RAS Peptide Profiles in Arterial Hypertension

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Andrea

„Gut Ding will Weile haben.“

Publius Ovidius Naso (43 v. Chr. - 17 n. Chr.), römischer Epiker

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Summary

The classical endocrine renin-angiotensin aldosterone system (RAS) plays an important role in blood pressure and fluid balance regulation. Important effectors of the RAS are small bioactive peptides like angiotensin II (Ang 1-8) or Ang 1-7. The possibility to reliably quantify the low abundance RAS peptides in serum using a mass spectrometry based method opened up new opportunities to investigate their role as possible biomarkers in patients treated with cardiovascular drugs that modulate the RAS cascade. In a pilot study in healthy individuals, RAS peptide concentrations were quantified under normal conditions and after administration of approved drugs that inhibit key enzymes of the RAS (renin, angiotensin-converting enzyme) or block the effect of Ang 1-8 (angiotensin receptor antagonists). Changes reflecting the mechanism of action of the different drugs were evident within a few hours after single dose administration. Based on these promising pilot data, the goal of the subsequent three clinical studies performed in the framework of this dissertation was to systematically characterize the RAS peptide profiles in normotensive healthy subjects as well as in patients with arterial hypertension. The work should contribute to a better understanding of the RAS peptides and investigate their possible role as biomarkers for patients under treatment with RAS modulating drugs.

In the first study, we assessed RAS peptide concentrations after single and repeated oral administration of the renin inhibitor aliskiren, the ACE inhibitor enalapril, and the angiotensin receptor antagonist losartan in healthy normotensive subjects. RAS peptide profiles showed drug-specific changes which directly reflected the mechanism of action of the different RAS inhibitors after single and multiple dose treatment for one week. While inhibition of renin decreased downstream RAS peptide concentrations, the ACE inhibitor caused a decrease of the ACE products Ang 1-8 and Ang 1-5. The angiotensin receptor antagonist on the other hand caused an increase of upstream Ang 1-10 and Ang 1-8 mainly by enhanced renin-feedback. Overall, the data suggested that RAS peptide profiles could be of value for the assessment of patients under RAS inhibitor therapy.

In the second study, the potential of drug-specific profiles found in healthy normotensive subjects as possible biomarkers were assessed in patients before and after start of monotherapy for arterial hypertension. Patients were randomized to four groups and RAS peptide concentrations were assessed after 4 weeks of consecutive oral treatment with intermediate or high doses of the ACE inhibitor

perindopril, the angiotensin receptor antagonist olmesartan, the calcium channel antagonist amlodipine or the thiazide diuretic hydrochlorothiazide. These four drugs are used as first line treatments in clinical practice. After treatment with the ACE inhibitor and the angiotensin receptor antagonist, RAS peptide profiles again showed the drug-specific changes observed in healthy normotensive subjects. The changes observed with the calcium channel antagonist and the thiazide diuretic were similar but less pronounced as for the ones observed after an angiotensin receptor antagonist treatment. The study showed that the drug-specific changes are preserved for at least 8 weeks of treatment.

Next it was of interest whether drug-specific changes could also be detected in patients under combination drug treatment. In the third study we therefore investigated RAS peptide profiles in patients with uncontrolled arterial hypertension, as such patients per definition are treated with at least three different antihypertensive drugs. With insufficient therapeutic response despite receiving combination drug treatment, patients with treatment resistant arterial hypertension belong to the most complex patient group. However, these patients are at great risk of stroke, myocardial infarction, heart failure, and/or chronic kidney disease and the benefits of successful treatment are substantial. Even in this heterogeneous patient group, the drug-specific RAS peptide profile changes observed in healthy normotensive subjects and patients under antihypertensive monotherapy were maintained. Data generated by this study suggest that RAS peptide profiles might also be useful in other patient populations treated with cardiovascular drug combinations.

