Neprilysin inhibition for pulmonary arterial hypertension: a randomized, double-blind, placebo-controlled, proof-of-concept trial

Running title: COMbination therapy in PAH with RacEcadotril (COMPARE)

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ABSTRACT

Background and purpose: Pulmonary arterial hypertension (PAH) is an incurable, incapacitating disorder resulting from increased pulmonary vascular resistance, pulmonary arterial remodeling and right ventricular failure. In pre-clinical models, combination of a phosphodiesterase 5 inhibitor (PDE5i) with a neprilysin inhibitor augments natriuretic peptide bioactivity, promotes cyclic GMP signaling, and reverses the structural and hemodynamic deficits that characterize PAH. Herein, we conducted a randomized, double-blind, placebo-controlled trial to assess the efficacy and safety of repurposing the neprilysin inhibitor, racecadotril, in PAH.

Experimental approach: 21 PAH patients stable on PDE5i therapy were recruited. Acute hemodynamic and biochemical changes following a single dose of racecadotril or matching placebo were determined; this was followed by a 14 day safety and efficacy evaluation. The primary endpoint in both steps was circulating atrial natriuretic peptide (ANP) concentration (Δ_{max}), with secondary outcomes including pulmonary and systemic hemodynamics plus mechanistic biomarkers.

Key results: Acute administration of racecadotril (100mg) resulted in a 79% (95%CI, +6,+203) increase in the plasma ANP concentration and a 106% (+28,+229) increase in plasma cyclic GMP levels, with a concomitant 14% (-24,-3) fall in pulmonary vascular resistance. Racecadotril (100mg; tid) treatment for 14 days resulted in a 19% (-18,+72) rise in plasma ANP concentration. Neither acute (-16%, -28,+1) nor chronic (-4%, -21,+16) administration of racecadotril resulted in a significant drop in MABP or any serious adverse effects.

Conclusions and implications: This Phase IIa evaluation provides proof-of-principle evidence that neprilysin inhibitors may have therapeutic utility in PAH and warrants a larger-scale prospective trial.

KEYWORDS

Natriuretic peptide, Neutral endopeptidase, Cyclic GMP, Guanylyl cyclase, Phosphodiesterase

BULLET POINT SUMMARY

What is already known

- Drugs that elevate cyclic GMP (i.e. PDE5 inhibitors) have proven efficacy in pulmonary arterial hypertension (PAH)
- Neprilysin inhibitors augment natriuretic peptide-driven cyclic GMP signalling and are licensed for use in heart failure

What this study adds

- Combining PDE5 and neprilysin inhibitors produces additive beneficial haemodynamic effects in PAH patients

Clinical significance

- These data support a larger prospective trial evaluating the repurposing of neprilysin inhibitors for PAH

NON-STANDARD ABBREVIATIONS

6MWD	6 minute walk distance
ACE	Angiotensin converting enzyme
ALT	Alanine transaminase
ANP	Atrial natriuretic peptide
APTT	Activated partial thromboplastin time
AST	Aspartate transaminase
BNP	Brain natriuretic peptide
cGMP	Cyclic guanosine-3',5'-monophosphate
CHD	Congenital heart disease
CNP	C-type natriuretic peptide
CO	Cardiac output
CTD	Connective tissue disease
eGFR	Estimated glomerular filtration rate
ERA	Endothelin receptor antagonist
ET-1	Endothelin-1
GC-1	NO-sensitive guanylyl cyclase α1β1 isoform

GC-2	NO-sensitive guanylyl cyclase α2β1 isoform
GC-A	Natriuretic peptide-sensitive guanylyl cyclase-A
Hb	Haemoglobin
HR	Heart rate
IDMC	Independent data monitoring committee
IMP	Investigational medicinal product
LA	Left atrium
LVEF	Left ventricular ejection fraction
MABP	Mean arterial blood pressure
mPAP	Mean pulmonary artery pressure
NEP	Neprilysin/neutral endopeptidase
NOx	$[NO_2^-] + [NO_3^-]$
PAH	Pulmonary arterial hypertension
PCWP	Pulmonary capillary wedge pressure
PDE5i	Phosphodiesterase 5 inhibitor
PH	Pulmonary hypertension
PT	Prothrombin time
PVR	Pulmonary vascular resistance
RA	Right atrium
RBC	Red blood cell count
RHC	Right heart catheterization
RV	Right ventricle
RVH	Right ventricular hypertrophy
SV	Stroke volume
TAPSE	Tricuspid annular plane systolic excursion
TRV	Tricuspid regurgitant velocity
TSC	Trial steering committee
ULN	Upper limit of normal
WBC	White blood cell count

INTRODUCTION

Pulmonary hypertension (PH) is progressive, debilitating disorder with high associated morbidity and mortality. It is characterized by increased pulmonary vascular resistance (PVR) and a degenerative remodeling of the pulmonary arterial tree, leading initially to right ventricular (RV) failure and death. A cure remains elusive and contemporary strategies to improve therapy have focused on developing drug combinations that synergize in the pulmonary circulation, improving hemodynamics and reversing structural remodeling (Ghofrani et al., 2002; Hoeper, Faulenbach, Golpon, Winkler, Welte & Niedermeyer, 2004; Humbert et al., 2004).

Targeting cyclic GMP (cGMP) is effective in treating PH, exemplified by the clinical use of phosphodiesterase 5 inhibitors (PDE5i) (Galie et al., 2005) and soluble guanylyl cyclase (NO-sensitive GC-1 & GC-2; 'sGC') stimulators (Ghofrani et al., 2013). However, these approaches only slow disease progression rather than offering resolution. Pre-clinical studies exploring cGMP signaling in the pulmonary circulation have provided compelling evidence to support atrial natriuretic peptide (ANP) and/or brain natriuretic peptide (BNP) -driven cGMP production in optimizing cGMP-based therapy founded on PDE inhibition, both in terms of efficacy and (pulmonary) selectivity. For example, the beneficial effects of PDE5i in experimental models of chronic hypoxia-induced PH are blunted in animals lacking GC-A (the cognate receptor for ANP and BNP) (Zhao, Mason, Strange, Walker & Wilkins, 2003); a similarly exacerbated phenotype in observed in GC-A$^{-/-}$ animals with bleomycin-triggered pulmonary fibrosis and secondary PH, with an associated loss of efficacy of PDE5i (Baliga, Scotton, Trinder, Chambers, MacAllister & Hobbs, 2014). Furthermore, infusion of natriuretic peptides in the presence of the PDE5i sildenafil, synergistically reduces pulmonary artery pressure in hypoxia-induced PH (Preston, Hill, Gambardella, Warburton & Klinger, 2004); a similar interaction has been reported between urodilatin (a renal-specific ANP variant) and the PDE5i dipyridamole (Schermuly et al., 2001). Similarly, infusion of monoclonal antibodies neutralizing ANP stimulates the development of PH in response to hypoxia (Raffestin et al., 1992), while exogenously administered or adenovirus-mediated natriuretic peptide supplementation protects against PH and the accompanying RVH (Jin, Yang, Chen, Jackson & Oparil, 1988; Klinger et al., 1993; Louzier et al., 2001). Such findings suggest that the mechanism of pulmonary selectivity of PDE5i depends on the

bioactivity of natriuretic peptides and, additionally, that in PH release of natriuretic peptides represents a cytoprotective mechanism that slows disease progression.

One potential mechanism that might be utilized pharmacologically in PH to promote this protective role of natriuretic peptides is to inhibit the enzyme neprilysin (or neutral endopeptidase, NEP). NEP is a membrane bound zinc metallopeptidase responsible for the metabolism and inactivation of an array of vasodilator (e.g. natriuretic peptides, adrenomedullin, vasoactive intestinal peptide) and vasoconstrictor (e.g. Angiotensin II, Endothelin-1 (ET-1)) peptides (Erdos & Skidgel, 1989; Kenny & Stephenson, 1988). Indeed, pre-clinical evidence indicates that $NEP^{-/-}$ mice are less susceptible to hypoxia-induced pulmonary edema (Irwin, Patot, Tucker & Bowen, 2005) and that inhibition of NEP attenuates hypoxia-induced PH by potentiating the action of natriuretic peptides (Klinger, Petit, Warburton, Wrenn, Arnal & Hill, 1993; Thompson, Sheedy & Morice, 1994; Winter, Zhao, Krausz & Hughes, 1991). These observations fit with the up-regulation of NEP in the pulmonary circulation during acute lung injury and heart failure (Abassi, Kotob, Golomb, Pieruzzi & Keiser, 1995; Hashimoto, Amaya, Oh-Hashi, Kiuchi & Hashimoto, 2010).

We exploited this 'natriuretic peptide-centric' approach to evaluate the therapeutic potential of a PDE5i and NEPi combination in animal models of PH (Baliga, Scotton, Trinder, Chambers, MacAllister & Hobbs, 2014; Baliga et al., 2008). Our data showed that this dual therapy is superior to either drug alone in hypoxia-induced PH and PH secondary to pulmonary fibrosis. This held true for both hemodynamic (e.g. pulmonary artery pressure) and structural (e.g. right ventricular hypertrophy, pulmonary arterial remodeling) indices; importantly however, combination therapy did not significantly affect systemic blood pressure, confirming selective targeting of the pulmonary vasculature (Baliga, Scotton, Trinder, Chambers, MacAllister & Hobbs, 2014; Baliga et al., 2008). Thus, by combining a PDE5i and a NEPi it is possible to harness further the beneficial effects of natriuretic peptide-cGMP signaling, thereby optimizing pulmonary efficacy and selectivity.

A clear advantage of evaluating this novel PDE5i/NEPi combination in PH patients is the availability of existing licensed medications; the PDE5i sildenafil and tadalafil are prescribed for the treatment of PAH and the NEPi, racecadotril, is also licensed for use in secretory diarrhea. In accord, this report describes a randomized, double-blind, placebo controlled trial to assess the safety and efficacy of repurposing racecadotril in PAH patients stable on PDE5i therapy.

