine-related hypotension after cardiopulmonary bypass. *Clinical pharmacology and therapeutics*. 2005;78:477-85.

63. Marney AM, Ma J, Luther JM, Ikizler TA and Brown NJ. Endogenous bradykinin contributes to increased plasminogen activator inhibitor 1 antigen following hemodialysis. *Journal of the American Society of Nephrology : JASN*. 2009;20:2246-52.

64. LeFebvre J, Shintani A, Gebretsadik T, Petro JR, Murphey LJ and Brown NJ. Bradykinin B(2) receptor does not contribute to blood pressure lowering during AT(1) receptor blockade. *The Journal of pharmacology and experimental therapeutics*. 2007;320:1261-7.

65. Pretorius M, McFarlane JA, Vaughan DE, Brown NJ and Murphey LJ. Angiotensin-converting enzyme inhibition and smoking potentiate the kinin response to cardiopulmonary bypass. *Clinical pharmacology and therapeutics*. 2004;76:379-87.

66. Bas M, Greve J, Strassen U, Khosravani F, Hoffmann TK and Kojda G. Angioedema induced by cardiovascular drugs: new players join old friends. *Allergy*. 2015;70:1196-200.

67. Kristensen SL, Preiss D, Jhund PS, Squire I, Cardoso JS, Merkely B, Martinez F, Starling RC, Desai AS, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile MR, McMurray JJ and Packer M. Risk Related to Pre-Diabetes Mellitus and Diabetes Mellitus in Heart Failure With Reduced Ejection Fraction: Insights From Prospective Comparison of ARNI With ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure Trial. *Circulation Heart failure*. 2016;9.

68. Gu J, Noe A, Chandra P, Al-Fayoumi S, Ligueros-Saylan M, Sarangapani R, Maahs S, Ksander G, Rigel DF, Jeng AY, Lin TH, Zheng W and Dole WP. Pharmacokinetics and pharmacodynamics of LCZ696, a novel dual-acting angiotensin receptor-neprilysin inhibitor (ARNi). *Journal of clinical pharmacology*. 2010;50:401-14.

69. Bergman AJ, Stevens C, Zhou Y, Yi B, Laethem M, De Smet M, Snyder K, Hilliard D, Tanaka W, Zeng W, Tanen M, Wang AQ, Chen L, Winchell G, Davies MJ, Ramael S, Wagner JA and Herman GA. Pharmacokinetic and pharmacodynamic properties of multiple oral doses of sitagliptin, a dipeptidyl peptidase-IV inhibitor: a double-blind, randomized, placebo-controlled study in healthy male volunteers. *Clinical therapeutics*. 2006;28:55-72.

70. Pretorius MM, Gainer JV, Van Guilder GP, Coelho EB, Luther JM, Fong P, Rosenbaum DD, Malave HA, Yu C, Ritchie MD, Vaughan DE and Brown NJ. The bradykinin type 2 receptor BE1 polymorphism and ethnicity influence systolic blood pressure and vascular resistance. *Clinical pharmacology and therapeutics*. 2008;83:122-9.

71. Ozkor MA, Rahman AM, Murrow JR, Kavtaradze N, Lin J, Manatunga A, Hayek S and Quyyumi AA. Differences in vascular nitric oxide and endothelium-derived hyperpolarizing factor bioavailability in blacks and whites. *Arteriosclerosis, thrombosis, and vascular biology*. 2014;34:1320-7.

72. Pretorius M, Luther JM, Murphey LJ, Vaughan DE and Brown NJ. Angiotensin-converting enzyme inhibition increases basal vascular tissue plasminogen activator release in women but not in men. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2435-40.

73. Ruilope LM, Dukat A, Bohm M, Lacourciere Y, Gong J and Lefkowitz MP. Blood-pressure reduction with LCZ696, a novel dual-acting inhibitor of the angiotensin II receptor and neprilysin: a randomised, double-blind, placebo-controlled, active comparator study. *Lancet (London, England)*. 2010;375:1255-66.

74. Brown NJ, Agirbasli M and Vaughan DE. Comparative effect of angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor antagonism on plasma fibrinolytic balance in humans. *Hypertension*. 1999;34:285-90.

75. Gamboa JL, Pretorius M, Todd-Tzanetos DR, Luther JM, Yu C, Ikizler TA and Brown NJ. Comparative effects of angiotensin-converting enzyme inhibition and angiotensin-receptor blockade on inflammation during hemodialysis. *Journal of the American Society of Nephrology : JASN*. 2012;23:334-42.

76. Oya H, Nagaya N, Furuichi S, Nishikimi T, Ueno K, Nakanishi N, Yamagishi M, Kangawa K and Miyatake K. Comparison of intravenous adrenomedullin with atrial natriuretic peptide in patients with congestive heart failure. *The American journal of cardiology*. 2000;86:94-8.

77. Lisy O, Jougasaki M, Schirger JA, Chen HH, Barclay PT and Burnett JC, Jr. Neutral endopeptidase inhibition potentiates the natriuretic actions of adrenomedullin. *The American journal of physiology*. 1998;275:F410-4.

78. Brown NJ, Byiers S, Carr D, Maldonado M and Warner BA. Dipeptidyl peptidase-IV inhibitor use associated with increased risk of ACE inhibitor-associated angioedema. *Hypertension*. 2009;54:516-23.

79. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mosenzon O, McGuire DK, Ray KK, Leiter LA and Raz I. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *The New England journal of medicine*. 2013;369:1317-26.