In conclusion, data generated by our studies showed a correlation between RAS peptide concentrations and treatment with an ACE inhibitor or angiotensin receptor antagonist in normotensive healthy subjects as well as in patients with arterial hypertension under monotherapy or in patients with uncontrolled hypertension with a combination drug treatment. Taken together, RAS peptide profiles as biomarkers for the assessment of drug adherence and (in combination with drug concentrations in plasma) true drug resistance as well as their potential usefulness in the guidance of antihypertensive treatment in patients with insufficient treatment response merits further investigation.

Abbreviations

ACE	Angiotensin-I-converting enzyme
ACE2	Angiotensin-I-converting enzyme 2
Ang	angiotensin
Ang 1-10	angiotensin 1-10, Ang 1-10, angiotensin I
Ang 1-8	angiotensin 1-8, Ang 1-8, angiotensin II
AP	aminopeptidase
ARB	angiotensin receptor antagonist, angiotensin receptor blocker
AT1	Angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
BP	blood pressure
CCB	calcium channel antagonist, calcium channel blocker
CV	cardiovascular
CVD	cardiovascular disease
DAP	dipeptidyl aminopeptidase
DBP	diastolic blood pressure
EKNZ	Ethikkommission Zentral- und Nordwestschweiz, EKNZ
ESH	European Society of Hypertension
HCT, HCTZ	hydrochlorothiazide
HPLC	high performance liquid chromatography
HR	heart rate
LLOQ	lower limit of quantification
MS	mass spectrometry
NEP	neutral endopeptidase
RAS	renin-angiotensin system
SBP	systolic blood pressure

1 Introduction

1.1 Arterial Hypertension

Arterial hypertension is defined as a systolic blood pressure (SBP) ≥140 mmHg or a diastolic blood pressure (DBP) ≥90 mmHg.[1, 2] Arterial hypertension is an important cardiovascular (CV) risk factor, leading to heart attacks and strokes. The incidence of arterial hypertension increases with age. Worldwide approximately one billion people are affected and about nine million people die from it every year. The prevalence of arterial hypertension increases from 18 to 30% in America, Western Pacific Region, Europe, South-East Asia Region, Eastern Mediterranean Region and Africa. Although there is no huge difference in mean prevalence between developed and developing countries, mortality rates of cardiovascular disease (CVD) are higher in low-income countries than those of industrialized nations.[3] In Switzerland, limited data are available on the prevalence of hypertension as there is no survey covering whole Switzerland. According to data provided by the Federal Office of Statistics in Switzerland, the overall prevalence of hypertension was around 18% in 2012, with a substantial increase with age. Compared to data from 1992, there was a noticeable increase in prevalence over the two past decades, especially in men. From 1992 to 2012, prevalence of arterial hypertension in Swiss men aged 55 – 64 years, 65 – 74 years and older than 75 years, rose from 21.9%, 32.0% and 30.3% to 31.1%, 48.5% and 50.3%, in Swiss women from 21.8%, 35.7% and 41.3% to 23.2%, 38.5% and 56.9%, respectively (Source: Federal Statistical Office, Swiss Health Interview Survey 2012). Furthermore, CVD are the most common cause of death in Switzerland, accounting for 30.6% of deaths in men and 34.8% of deaths in women (Source: Federal Statistical Office, Swiss causes of death statistics 2014).

1.1.1 Management of Arterial Hypertension

At the University Hospital Basel, antihypertensive drug treatment follows current ESH guidelines for management of arterial hypertension.[4] This includes confirmation of diagnosis of hypertension by a 24 hour ambulatory blood pressure monitoring, detection of causes of secondary hypertension and assessment of total CV risk which is determined by blood pressure (BP) level, CV risk factors, asymptomatic organ damage, the presence of diabetes, asymptomatic CVD or

chronic kidney disease. Depending on BP level and total CV risk, antihypertensive therapy is started with a single agent or with a two-drug combination. In case of mild BP elevation and low or moderate total CV risk, initial treatment is a monotherapy at moderate dose with a subsequent increase in drug dose if BP goal is not achieved. Target BP should be at least < 140/90 mmHg in all hypertensive patients. If BP goal is not achieved with a full dose monotherapy, a two-drug combination is required. In case of marked BP elevation or when total CV risk is high or very high with mild BP elevation a two-drug combination at low doses should be preferred for initial treatment. If BP goal is not achieved with a two-drug combination at full dose, a third drug is added. Suitable and recommended antihypertensive drug classes for treatment initiation are angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists, calcium antagonists, diuretics and beta-receptor antagonists. Lifestyle modification such as healthy diet, sodium and alcohol restriction, regular physical activity, smoking cessation and weight reduction where necessary should always be performed in addition to any antihypertensive drug treatment.