METHODS

Study design and participants

This study was a single-center, double-blind, phase IIa, randomized, placebo-controlled trial conducted in two stages (with planned interim and final analyses), to determine whether administration of the NEPi, racecadotril, to patients with pulmonary arterial hypertension (PAH; WHO Group 1) on PDE5i therapy, increases plasma [ANP] and favorably alters pulmonary hemodynamics without affecting mean arterial blood pressure (MABP). The trial was approved by a local independent research ethics committee (NRES Committee London – Westminster; ref: 13/LO/0387), the Medicines and Healthcare Products Regulatory Agency (ref: 20363/0320/001-0003), documented in approved registries (EudraCT#2012-003921-13), and conducted in accordance with the Declaration of Helsinki (1996) and the principles of the International Conference on Harmonization-Good Clinical Practice guidelines. Trial management was undertaken by the UCL Comprehensive Clinical Trials Unit (CCTU) and an Independent Trial Steering Committee (TSC) and Independent Data Monitoring Committee (IDMC) provided trial oversight. Eligible patients at the Royal Free London NHS Foundation Trust who gave written informed consent were included in the trial.

Step 1

After consenting, patients considered for Step 1 of the trial underwent right heart catheterization (RHC) as routine care. Patients who met the inclusion criteria were randomized to receive a single dose of racecadotril (100mg; p.o.) or matching placebo (concurrently with their existing PDE5i therapy). The access sheath to the catheter was maintained in position so that pulmonary and systemic hemodynamic measurements could be recorded at 1.5 and 2 h following administration of racecadotril or placebo, after which it was removed. Venous blood samples were also collected at baseline, 0, 1, 2, 3 & 6 h following administration of racecadotril or placebo for the assessment of biochemical endpoints.

Step 2

Patients were randomized to receive racecadotril (100mg, tid; p.o.) or matching placebo for 14 days in addition to (and concomitantly with) their current PH-directed

therapy. Systemic blood pressure measurements and venous blood samples were taken at baseline (day 0) and day 14. A telephone conversation at day 7 was undertaken to assess the occurrence of any adverse events.

Endpoints

Step 1

The *primary endpoint* was the maximum change (Δ_{max}) in plasma [ANP]. *Secondary endpoints* comprised a range of pulmonary & systemic hemodynamic indices: Δ_{max} mean pulmonary artery pressure (mPAP), Δ_{max} pulmonary vascular resistance (PVR), Δ_{max} pulmonary capillary wedge pressure (PCWP), Δ_{max} mean arterial blood pressure (MABP), Δ_{max} plasma [BNP], Δ_{max} plasma [NT-proBNP], Δ_{max} plasma [C-type natriuretic peptide (CNP)], Δ_{max} plasma [ET-1], Δ_{max} plasma [cGMP], Δ_{max} plasma [NO_x]

Step 2

The *primary co-endpoints* were safety (adverse effects; assessed for seriousness, severity and causality by on-site clinicians and reported to the IDMC) and Δ_{max} plasma [ANP].

Secondary endpoints comprised a range of systemic hemodynamic indices: Δ_{max} MABP, Δ_{max} plasma [BNP], Δ_{max} plasma [NT-proBNP], Δ_{max} plasma [CNP], Δ_{max} plasma [ET-1], Δ_{max} plasma [cGMP], Δ_{max} plasma [NO_x]

Study drug

The NEPi racecadotril (Hidrasec®, Tiorfan®; benzyl *N*-[3-(acetylthio)-2-benzylpropanoyl]glycinate) is approved in Europe and South America for the treatment of acute diarrhea. Racecadotril reduces intestinal hyper-secretion of water and electrolytes, without affecting basal secretion and has no effect in the normal intestine. When given orally, NEP inhibition is solely peripheral; racecadotril does not affect central NEP activity. It has a reassuring safety record (>1million adult exposures), with rarely reported (<2%) side effects including drowsiness, nausea, dizziness and headaches. In adults, single doses of 2g (i.e. 20 times the therapeutic dose for the treatment of acute diarrhea), have been administered in clinical trials for up to 3 months without causing any harmful effects (Lecomte, 2000). Racecadotril has a rapid onset of action, with maximal inhibition of NEP at 60 mins following a single dose of

100mg (p.o.) in humans (Lecomte, 2000); NEP activity returns to baseline within 8 hours (the biological half-life is approximately 3.5 hours). This PK profile translates to significantly increased plasma ANP levels within 2 h, returning to baseline at approximately 6 h (Kahn et al., 1990). Racecadotril does not induce or inhibit CYP450 enzymes and the principal route of elimination is renal.

Inclusion criteria

1. WHO Group I pulmonary arterial hypertension (i.e. idiopathic, familial or associated with connective tissue diseases)
2. 18-80 years old
3. Technically satisfactory RHC (Step 1 only)
4. Taking sildenafil (20-100 mg; t.i.d.) or tadalafil (20-40mg; o.d.) for at least 1 month
5. No changes to any PH-specific therapies for 1 month
6. Six minute walk distance of >150 m
7. Not pregnant (women only)
8. Able to provide consent for the trial

Exclusion criteria

1. Known sensitivity to racecadotril or its excipients
2. Clinical diagnosis of liver cirrhosis or ALT/AST >2xULN
3. Kidney disease with an estimated glomerular filtration rate (eGFR) of <50 ml/min
4. History of angioedema
5. Systolic blood pressure <85 mmHg
6. Known history of drug or alcohol abuse within six months of enrolment
7. Participation in a clinical study involving another investigational drug
8. Women who are breastfeeding
9. Taking an angiotensin converting enzyme (ACE) inhibitor
10. Any clinical condition for which the investigator would consider the patient unsuitable for the trial

Biochemical assays

All clinical biochemistry (including NT-proBNP) and hematology analyses were conducted at the Royal Free London NHS Foundation Trust. Plasma natriuretic peptide, ET-1 and cGMP concentrations were determined using commercially available enzyme immunoassay (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany). Plasma samples were also analyzed for nitrite (NO_2^-) and nitrate (NO_3^-), as an index of endogenous NO production (total NO_x), using chemiluminescence as described previously (Ignarro, Fukuto, Griscavage, Rogers & Byrns, 1993).

Sample size

Based on previous clinical studies (Berglund, Nyquist, Beermann, Jensen-Urstad & Theodorsson, 1994; Bruins et al., 2004; Tan, Kloppenborg & Benraad, 1989) the between patient standard deviation of the percentage change from baseline in ANP (i.e. primary outcome measure) was estimated as 12.2. Sample size calculations used a two-sided 5% significance level, 80% power and a 2:1 active:placebo randomization ratio.

Step 1

Within each block of 6 patients (in step 1a and step 1b), a sample size of 4 patients on active therapy and 2 patients on placebo enabled detection of a difference between the two groups in the mean % change in ANP of 50%. Combination of the placebo groups from the two blocks of 6 patients, with adjustment for multiple comparisons using the Bonferroni procedure, permitted detection of a difference between placebo and racecadotril in the mean % change in ANP of 21%. By way of precedent, in chronic heart failure 30 mg sinorphan (L-isomer of acetorphan; equivalent to 60 mg of racecadotril) increases plasma ANP by 100% (Kahn et al., 1990) and in cirrhotic patients 100 mg sinorphan elicits a 180% increase in plasma ANP (Dussaule et al., 1991). In PH patients, a 21% increase was therefore considered conservative.

Step 2

Using a 2:1 active:placebo ratio, a sample size of 8 patients on active therapy and 4 patients on placebo enabled detect of a difference between the two groups in the mean % change in ANP of 21%. A 2:1 ratio also provide twice as much

information on the safety profile of the drug combination, with minimal effect on the difference that would be able to be distinguished.

Early patient safety and biological activity adaptive design

Step 1 of the study was designed with an adaptive early recruitment phase to allow early termination in case of lack of biological activity of racecadotril or harms. Step 1 was further broken down into two steps:

Step1a

After the first 6 patients received a single dose of 100mg of racecadotril or placebo, the trial stopped recruitment and key clinical parameters and safety data were reviewed in an unblinded fashion by the IDMC who were asked to consider whether a dose of racecadotril has been identified that:

i. on average increases ANP by a minimum of 20% and
ii. on average decreases PVR by a minimum of 10% and
iii. on average decreases SBP by no more than 10%.

based on whether the 95% confidence interval for the relevant (racecadotril – control) difference excluded any of these pre-specified clinically important changes. The recommendation was to continue recruiting patients without increasing the investigational medicinal product (IMP) dose due to the apparent pharmacodynamic effect.

Step1b

An additional 6 patients were recruited into the study and administered a single dose of 100mg of racecadotril or placebo followed by the IDMC data review in a similar fashion to Step1a. The IDMC recommended proceeding to Step 2 of the trial, recruiting patients at the 100mg dose of racecadotril.

Randomization and blinding

Patients were randomly assigned in a 2:1 ratio to received 100mg of racecadotril or matching placebo using random permuted blocks. The allocation sequence was computer-generated by the trial statistician and concealment of allocation was ensured

by the use of an identical inert placebo, with security in place to ensure allocation of unblinded codes could not be accessed by anyone in the trial team other than the statistician and the pharmacist (for manufacturing and labeling purposes).

Statistical analyses

All statistical tests were two-sided with a significance level of 5%. All continuous efficacy outcomes were log transformed for the statistical analyses. Results were back transformed and are presented as geometric means (GM) and 95% confidence intervals (CI), or ratios and 95% CI. All statistical analysis was based on a pre-specified Statistical Analysis Plan which was reviewed by the TSC and IDMC. All statistical analyses were performed using Stata/IC (Ver.14.2 RRID:SCR_012763; StataCorp, College Station, TX, 77845 USA).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

RESULTS

Step 1

Overview

17 patients were screened at the Royal Free London NHS Foundation Trust, between February 2014 and December 2015, for entry to Step 1 of the trial. A total of 15 participants were randomized; Of these, two participants did not receive racecadotril (one due to technical difficulties with the RHC and the other was ruled unfit to receive IMP) and one participant was withdrawn & replaced (for logistical reasons). This is depicted in the CONSORT flow chart (***Figure 1***). The analysis population for Step1 comprises 9 patients allocated to racecadotril and 4 allocated to placebo.

Characteristics of the study population

All baseline and procedural characteristics were similar between groups, except the plasma [CNP] which by chance was higher in the placebo group (***Table 1***). The mean age of the trial participants was 57 years, with 77% (10/13) female. All participants had significantly raised mPAP (45.54±2.8mmHg) and PVR (514.5±39.9dynes/sec/cm^{-5}) consistent with a PAH diagnosis.

Primary endpoint

Administration of racecadotril caused an increase in plasma [ANP] that peaked at 2 h and returned to baseline by 6 h (***Figure 2***). This pharmacokinetic profile mirrored that observed in previous studies evaluating racecadotril in left heart failure patients and closely aligns with the biological half-life of the drug (Dussaule et al., 1991; Kahn et al., 1990; Lecomte, 2000). Racecadotril caused a significant increase (79%) in the maximum plasma [ANP] whereas a small decrease was observed in the placebo arm (albeit two patients receiving placebo exhibited an increase in plasma [ANP]; (***Figure 2***). These data suggest that, akin to previous studies in heart failure, NEP inhibition causes a significant rise in circulating ANP levels.

Secondary endpoints

The increases in plasma [ANP] in patients receiving racecadotril was mirrored temporally and in magnitude by plasma cGMP concentrations (***Figure 2***). Thus, cGMP levels peaked at the 2 h timepoint and were 106% higher compared to baseline, returning to pre-IMP concentrations after 6 h (***Figure 2***). Indeed, plasma [cGMP] was significantly increased in the racecadotril arm *per se* and in comparison to placebo (***Figure 2***). These data imply that the temporal elevation in circulating ANP concentrations results in a commensurate increase in cGMP formation (via activation of GC-A).

The parallel rise in plasma levels of ANP and cGMP in response to racecadotril exerted a positive influence on pulmonary hemodynamics without having an overt effect on the systemic circulation. In this setting, PVR and PCWP were reduced in patients receiving racecadotril compared to placebo (although one of four patients in the placebo arm also exhibited a reduction in PVR and PCWP), and the peak response on pulmonary hemodynamics (2 h) matched the maximum rise in both plasma [ANP] and [cGMP] (***Figure 3***); indeed, there was a significant correlation between increases

in plasma [ANP] and plasma [cGMP] with reductions in PVR (***Figure 2***), tendering some elementary indication of causality. A similar trend was observed with mPAP (***Figure 4***) in the absence of any overt change in CO or HR (***Table 2***). However, there was no significant effect of racecadotril on MABP between the active and control arms (***Figure 4***), with MABP falling modestly over time in both groups.

Importantly, plasma [ET-1] was not changed following exposure to racecadotril (***Figure 5***). This is a key safety readout since NEP metabolizes a number of vasoactive peptides, including ET-1 (Llorens-Cortes, Huang, Vicart, Gasc, Paulin & Corvol, 1992), which is known to be a key driver of pathology in PAH and the target of existing therapy (Luscher & Barton, 2000; Stewart, Levy, Cernacek & Langleben, 1991; Williamson et al., 2000). Additional mechanistic biomarkers were also unaltered following racecadotril administration in comparison to placebo. This included plasma concentrations of other members of the natriuretic peptide family including BNP (***Table 2***), NT-proBNP (***Figure 5***) and CNP (***Table 2***), and plasma NO_x (as an index of NO bioactivity) (***Table 2***). The lack of effect of NEP inhibition on these biomarkers intimates that the rise in plasma [cGMP] and accompanying reductions in PVR and PCWP are solely mediated by increased ANP bioactivity.

Safety

No serious adverse events (SAE) or serious adverse reactions (SAR) were reported in Step 1 of the trial. Only one patient allocated to racecadotril reported (two) minor AEs that were potentially drug-related; vomiting and epistaxis. Full details of the AEs reported by severity grade, seriousness criteria, causality and trial step can be found in ***Table 5***.

Step 2

Overview

A total of 10 patients were screened at the Royal Free London NHS Foundation Trust, between May 2016 and October 2016, for possible entry to Step 2 of the trial. Of these, 2 were excluded before they were randomized; one patient was unable to attend further visits, and a second individual declined to participate after initially consenting. Due to logistical difficulties in recruiting patients to this phase of the study only 8 participants were randomized; this fell short of the necessary 12 individuals, randomized 1:2 to the placebo and racecadotril arms, to be powered to detect a 50%

difference in plasma [ANP], the primary endpoint. This is depicted in the CONSORT flow chart (***Figure 1***). The analysis population for Step 2 comprises 5 patients allocated to racecadotril and 3 allocated to placebo.

Characteristics of the study population & treatment compliance

All baseline and procedural characteristics were similar between groups, except by chance the plasma [ANP], plasma [CNP], plasma [cGMP] and plasma [NO_x] were higher in the placebo group (***Table 3***). The mean age of the trial participants was 68 years, with 88% (7/8) female.

All patients were treatment compliant; that is, they took at least 30 of the 42 tablets during the repeat dosage schedule of 12-14 days. The median number of capsules taken in the placebo and racecadotril groups was 39 (IQR 39 to 40) and 40 (IQR 37 to 41), respectively.

Primary endpoint

Administration of racecadotril for 14 days caused ~25% increase in plasma [ANP], whereas those individuals receiving placebo saw a drop in their plasma [ANP] of ~10%; however, an intra-patient analysis did not reveal a significant increase (P=0.19), despite an apparent trend (***Figure 6***). The clear increase in circulating ANP levels brought about by NEP inhibition in Step 1 was therefore not maintained to the same extent over a period of two weeks in response to t.i.d administration of the same dose of racecadotril.

Secondary endpoints

Despite the inability to detect an increase in plasma [ANP] following chronic dosing, arguably the most important goal of Step 2 was to assess the safety of daily use of racecadotril in patients with PH. Administration of racecadotril over 14 days did not alter MABP, substantiating the acute lack of effect in Step 1 (***Figure 6***); moreover, chronic administration of racecadotril did not cause any SAEs (see below).

Additional mechanistic biomarkers (e.g. cGMP, BNP, NT-proBNP, CNP & NOx) were unaltered following racecadotril administration in comparison to placebo (***Table 4***). Again, NEP inhibition did not alter plasma [ET-1] levels (***Table 4***), which is critical to any potential therapeutic application of the drug in PAH.

Safety

No serious adverse events (SAE) or serious adverse reactions (SAR) were reported in Step 2 of the trial. ***Table 5*** shows there were a total of 14 minor adverse events (AEs) reported by patients allocated to racecadotril, and thought to be potentially drug-related, included vomiting, epistaxis, dizziness and headache. The difference in the proportion of patients reporting at least one AE between the two arms (i.e. 0.33 in the placebo group vs. 0.80 in the racecadotril) was not statistically significant (p=0.464; Fisher's exact test).

DISCUSSION

The introduction of PDE5i and sGC stimulators, both of which promote cGMP-dependent signaling, has significantly improved the treatment of PAH (Galie et al., 2005; Ghofrani et al., 2013). However, a significant cohort of PAH patients do not respond well to these interventions, or experience a diminution of efficacy over time (Galie, Manes, Negro, Palazzini, Bacchi-Reggiani & Branzi, 2009). This is particularly true of PDE5i since blockade of cGMP breakdown is inexorably dependent on endogenous NO and/or natriuretic peptide signaling to drive efficacy (i.e. endogenous input to the system), which often wane with disease progression. The development of sGC stimulators has negated this decline in efficacy to a certain extent, circumventing the reliance on endogenous NO signaling; this is demonstrated, arguably, by maintained improvement in 6MWD, WHO functional class and NT-proBNP levels two years after initiation of treatment (Rubin et al., 2015). However, pharmacological activation of sGC appears not to provide any pulmonary specificity, eliciting a generic vasodilator influence which can result in dose-limiting systemic hypotension or drug discontinuation for related adverse effects (Galie, Muller, Scalise & Grunig, 2015; Ghofrani et al., 2016; Rubin et al., 2015). Thus, identification of drug combinations which trigger cGMP signaling preferentially in the pulmonary vasculature and RV have potential to optimally harness the therapeutic potential of cGMP above and beyond existing therapy, and further improve the treatment of PH.

Targeting natriuretic peptide signaling may address this aspiration. Evidence from pre-clinical and clinical studies suggests that the therapeutic efficacy of PDE5i in PH is primarily dependent on promoting natriuretic peptide bioactivity rather than NO (Baliga, Scotton, Trinder, Chambers, MacAllister & Hobbs, 2014; Jin, Yang, Chen,

Jackson & Oparil, 1988; Klinger et al., 1993; Louzier et al., 2001; Zhao, Mason, Strange, Walker & Wilkins, 2003). This concept provides a clear rationale for evaluating combination therapy with PDE5i and NEPi (which slow natriuretic peptide inactivation) in PH patients. Indeed, a precedent for such a therapeutic strategy exists in left-sided heart failure; the dual NEPi (sacubitril)-angiotensin receptor blocker (valsartan), LCZ696, has been demonstrated to reduce mortality and hospitalizations by approximately 20% in a large scale RCT (McMurray et al., 2014).

Herein, acute administration of racecadotril to PH patients stable on PDE5i therapy caused a marked augmentation of circulating ANP concentrations that peaked at 2h and returned to baseline within 6h. This pharmacodynamic profile closely matches the plasma half-life of the drug (6-8 h). The circulating levels of cGMP followed an almost identical time-course and magnitude. These commensurate increases provide good evidence of the anticipated efficacy of NEPi in the PAH patients population, increasing ANP/GC-A/cGMP signaling for therapeutic gain. Accordingly, acute administration of racecadotril produced a commensurate effect on pulmonary hemodynamics (i.e. PVR and PCWP), but not the systemic vasculature, as predicted by pre-clinical models (a phenomenon also observed following administration of NEPi to healthy volunteers (Ando, Rahman, Butler, Senn & Floras, 1995)). The time-course of reduction in pulmonary hemodynamic variables also matched the biological half-life of the drug and the temporal profile of the enhancement of ANP and cGMP. Yet, there was little or no effect on MABP across the entire time-course of the acute study, which was corroborated by observations from the 14 day evaluation in which systemic hemodynamics remained unchanged between the placebo and active arms. These findings give credence to thesis that combination of PDE5i and NEPi produces a pulmonary-specific effect on hemodynamics.

Importantly, administration of racecadotril was not associated with an overt increase in circulating ET-1 concentrations. This is an important finding since NEP is thought to underpin a principle route of ET-1 breakdown & inactivation (Llorens-Cortes, Huang, Vicart, Gasc, Paulin & Corvol, 1992). ET-1 is well-established to contribute to disease progression in PH and pharmacological blockade of its cognate receptor(s), particularly the ET_A subtype, is effective in treating the disease (Luscher & Barton, 2000; Stewart, Levy, Cernacek & Langleben, 1991; Williamson et al., 2000). Thus, at least in this relatively short-term evaluation, the efficacy of NEP inhibition does not appear to be limited by detrimental augmentation of ET-1 bioactivity.