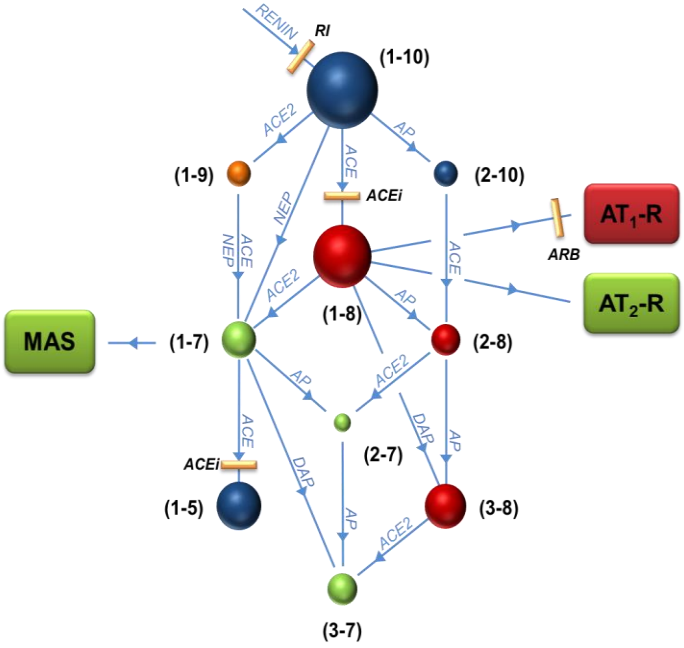
1.1.2 Treatment-Resistant Hypertension

Resistant hypertension is defined as blood pressure that remains above goal in spite of concurrent use of 3 antihypertensive agents at optimal doses, including a diuretic or BP is controlled with four or more medications.[5] The true prevalence of treatment-resistant hypertension is difficult to quantify because differentiation between true resistant and pseudo-resistant hypertension is difficult. True resistance refers to a true drug resistance, where reasons for a pseudo-resistant hypertension are white-coat hypertension, BP elevating co-medications, poor adherence, insufficient drug therapy, or secondary causes such as obstructive sleep apnea or primary aldosteronism. Although the true prevalence of apparent resistance is unknown, several studies indicate that a considerable number of patients do not reach BP goals and thus uncontrolled hypertension is a clinical problem.[5-8] Patients with uncontrolled arterial hypertension are at greater risk of stroke, myocardial infarction, heart failure, and/or chronic kidney disease and the benefits of successful treatment are substantial.[9, 10] Moreover, literature indicates that non-adherence is one of the major causes of uncontrolled hypertension and therefore treatment failure.[11, 12] In patients referred from primary care physicians to a tertiary centre for further work-up of uncontrolled hypertension, partial or complete non-adherence was found in more than half of

the patients.[13] Detection and treatment of uncontrolled hypertension due to non-adherence is important to improve the management of these patients but few tools exist to accurately and routinely detect it.