Interestingly, the plasma concentrations of BNP and NT-proBNP did not show a similar significant change in patients receiving racecadotril, which likely reflects the reduced susceptibility of BNP to breakdown by NEP (compared to ANP; (Watanabe, Nakajima, Shimamori & Fujimoto, 1997). CNP levels were also unaltered in the face of NEP blockade; this is perhaps surprising since this is the preferred natriuretic peptide substrate for NEP (Watanabe, Nakajima, Shimamori & Fujimoto, 1997). However, CNP acts primarily in a paracrine fashion, and its systemic concentrations might not reflect tissue levels(Potter, 2011). Finally, and as expected, the circulating NO_X concentrations were not altered by NEP inhibition, confirming that the increases in cGMP observed in patients receiving racecadotril were exclusively due to upregulation of ANP/GC-A signaling, and not through activation of NO–driven pathways.

Longer term (14 day) treatment with racecadotril did not appear to give rise to equivalent increases in circulating ANP levels as achieved by acute administration. This reduced efficacy is likely underpinned by two explanations. First, the study was powered to detect an intra-patient difference in plasma [ANP] of 21%, so since recruitment fell short of the desired total in Step 2 a larger prospective study would be required to corroborate a beneficial effect of this magnitude. Second, the rapid pharmacokinetic and pharmacodynamic profile of racecadotril with respect to plasma [ANP] demonstrated in Step 1 implies that time of administration is critical to detection of elevated levels. Whilst patients were requested to take their final dose of racecadotril on the morning of their 14 day assessment (concurrently with their PDE5i therapy), the time between consumption and hospital evaluation varied greatly and did not coincide with the peak plasma [ANP] identified in Step 1. However, previous clinical studies in heart failure have demonstrated that NEP inhibition (using ecadotril, omapatrilat or LCZ696) maintains elevated [ANP] and/or [cGMP] chronically, for up to eight months (Campese et al., 2001; Cleland & Swedberg, 1998; McMurray et al., 2014; Packer et al., 2015), tendering reassurance that NEPi are likely to exert a similar longer-term pharmacological action in the PH patient cohort. Additionally, this trial did not investigate the dose-response relationship for racecadotril in PAH patients, rather the study utilized the licensed dose of this NEPi for safety and repurposing intentions. Thus, it is possible that the maximum pharmacodynamic effect has not been reached and higher concentrations of racecadotril would exert greater beneficial activity. Further optimization in this context is warranted. Regardless, it should be noted that the fall in PVR produced by NEP inhibition herein was on top of that provided by

existing PDE5i therapy, suggesting current cGMP-centric drugs can be further enhanced.

With respect to safety, racecadotril has a reassuring profile with more than 1 million patient exposures without overt evidence of serious side effects. Whilst there were numerically more adverse events reported by patients taking racecadotril versus placebo, there was not a statistical difference between the two groups; moreover, the adverse events reported by those taking racecadotril were mild and largely expected based on the vascular (vasodilator) and intestinal (diminution of Cl^- & water secretion) effects of NEP inhibition, including headache, dizziness & constipation. These observations give reassurance that chronic use of NEPi in the PAH population will be well-tolerated and safe, although a longer-term study will be needed to ratify this.

In sum, this study provides proof-of-concept clinical evidence of the therapeutic potential of repurposing NEP inhibition in PAH. The beneficial effects of NEPi are dependent on endogenous natriuretic peptide bioactivity, synergize with PDE5i, and exhibit a pulmonary-specific action. The dual mechanism of action inherent to a PDE5i/NEPi combination is unique in terms of existing PH therapy (which target one step in the cGMP signaling cascade) and therefore holds a theoretical advantage in treating the disease. These findings warrant a larger-scale, prospective study with this combination therapy in PH patients to determine if efficacy, selectivity and safety are maintained over a longer period with respect to pulmonary hemodynamics, RV function, exercise capacity, and quality of life.

FUNDING

This work was supported by a British Heart Foundation Project Grant (PG/11/88/28992) and the National Institutes for Health Research, Comprehensive Biomedical Research Centre award to UCL.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the contribution made by the members of the trial steering committee and independent data monitoring committee, Prof. Martin Wilkins, Prof. Mark Griffiths & Dr. Timothy Collier.

CONFLICT OF INTEREST

BS has been a consultant/advisory board member for GSK & Actelion. JC has been a consultant/advisory board member for Actelion, GSK, Bayer, United Therapeutics, Endotronic & Pfizer. AH has been a consultant/advisory board member for Bayer AG, Serodus ASA & Palatin Technologies Inc.

AUTHOR CONTRIBUTIONS

All authors made a significant contribution to the experimental design, data acquisition and analysis/interpretation, were involved with drafting or critically appraising the manuscript, gave approval for submission, and are familiar with all aspects of the study.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

FIGURES

Figure 1. CONSORT flow chart for Step 1 (left panel) and Step 2 (right panel)

Figure 2. Time course (left panel), absolute change (middle panels), and maximum percentage change from baseline (right panel) in plasma atrial natriuretic peptide concentration ([ANP]; ***A***) or cyclic guanosine-3',5'-monophosphate concentration ([cGMP]; ***B***) in PAH patients receiving racecadotril (100mg) or matching placebo. Data are represented as geometric mean ± 95% CI. n=4 (placebo) and n=9 (racecadotril). Data were analyzed by paired t-test for absolute intra-patient change (i.e. before and after treatment) and by unpaired t-test for inter-group comparison (i.e. placebo v racecadotril) of Δ_{max} (%). Correlation between maximum percentage change in PVR and maximum percentage change in plasma [ANP] or plasma [cGMP] (***C***). n=4 (placebo) and n=8 (racecadotril). Data were analyzed by Pearson correlation. In all cases $P<0.05$ was considered statistically significant.

Figure 3. Time course (left panel), absolute change (middle panels), and maximum percentage change from baseline (right panel) in pulmonary vascular resistance (PVR; ***A***) or pulmonary capillary wedge pressure (PCWP; ***B***) in PAH patients receiving racecadotril (100mg) or matching placebo. Data are represented as geometric mean ± 95% CI. n=4 (placebo) and n=8 (racecadotril). Data were analyzed by paired t-test for absolute intra-patient change (i.e. before and after treatment) and by unpaired t-test for inter-group comparison (i.e. placebo v racecadotril) of Δ_{max} (%). $P<0.05$ was considered statistically significant.

Figure 4. Time course (left panel), absolute change (middle panels), and maximum percentage change from baseline (right panel) in mean pulmonary artery pressure (mPAP; ***A***) or mean arterial blood pressure (MABP; ***B***) in PAH patients receiving racecadotril (100mg) or matching placebo. Data are represented as geometric mean ± 95% CI. n=4 (placebo) and n=9 (racecadotril). Data were analyzed by paired t-test

for absolute intra-patient change (i.e. before and after treatment) and by unpaired t-test for inter-group comparison (i.e. placebo v racecadotril) of Δ_{max} (%). *P*<0.05 was considered statistically significant.

Figure 5. Time course (left panel), absolute change (middle panels), and maximum percentage change from baseline (right panel) in plasma endothelin-1 concentration ([ET-1]; ***A***) or N-terminal-pro-brain natriuretic peptide concentration ([NT-proBNP]; ***B***) in PAH patients receiving racecadotril (100mg) or matching placebo. Data are represented as geometric mean ± 95% CI. n=4 (placebo) and n=9 (racecadotril). Data were analyzed by paired t-test for absolute intra-patient change (i.e. before and after treatment) and by unpaired t-test for inter-group comparison (i.e. placebo v racecadotril) of Δ_{max} (%). *P*<0.05 was considered statistically significant.

Figure 6. Absolute change (left & middle panels) and maximum percentage change from baseline (right panel) in mean arterial blood pressure (MABP; ***A***) or plasma atrial natriuretic peptide concentration ([ANP]; ***B***) in PAH patients receiving racecadotril (100mg; tid; 14 days) or matching placebo. Data are represented as geometric mean ± 95% CI. n=3 (placebo) and n=5 (racecadotril). Data were analyzed by paired t-test for absolute intra-patient change (i.e. before and after treatment) and by unpaired t-test for inter-group comparison (i.e. placebo v racecadotril) of Δ_{max} (%). *P*<0.05 was considered statistically significant.

Table 1. Baseline characteristics of study population – Step 1

Characteristic		Placebo (n=4)	Racecadotril (n=9)
Demographics			
Age (years)	Mean (SD)	58 (10)	57 (14)
Sex	(M/F)	0/4	3/6
Ethnicity			
Caucasian	Number (%)	4 (100)	8 (89)
Black African	Number (%)	0 (0)	1 (11)
Weight (kg)	Mean (SD)	72.5 (24.3)	76.8 (8.3)
Height (m)	Mean (SD)	1.65 (0.06)	1.70 (0.14)
Body Mass Index (kg/m^2)	Mean (SD)	28.3 (7.7)	26.9 (4.7)
Clinical biochemistry			
ALT (U/L)	GM (95% CI)	16 (12 to 21)	19 (15 to 24)
AST (U/L)	GM (95% CI)	18 (12 to 27)	23 (19 to 28)
eGFR (mL/min)	GM (95% CI)	63 (50 to 79)	72 (62 to 82)
Hb (g/L)	GM (95% CI)	137 (120 to 156)	129 (113 to 146)
WBC ($x10^9/L$)	GM (95% CI)	8 (4 to 15)	7 (6 to 8)
RBC ($x10^{12}/L$)	GM (95% CI)	4.62 (4.20 to 5.08)	4.33 (3.87 to 4.83)
PT (s)	GM (95% CI)	11 (10 to 13)	16 (12 to 23)
APTT (s)	GM (95% CI)	30 (27 to 33)	36 (33 to 39)
Disease characteristics			
Time since diagnosis (months)	Mean (SD)	11.3 (0.6 to 198.9)	24.3 (12.1 to 48.8)
PH Group			
1.1 Idiopathic	Number (%)	1 (25)	3 (33)
1.4.1 CTD	Number (%)	2 (75)	6 (67)
1.4.4 CHD	Number (%)	1 (25)	0 (0)
WHO Functional Class			
I	Number (%)	0 (0)	2 (22)
II	Number (%)	2 (50)	1 (11)
III	Number (%)	2 (50)	6 (67)
6MWD (m)	GM (95% CI)	364 (198 to 669)	402 (335 to 483)
Borg dyspnea score	Mean (SD)	11.8 (3.2)	10.8 (3.9)
Concurrent PH therapy			
Sildenafil	Number (%)	4 (100)	10 (89)
Tadalafil	Number (%)	0 (0)	1 (11)
ERA	Number (%)	1 (25)	8 (89)

Prostacyclin analogue	Number (%)	0 (0)	1 (11)
Diuretic	Number (%)	1 (25)	6 (67)
Primary endpoint			
Plasma [ANP] (nM)	GM (95% CI)	0.11 (0.06 to 0.20)	0.08 (0.05 to 0.12)
Systemic haemodynamics			
MABP (mmHg)	GM (95% CI)	95 (79 to 115)	89 (81 to 98)
SBP (mmHg)	GM (95% CI)	133 (111 to 159)	122 (112 to 133)
DBP (mmHg)	GM (95% CI)	76 (60 to 95)	72 (64 to 81)
SaO_2 (%)	GM (95% CI)	94 (89 to 100)	95 (93 to 98)
Pulmonary haemodynamics			
mPAP (mmHg)	GM (95% CI)	48 (37 to 62)	43 (36 to 51)
PVR (dynes/sec/cm^{-5})	GM (95% CI)	549 (327 to 921)	470 (368 to 602)
PCWP (mmHg)	GM (95% CI)	10 (6 to 19)	11 (10 to 13)
TAPSE (mm)	GM (95% CI)	22.6 (19.5 to 26.1)	19.0 (15.7 to 22.9)
TRV (m/s)	GM (95% CI)	4.1 (2.4 to 6.8)	3.2 (2.8 to 3.8)
Cardiac indices			
HR (bpm)	GM (95% CI)	85 (63 to 116)	72 (64 to 80)
CO (L/min)	GM (95% CI)	5.4 (2.9 to 10.3)	5.4 (4.3 to 6.7)
SV (mL)	GM (95% CI)	64 (42 to 97)	75 (59 to 95)
LVEF (%)	GM (95% CI)	57 (53 to 61)	56 (55 to 58)
LA area (cm^2)	GM (95% CI)	15.6 (11.7 to 20.7)	18.2 (15.2 to 21.9)
RA area (cm^2)	GM (95% CI)	19.1 (13.3 to 27.5)	19.7 (15.0 to 25.8)
RV diameter (cm)	GM (95% CI)	3.9 (3.0 to 5.2)	3.7 (3.3 to 4.2)
Pericardial effusion	Number (%)	0 (0)	2 (22)
Biomarkers			
Plasma [BNP] (nM)	GM (95% CI)	0.007 (0.00002 to 2.01)	0.004 (0.0004 to 0.04)
Plasma [NT-proBNP] (nM)	GM (95% CI)	38 (10 to 137)	40 (18 to 89)
Plasma [CNP] (pM)	GM (95% CI)	706 (0 to 1642008)	38 (1 to 2214)
Plasma [cGMP] (nM)	GM (95% CI)	41 (14 to 119)	17 (8 to 36)
Plasma [ET-1] (nM)	GM (95% CI)	1.96 (1.14 to 3.36)	1.86 (1.62 to 2.14)
Plasma [NO_x] (nM)	GM (95% CI)	36274 (14389 to 91445)	31642 (15836 to 63223)

SD=Standard Deviation, GM=Geometric Mean, ALT=Alanine transaminase, AST=Aspartate transaminase, eGFR=estimated glomerular filtration rate, Hb=haemoglobin, WBC=white blood cell count, RBC=red blood cell count, PT=Prothrombin time, APTT=activated partial thromboplastin time, CTD=connective tissue disease, CHD=congenital heart disease, 6MWD=6 minute walk distance, ERA=endothelin receptor antagonist, mPAP=mean pulmonary artery pressure, PVR=pulmonary vascular resistance, PCWP=pulmonary capillary wedge pressure, TAPSE=Tricuspid annular plane systolic excursion, TRV=Tricuspid regurgitant velocity, HR=heart rate, CO=cardiac output, SV=stroke volume, LVEF=Left ventricular ejection fraction, LA=Left atrium, RA=right atrium, RV=Right ventricle, BNP=Brain natriuretic peptide, NT-proBNP=N-terminal-proBNP, CNP=C-type natriuretic peptide, cGMP=cyclic guanosine-3',5'-monophosphate, ET-1=Endothelin-1, $NO_x=[NO_2^-]+[NO_3^-]$

Table 2. Additional hemodynamic and biomarker parameters– Step 1

Characteristic	Placebo (n=4)	Racecadotril (n=9)	P-value
Systemic hemodynamics			
ΔSBP (mmHg)	-6 (-54 to 94)	-15 (-25 to -3)	0.18
ΔDBP (mmHg)	-16 (-60 to 74)	-24 (-37 to -9)	0.44
ΔHR (bpm)	-22 (-36 to -6)	-4 (-24 to 21)	0.64
ΔCO (L/min)	0.2 (-0.3 to 0.6)	0.3 (-0.1 to 0.6)	0.56
ΔSV (mL)	29 (6 to 58)	33 (20 to 46)	0.56
ΔSaO_2 (%)	-1 (-12 to 12)	-1 (-6 to 3)	0.99
Biomarkers			
ΔPlasma [BNP] (nM)	155 (-84 to 4367)	32 (-79 to 714)	0.37
ΔPlasma [CNP] (pM)	-1 (-96 to 2466)	-44 (-87 to 154)	0.36
ΔPlasma [NO_x] (nM)	-28 (-49 to 0)	-47 (-77 to 22)	0.56

Data are presented as change in Geometric Mean (95% CI)

Table 3. Baseline characteristics of study population – Step 2

Characteristics		**Placebo (n=3)**	**Racecadotril (n=5)**
Demographics			
Age (years)	Mean (SD)	67 (3)	69 (7)
Sex	(M/F)	1/2	0/5
Ethnicity			
Caucasian	Number (%)	3 (100)	4 (80)
Black African	Number (%)	0 (0)	1 (20)
Weight (kg)	Mean (SD)	77.0 (20.3)	77.0 (20.3)
Height (m)	Mean (SD)	1.67 (0.07)	1.63 (0.07)
Body Mass Index (kg/m^2)	Mean (SD)	27.5 (6.1)	29.3 (7.8)
Clinical biochemistry			
ALT (U/L)	GM (95% CI)	19 (13 to 26)	24 (11 to 53)
AST (U/L)	GM (95% CI)	21 (14 to 31)	23 (11 to 46)
eGFR (mL/min)	Median (IQR)	69 (71 to 90)	73 (73 to 90)
Hb (g/L)	GM (95% CI)	120 (76 to 188)	127 (114 to 142)
WBC ($x10^9/L$)	GM (95% CI)	6 (4 to 11)	5 (4 to 6)
RBC ($x10^{12}/L$)	GM (95% CI)	4.26 (3.02 to 5.99)	4.51 (3.76 to 5.42)
PT (s)	GM (95% CI)	11 (11 to 13)	17 (9 to 31)
APTT (s)	GM (95% CI)	36 (25 to 52)	37 (31 to 44)
Disease characteristics			
Time since diagnosis (months)	Mean (SD)	35.0 (27.8)	13.4 (11.2)
PH Group			
1.1 Idiopathic	Number (%)	0 (0)	1 (20)
1.4.1 CTD	Number (%)	3 (100)	4 (80)
1.4.4 CHD	Number (%)	0 (0)	0 (0)
WHO Functional Class			
I	Number (%)	1 (33)	0 (0)
II	Number (%)	0 (0)	2 (40)
III	Number (%)	2 (67)	3 (60)
6MWD (m)	GM (95% CI)	443 (201 to 975)	315 (191 to 522)
Borg dyspnea score	Mean (SD)	10.7 (3.2)	13.0 (3.7)
Concurrent PH therapy			
Sildenafil	Number (%)	3 (100)	5 (100)
Tadalafil	Number (%)	0 (0)	0 (0)

ERA	Number (%)	0 (0)	2 (40)
Prostacyclin analogue	Number (%)	0 (0)	0 (0)
Diuretic	Number (%)	3 (100)	3 (60)
Primary endpoint			
Plasma [ANP] (nM)	GM (95% CI)	0.20 (0.17 to 0.24)	0.16 (0.08 to 0.34)
Systemic hemodynamics			
MABP (mmHg)	GM (95% CI)	73 (34 to 156)	70 (55 to 90)
SBP (mmHg)	GM (95% CI)	115 (78 to 170)	121 (105 to 141)
DBP (mmHg)	GM (95% CI)	71 (46 to 111)	68 (54 to 86)
SaO_2 (%)	GM (95% CI)	97 (91 to 103)	95 (93 to 97)
Pulmonary haemodynamics			
TAPSE (mm)	GM (95% CI)	23.7 (14.0 to 40.0)	17.6 (10.6 to 29.0)
TRV (m/s)	GM (95% CI)	2.61 (0.69 to 9.93)	3.62 (2.84 to 4.61)
Cardiac indices			
HR (bpm)	GM (95%CI)	87 (48 to 158)	88 (72 to 107)
CO (L/min)	GM (95% CI)	5.4 (2.9 to 10.3)	5.4 (4.3 to 6.7)
SV (mL)	GM (95% CI)	64 (42 to 97)	75 (59 to 95)
LVEF (%)	GM (95% CI)	58 (58 to 58)	57 (54 to 60)
LA area (cm^2)	GM (95% CI)	22.6 (19.1 to 26.8)	19.1 (11.7 to 31.3)
RA area (cm^2)	GM (95% CI)	24.3 (19.3 to 30.5)	18.0 (13.4 to 24.1)
RV diameter (cm)	GM (95% CI)	3.7 (2.8 to 5.0)	3.8 (3.2 to 4.5)
Pericardial effusion	Number (%)	1 (33)	1 (20)