1.2 Renin-Angiotensin System

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure.[14] Angiotensin II (Ang 1-8) is the main effector peptide of the classic RAS. It is formed by c-terminal cleavage of two amino acids from Angiotensin I (Ang 1-10) by the angiotensin-I-converting enzyme (ACE).[15] The effects of Ang 1-8 mediated by binding of Ang 1-8 to the angiotensin type I receptor (AT1) include vasoconstriction, stimulation of aldosterone and antidiuretic hormone release, inflammation, cardiac hypertrophy, vascular proliferation, and oxidative stress. This activation of the classical ACE – Ang II – AT1 axis of the RAS has been associated with hypertension. The Effects mediated by binding of Ang 1-8 to the angiotensin type II receptor (AT2), include counter-regulatory effects such as vasodilation and anti-inflammatory, pro-apoptotic, anti-proliferative and anti-oxidative stress effects.[16] The binding affinity of Ang II for the two AT receptors is comparable, but AT1 is ubiquitously expressed in the CV system while AT2 is low expressed in healthy adults and can be modulated by pathological states such as hypertension or stroke.[17] In 2000, a tissue specific human homologue of ACE, called angiotensin-I-converting enzyme 2 (ACE2) was described [15, 18] and subsequently the alternative axis ACE2 – Ang 1-7 – Mas has been established.[19] In this axis, Ang 1-7 is formed by cleavage of a single amino acid from the c-terminus of Ang 1-8 by ACE2.[15] The effects of Ang 1-7 mediated by binding of Ang 1-7 to the G-protein coupled receptor MAS [20] include opposing effects to the classic RAS axis, such as vasodilation, vascular protection and anti-proliferation as well as anti-fibrinogenic, anti-thrombogenic and anti-arrythmogenic effects.[20-22] Studies suggest a contribution of the ACE2 – Ang 1-7 – Mas axis in the evolution of hypertension and a reduction in the expression and activity of Ang 1-7 may be a critical factor in mediating the progression of CVD.[23] Figure 1 summarizes the minor and major degradation pathways of Ang 1-10.



ACE = Angiotensin-I-converting-enzyme 1
ACE2 = Angiotensin-I-converting-enzyme 2
AP = Aminopeptidase
NEP = Neutral endopeptidase
(1-8) = Angiotensin II, angiotensin 1-8
AT_1-R = Angiotensin II type 1 receptor
MAS = Mas receptor

RI = Blocked by renin inhibitors
ARB = Blocked by angiotensin receptor antagonists
ACEi = Blocked by ACE inhibitors

Figure 1: Degradation pathway of angiotensinogen. The numbers in brackets indicate the number of amino acids (figure provided by Marko Poglitsch, Attoquant Diagnostics GmbH).

According to their different mechanisms of action, CV drugs such as renin or ACE inhibitors, or angiotensin receptor antagonists will cause characteristic concentration changes of Ang 1-10, Ang 1-8 and possibly also of smaller downstream RAS peptides. In recent years, several MS based methods have been developed for the quantification of angiotensin peptides in human plasma.[24-28]

2 Objectives

The possibility of quantification of multiple RAS peptides by a MS based method may provide drug-type specific RAS peptide profiles that could e.g. be useful for the assessment of uncontrolled hypertension under RAS inhibitor treatment. In combination with measured drug concentrations, RAS peptide profiles could serve as a tool to differentiate between true drug resistance and non-adherence and also to investigate the response to antihypertensive drug treatment. In a pilot study in healthy individuals, where the RAS peptides were investigated under normal conditions and after single dose administration of approved drugs that inhibit key enzymes of the RAS (renin, angiotensin-converting enzyme) or block Ang 1-8 at the receptor (angiotensin receptor antagonists), changes reflecting the mechanism of action of these drugs were evident within a few hours after administration of a single dose.

The goals of this dissertation were therefore to investigate and characterize RAS peptide profiles in healthy normotensive subjects and later in different conditions in relation with high blood pressure. The work should contribute to a better understanding of RAS peptides and investigate their clinical usability as biomarkers of drug effect and or drug adherence.

The following section presents non-published data from this dissertation project, starting with the first project, assessing RAS peptide concentrations after single and repeated oral administration of the renin inhibitor aliskiren, the ACE inhibitor enalapril, and the angiotensin receptor antagonist losartan in healthy normotensive subjects. In a second project, the potential of drug-specific profiles found in healthy normotensive subjects were assessed in patients with a new diagnosis of arterial hypertension before and after start of antihypertensive monotherapy. RAS peptide profiles were assessed after 4 weeks of consecutive oral treatment with intermediate and high doses of four drugs used as first line treatments, namely the ACE inhibitor perindopril, the angiotensin receptor antagonist olmesartan, the calcium channel antagonist amlodipine or the diuretic hydrochlorothiazide. Preliminary data confirming drug-specific changes observed in healthy normotensive subjects led to the third project where the RAS peptide profiles were investigated in patients referred to the hospital for the work-up of uncontrolled arterial hypertension.

3 Results

3.1 RAS peptide profiles in healthy subjects

Angiotensin peptide profiles in non-salt-depleted healthy subjects after single and multiple dose treatment with aliskiren, enalapril and losartan

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Abstract

Background The renin-angiotensin system (RAS) plays an important role in blood pressure and fluid balance regulation. Important effectors of the RAS are small bioactive peptides like angiotensin II (Ang 1-8) or Ang 1-7. Frequently used cardiovascular drugs, such as renin or ACE inhibitors or angiotensin receptor antagonists act on key enzymes or receptors of the RAS, and are expected to cause drug-type specific changes of RAS peptide concentrations. The objective of this study was to assess RAS peptide concentrations after single and repeated oral administration of the renin inhibitor aliskiren, the ACE inhibitor enalapril, and the angiotensin receptor antagonist losartan in normotensive subjects.

Methods In a single-center, open-label, randomized, 3-way crossover study 12 healthy, normotensive male subjects received once-daily doses of 10 mg enalapril, 50 mg losartan, or 150 mg aliskiren in three sequential treatment periods. Concentrations of RAS inhibitors and RAS peptides were determined using a liquid chromatography-tandem mass spectrometry method before, and 2, 4, 8, and 24 hours after administration of a single dose and after 8 days of repeated once-daily dosing. Circulating RAS peptide concentrations were determined from aliquots containing protease inhibitors and equilibrium angiotensin concentrations from standard lithium heparin aliquots.

Results In untreated subjects, circulating RAS peptide concentrations were in the low picomolar range. Single dose treatment caused drug-specific changes of circulating angiotensin peptide profiles which directly reflect the mechanism of action of the different RAS inhibitors. The renin inhibitor aliskiren decreased downstream RAS peptide concentrations. The ACE inhibitor enalapril increased concentrations of the ACE substrates angiotensin I (Ang 1-10) and Ang 1-7. The angiotensin receptor antagonist losartan increased concentrations of Ang 1-10 and Ang 1-8. In RAS equilibrium analysis the drug-specific qualitative alterations of circulating RAS peptide profiles were preserved. In equilibrium profiles RAS peptide concentrations were higher and unmasked additional

drug-specific changes in Ang 2-10, Ang 2-8 and Ang 3-8 compared to circulating peptide profiles.

Conclusion Circulating and equilibrium RAS peptide profiles show drug-specific changes which directly reflect the mechanism of action of the different RAS inhibitors after single and multiple dose treatment for one week. Equilibrium samples contain higher RAS peptide concentrations and show additional drug-specific changes in small downstream peptides compared to circulating RAS peptide profiles. The present data suggest that RAS equilibrium peptide profiles could be of value for the stratification and monitoring of patients under RAS inhibitor therapy. Their potential as possible biomarkers in such patients will have to be assessed in subsequent studies.

Introduction

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and is involved in vascular injury and repair responses.[1] Angiotensin II (Ang 1-8) is the main effector peptide of the classic RAS. It is formed by C-terminal cleavage of two amino acids from Angiotensin I (Ang 1-10), a reaction catalyzed by the di-carboxypeptidase angiotensin-I-converting enzyme (ACE). The classic effects of Ang 1-8, such as vasoconstriction, stimulation of aldosterone and antidiuretic hormone release, and increase of renal sodium and water reabsorption are mediated by binding of Ang 1-8 to the angiotensin II type 1 receptor (AT1).