SD=Standard Deviation, GM=Geometric Mean, IQR=Interquartile Range, ALT=Alanine transaminase, AST=Aspartate transaminase, eGFR=estimated glomerular filtration rate, Hb=haemoglobin, WBC=white blood cell count, RBC=red blood cell count, PT=Prothrombin time, APTT=activated partial thromboplastin time, CTD=connective tissue disease, CHD=congenital heart disease, 6MWD=6 minute walk distance, ERA=endothelin receptor antagonist, mPAP=mean pulmonary artery pressure, PVR=pulmonary vascular resistance, PCWP=pulmonary capillary wedge pressure, TAPSE=Tricuspid annular plane systolic excursion, TRV=Tricuspid regurgitant velocity, HR=heart rate, CO=cardiac output, SV=stroke volume, LVEF=Left ventricular ejection fraction, LA=Left atrium, RA=right atrium, RV=Right ventricle, BNP=Brain natriuretic peptide, NT-proBNP=N-terminal-proBNP, CNP=C-type natriuretic peptide, cGMP=cyclic guanosine-3',5'-monophosphate, ET-1=Endothelin-1, NO_x=$[NO_2^-]$+$[NO_3^-]$

Table 4. Additional hemodynamic and biomarker parameters– Step 2

Characteristic	**Placebo (n=3)**	**Racecadotril (n=5)**	**P-value**
Hemodynamics			
ΔSBP (mmHg)	-10 (-42 to 41)	-4 (-24 to 19)	0.36
ΔDBP (mmHg)	-10 (-56 to 83)	-4 (-19 to 13)	0.70
ΔHR (bpm)	-7 (-38 to 40)	24 (5 to 46)	0.05
ΔSaO_2 (%)	0 (-7 to 6)	0 (-3 to 3)	0.97
Biomarkers			
ΔPlasma [BNP] (nM)	548 (-100 to 1195000)	-66 (-99 to 1336)	0.20
ΔPlasma [NT-proBNP] (nM)	16 (-75 to 440)	-12 (-20 to -3)	0.40
ΔPlasma [CNP] (pM)	-16 (-44 to 26)	-2 (-16 to 16)	0.16
ΔPlasma [cGMP] (nM)	-51 (-92 to 193)	34 (-64 to 395)	0.66
ΔPlasma [ET-1] (nM)	-21 (-84 to 278)	-20 (-37 to 1)	0.96
ΔPlasma [NO_x] (nM)	-23 (-79 to 186)	1 (-48 to 94)	0.96

Data are presented as change in Geometric Mean (95% CI)

Table 5. Adverse events by severity, seriousness and causality for each trial step

Trial step	Patient ID	Treatment group	Adverse event	Severity grade (0-5)	Seriousness	Outcome	Treatment related?
1	COM202	Racecadotril	Vomiting	2	Not serious	Resolved	Possibly
1	COM202	Racecadotril	Epistaxis	2	Not serious	Resolved	Possibly
2	COM121	Racecadotril	Headache	1	Not serious	Resolved	Possibly
2	COM121	Racecadotril	Diarrhoea	1	Not serious	Resolved	No
2	COM121	Racecadotril	Dizziness	1	Not serious	Resolved	Possibly
2	COM121	Racecadotril	Cough	1	Not serious	Resolved	Possibly
2	COM121	Racecadotril	Vomiting	1	Not serious	Resolved	Possibly
2	COM123	Racecadotril	Headache	1	Not serious	Resolved	Possibly
2	COM123	Racecadotril	Epistaxis	1	Not serious	Resolved	Possibly
2	COM123	Racecadotril	Stomach pain	1	Not serious	Resolved	Possibly
2	COM123	Racecadotril	Upper respiratory tract infection	2	Not serious	Resolved	Possibly
2	COM125	Racecadotril	Constipation	1	Not serious	Resolved	Possibly
2	COM126	Placebo	Diarrhoea	2	Not serious	Resolved	No
2	COM126	Placebo	Dry skin	1	Not serious	Resolved	No
2	COM127	Racecadotril	Hiccups	1	Not serious	Resolved	No
2	COM127	Racecadotril	Body cramps	1	Not serious	Resolved	No

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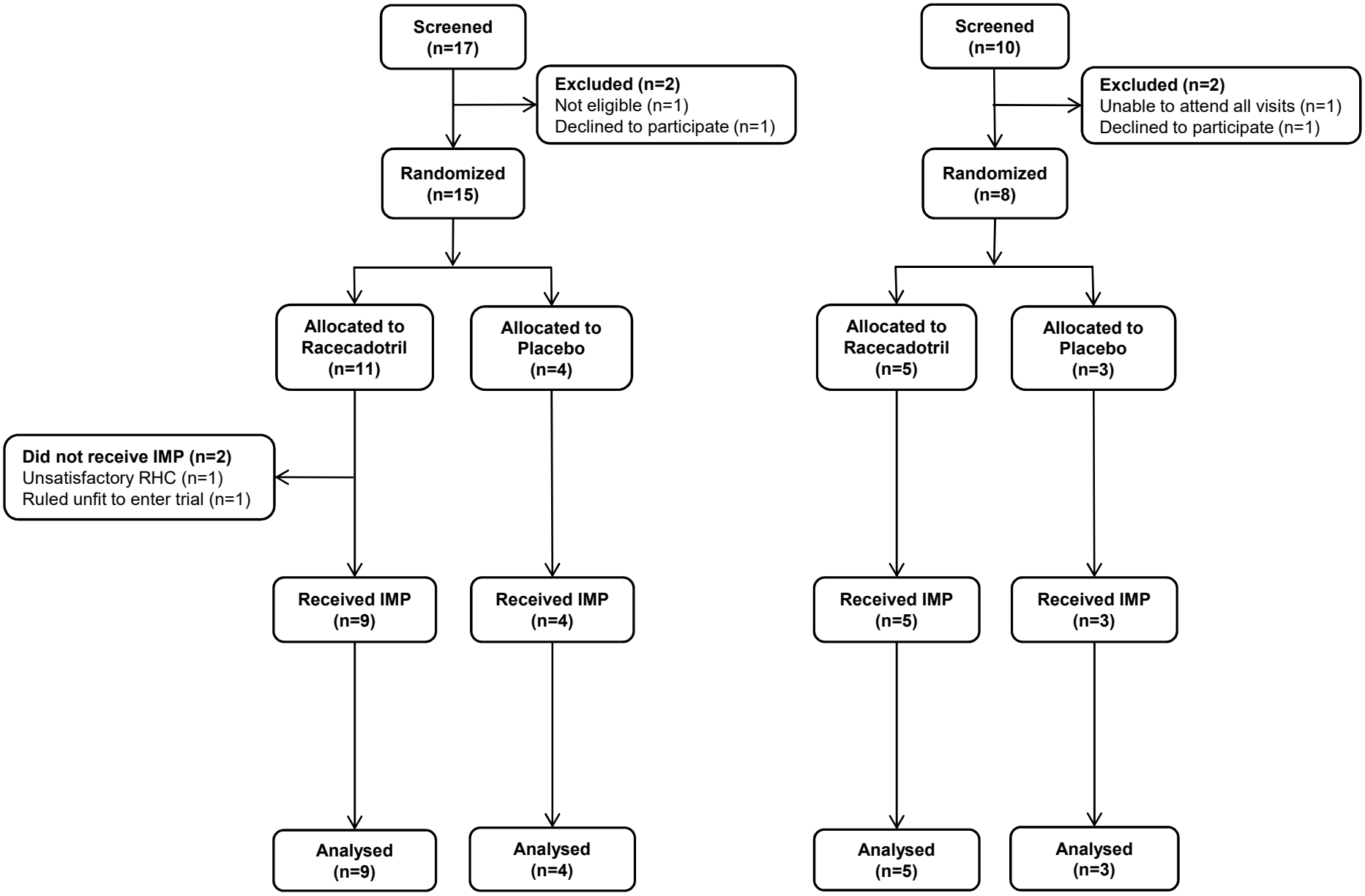
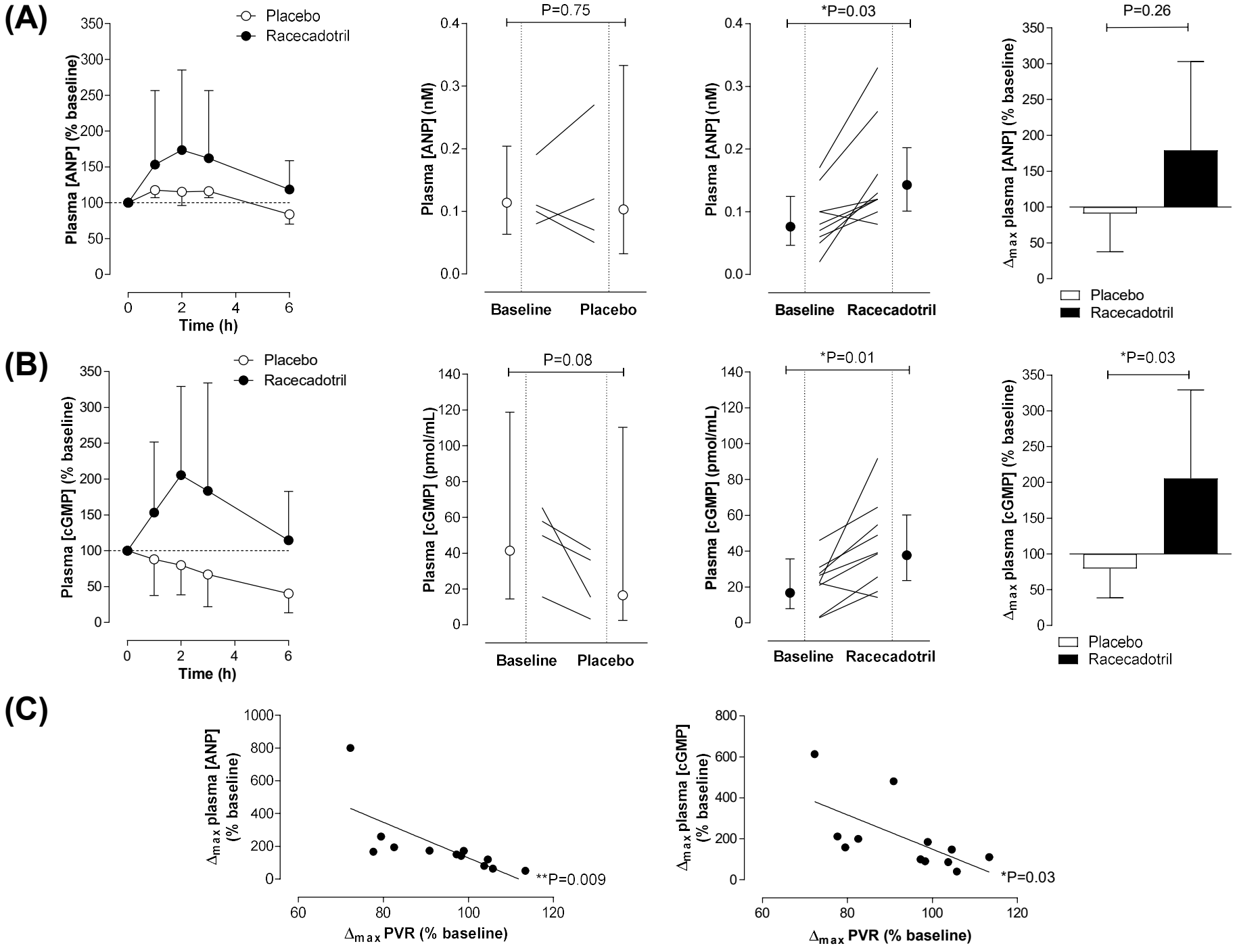
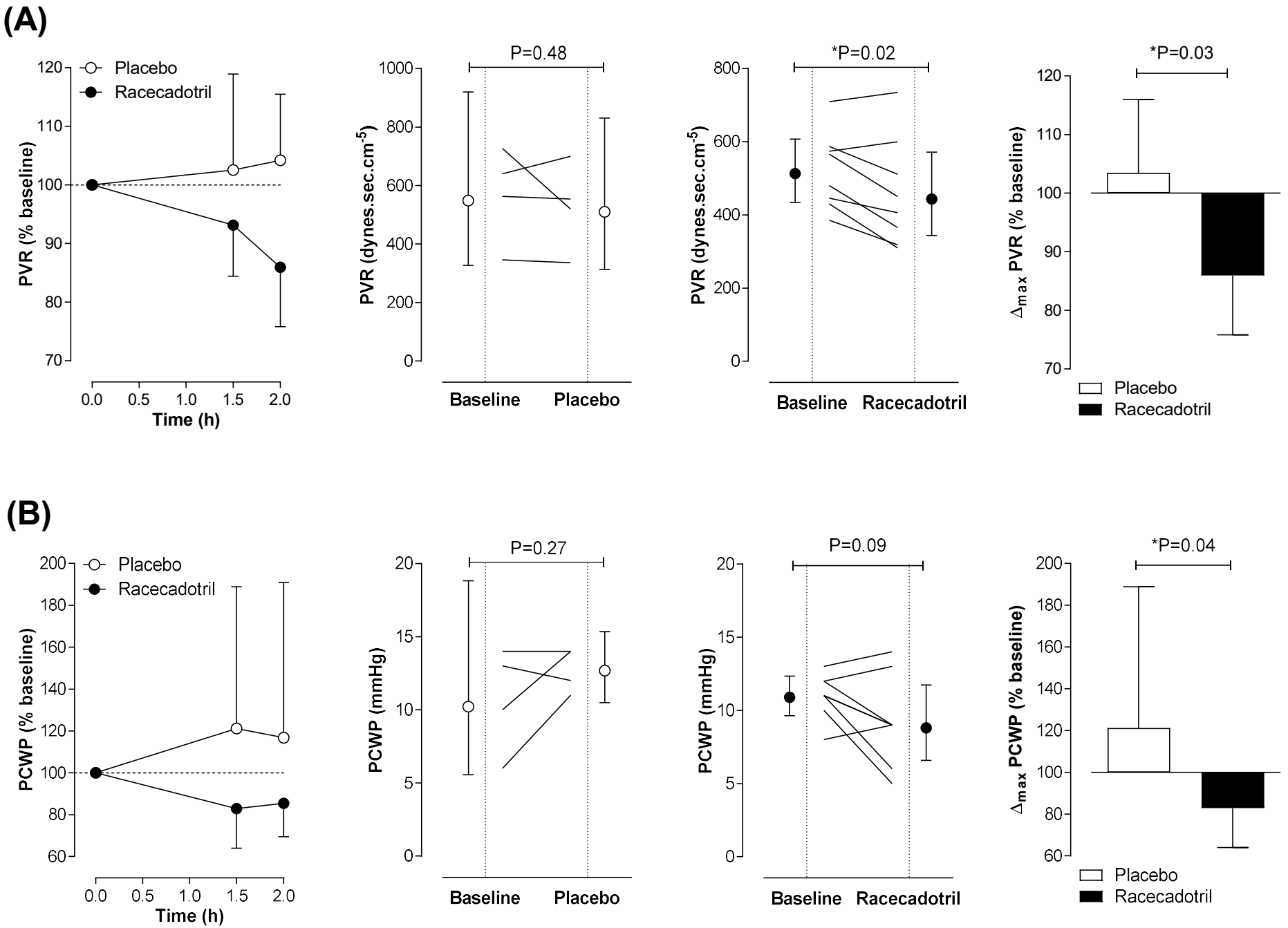
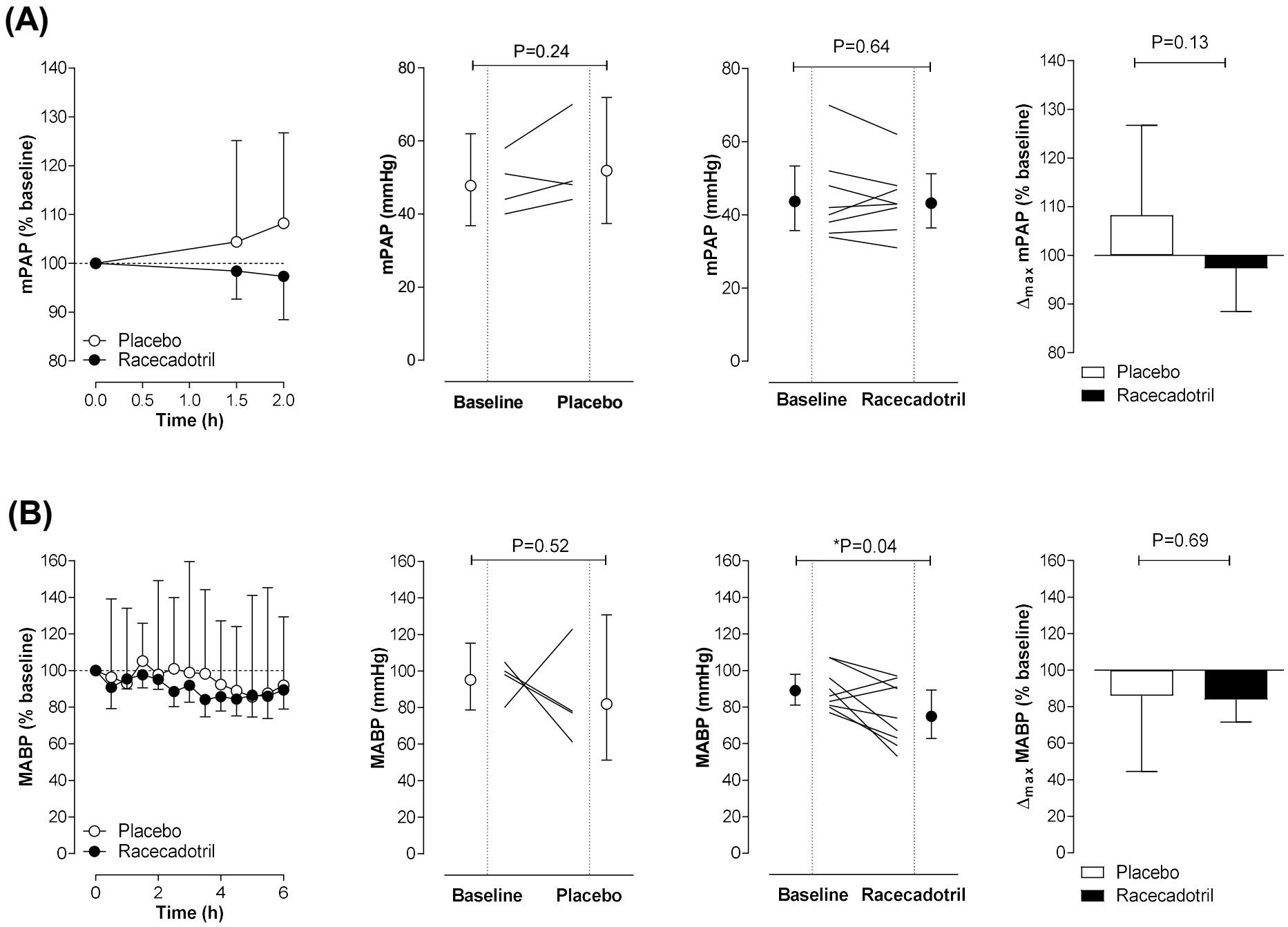
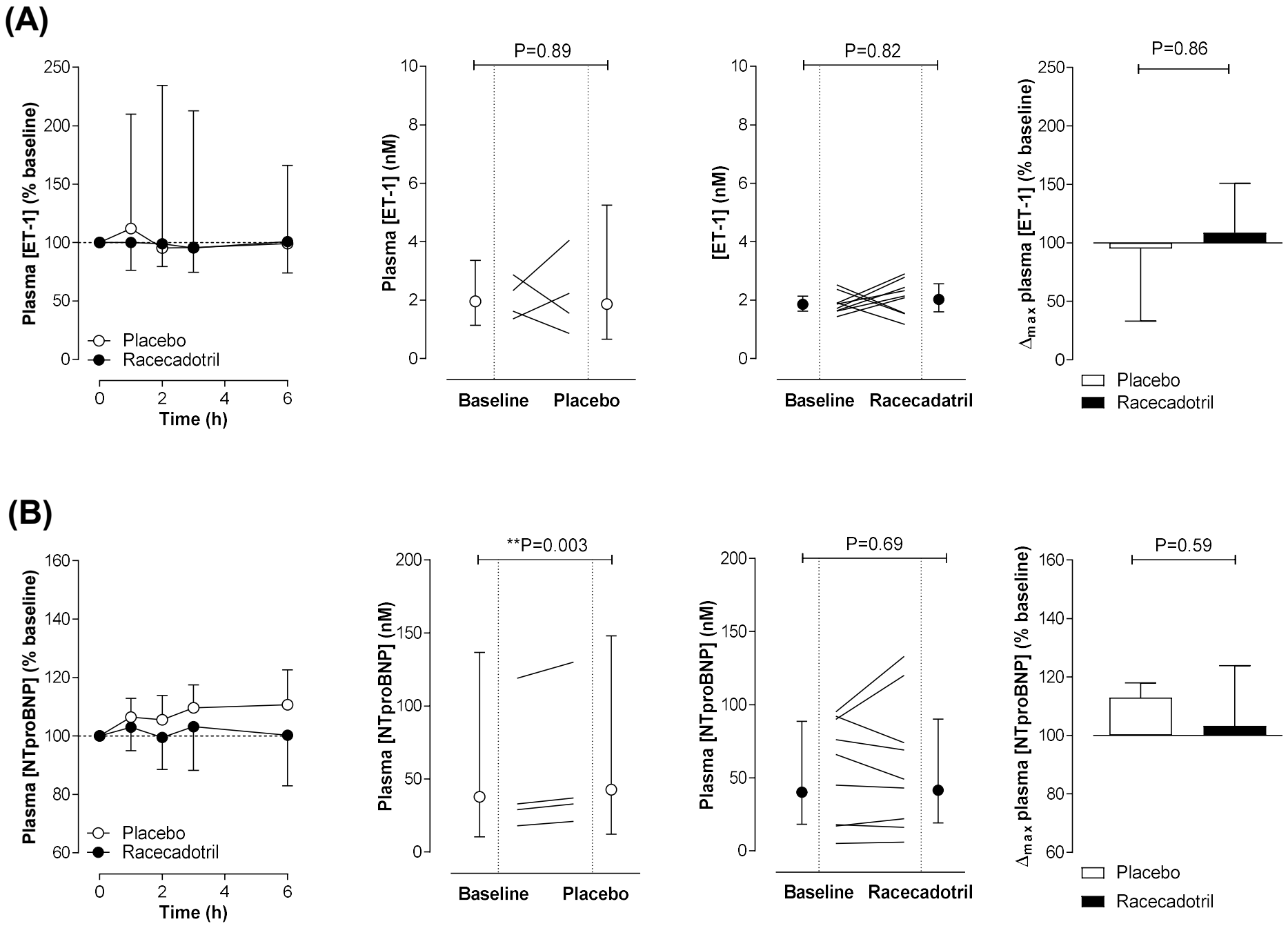
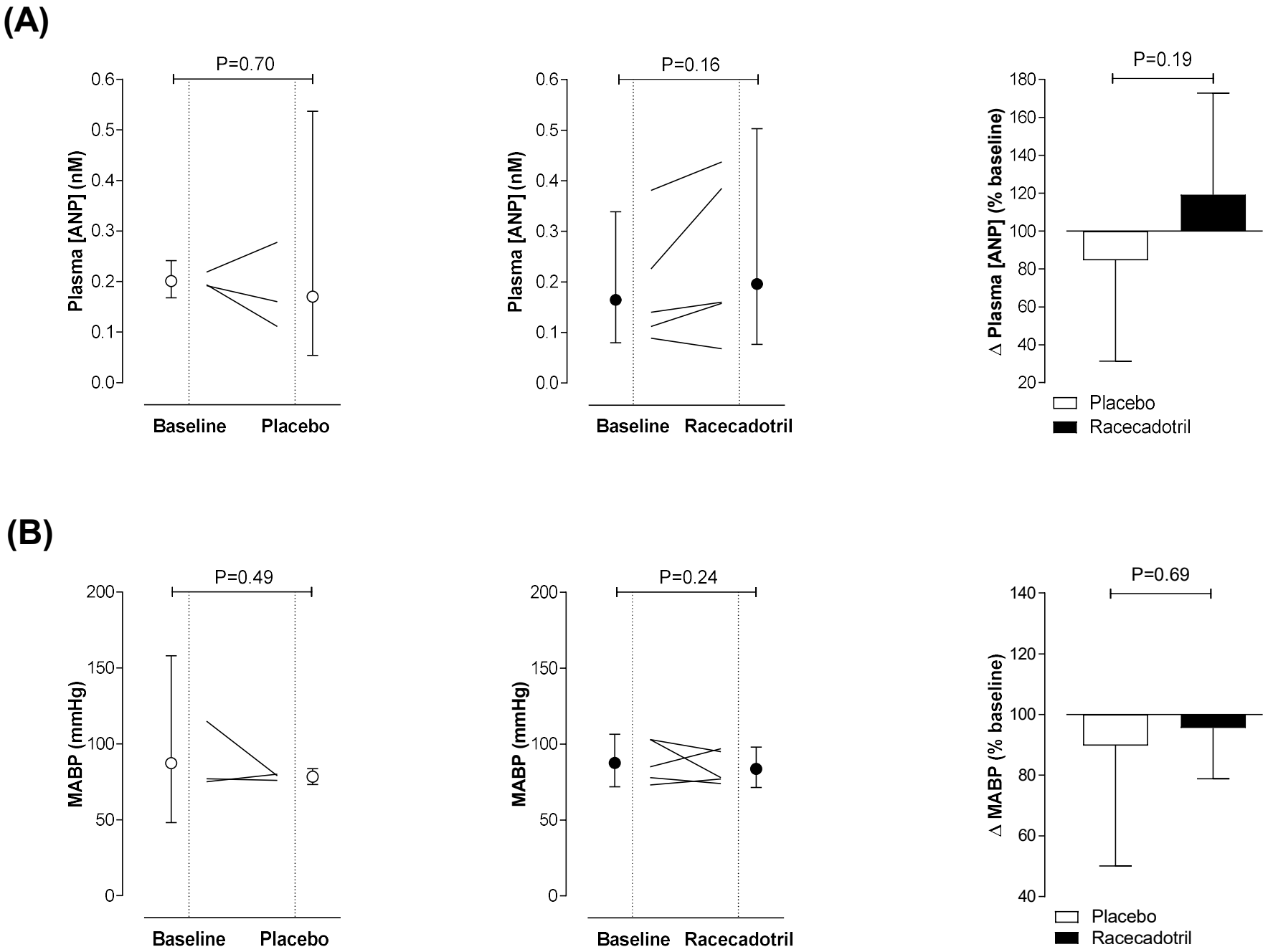

Screened
(n=17)
Excluded (n=2)
Not eligible (n=1)
Declined to participate (n=1)
Randomized
(n=15)
Allocated to
Racecadotril
(n=11)
Allocated to
Placebo
(n=4)
Did not receive IMP (n=2)
Unsatisfactory RHC (n=1)
Ruled unfit to enter trial (n=1)
Received IMP
(n=9)
Received IMP
(n=4)
Analysed
(n=9)
Analysed
(n=4)
Step 1
Screened
(n=10)
Excluded (n=2)
Unable to attend all visits (n=1)
Declined to participate (n=1)
Randomized
(n=8)
Allocated to
Racecadotril
(n=5)
Allocated to
Placebo
(n=3)
Received IMP
(n=5)
Received IMP
(n=3)
Analysed
(n=5)
Analysed
(n=3)
Step 2


(A)
Placebo
Racecadotril
Plasma [ANP] (% baseline)
Time (h)
P=0.75
Plasma [ANP] (nM)
Baseline
Placebo
*P=0.03
Plasma [ANP] (nM)
Baseline
Racecadotril
P=0.26
Δmax plasma [ANP] (% baseline)
Placebo
Racecadotril
(B)
Placebo
Racecadotril
Plasma [cGMP] (% baseline)
Time (h)
P=0.08
Plasma [cGMP] (pmol/mL)
Baseline
Placebo
*P=0.01
Plasma [cGMP] (pmol/mL)
Baseline
Racecadotril
*P=0.03
Δmax plasma [cGMP] (% baseline)
Placebo
Racecadotril
(C)
Δmax plasma [ANP] (% baseline)
**P=0.009
Δmax PVR (% baseline)
Δmax plasma [cGMP] (% baseline)
*P=0.03
Δmax PVR (% baseline)


(A)
Placebo
Racecadotril
PVR (% baseline)
Time (h)
PVR (dynes.sec.cm-5)
P=0.48
Baseline
Placebo
*P=0.02
Racecadotril
*P=0.03
Δmax PVR (% baseline)
(B)
PCWP (% baseline)
PCWP (mmHg)
P=0.27
P=0.09
*P=0.04
Δmax PCWP (% baseline)


(A)
mPAP (% baseline)
Placebo
Racecadotril
Time (h)
P=0.24
mPAP (mmHg)
Baseline
Placebo
P=0.64
mPAP (mmHg)
Baseline
Racecadotril
P=0.13
Δmax mPAP (% baseline)
Placebo
Racecadotril
(B)
MABP (% baseline)
Placebo
Racecadotril
Time (h)
P=0.52
MABP (mmHg)
Baseline
Placebo
*P=0.04
MABP (mmHg)
Baseline
Racecadotril
P=0.69
Δmax MABP (% baseline)
Placebo
Racecadotril


(A)
Plasma [ET-1] (% baseline)
250
200
150
100
50
0
Placebo
Racecadotril
0
2
4
6
Time (h)
P=0.89
Plasma [ET-1] (nM)
10
8
6
4
2
0
Baseline
Placebo
P=0.82
[ET-1] (nM)
10
8
6
4
2
0
Baseline
Racecadatril
P=0.86
Δmax plasma [ET-1] (% baseline)
250
200
150
100
50
0
Placebo
Racecadotril
(B)
Plasma [NTproBNP] (% baseline)
160
140
120
100
80
60
Placebo
Racecadotril
0
2
4
6
Time (h)
**P=0.003
Plasma [NTproBNP] (nM)
200
150
100
50
0
Baseline
Placebo
P=0.69
Plasma [NTproBNP] (nM)
200
150
100
50
0
Baseline
Racecadotril
P=0.59
Δmax plasma [NTproBNP] (% baseline)
160
140
120
100
80
60
Placebo
Racecadotril


(A)
P=0.70
Plasma [ANP] (nM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Baseline
Placebo
P=0.16
Plasma [ANP] (nM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Baseline
Racecadotril
P=0.19
Δ Plasma [ANP] (% baseline)
180
160
140
120
100
80
60
40
20
Placebo
Racecadotril
(B)
P=0.49
MABP (mmHg)
200
150
100
50
0
Baseline
Placebo
P=0.24
MABP (mmHg)
200
150
100
50
0
Baseline
Racecadotril
P=0.69
Δ MABP (% baseline)
140
120
100
80
60
40
Placebo
Racecadotril