In 2000, a tissue specific human homologue of ACE, called angiotensin-I-converting enzyme 2 (ACE2) was described.[2, 3] In contrast to ACE that removes a dipeptide, ACE2 only cleaves a single amino acid from the carboxy terminus of Ang 1-10 or Ang 1-8 to produce Ang 1-9 or Ang 1-7, respectively.[3] While for Ang 1-9 no specific receptor has been described yet, Ang 1-7 binds to MAS1, a G-protein coupled receptor.[4] This non-classic

ACE2 – Ang 1-7 – MAS1 axis is reported to mediate opposing effects to the classic RAS axis, such as vasodilation, vascular protection and anti-proliferation as well as anti-fibrinogenic, anti-thrombogenic and anti-arrythmogenic effects.[5-7] Ang 1-8 and Ang 1-7 are further cleaved by amino- and/or carboxypeptidases to Ang III (Ang 2-8)[8], Ang IV (Ang 3-8)[9], Ang 2-7[10, 11] or Ang 3-7.[12] While Ang 2-8 has effects similar to Ang 1-8 mediated by angiotensin receptors, Ang 3-8 is thought to have anti-inflammatory and anti-fibrotic effects by inhibiting the insulin-regulated membrane aminopeptidase (IRAP).[13, 14] Possible cardiovascular or CNS effects of the smaller fragments Ang 2-7 and Ang 3-7 are less well documented.[10, 12]

Reliable quantification of these small low abundance peptides is a technical challenge. The first quantifications of Ang 1-8 were performed with radioimmunoassays several decades ago.[15-17] The antibodies used were specific for the C-terminal sequence of Ang 1-8 and differentiated between Ang 1-10 and Ang 1-8 but cross-reacted with smaller angiotensin peptide fragments with identical C-terminal amino acid sequence as Ang 1-8.[18] Separation of the different angiotensin peptides using HPLC before performing the RIA improved the specificity of the method and allowed specific quantification of Ang 1-8 and other angiotensin metabolites.[19, 20] In recent years, several mass spectrometry based methods have been developed for the quantification of angiotensin peptides in human plasma, which introduced a high degree of specificity and the ability to control for sample preparation recovery by using stable isotope labeled peptides for internal standardization.[21-25]

Addition of a protease inhibitor cocktail during sample collection stabilizes peptide levels and allows quantification of circulating angiotensin peptide concentrations. Alternatively, without specific sampling requirements, equilibrium concentrations of RAS peptides can be determined in heparin plasma or serum samples.[26]

According to their different mechanisms of action, cardiovascular drugs such as renin or ACE inhibitors, or angiotensin receptor antagonists will cause characteristic concentration changes of Ang 1-10, Ang 1-8 and possibly also of smaller downstream RAS peptides. Simultaneous quantification of multiple RAS peptides may provide drug-type specific RAS

peptide profiles that could be useful for the assessment of patients under RAS inhibitor treatment. As a first step, we characterized circulating and equilibrium RAS peptide profiles after single and repeated doses of the renin inhibitor aliskiren, the ACE inhibitor enalapril and the angiotensin receptor antagonist losartan in healthy, normotensive subjects.

Methods

Clinical Study

A single-center, randomized, open-label, 3-way crossover study (ClinicalTrials.gov ID: NCT01771783) was performed at the Phase I Research Unit, University Hospital Basel, Switzerland. The study was approved by the local Ethics Committee (Ethikkommission Basel) and the national regulatory authorities (Swiss Agency for Therapeutic Products, Swissmedic) and conducted in accordance with the principles of the Declaration of Helsinki.

Healthy, normotensive male subjects were randomized to one of three treatment groups. In every treatment group, study subjects received eight oral doses of 10 mg enalapril, 50 mg losartan, and 150 mg aliskiren in a different sequence with a wash out period of at least 13 days between the different treatment periods. One day before starting with the first treatment period, blood samples for the assessment of baseline peptide profiles were collected. On day 1 and 8 of each treatment period the subjects were fasted for 10 h before until 4 h after administration of study medication. Standardized meals were served 4 h, 6 h and 10 h after study drug intake.

Venous blood samples were obtained from an indwelling venous catheter placed in the non-dominant forearm before and 1, 2, 4, 8, 12 and 24 h after drug administration on day 1 and on day 8 of each treatment period. For the analysis of circulating RAS peptide concentrations, blood was collected into tubes containing broad spectrum inhibitors against metalloproteases (ethylenediaminetetraacetic acid [EDTA], 1,10-phenanthroline), aspartic

proteases (pepstatin A), cysteine proteases (p-hydroxymercuribenzoic acid), serine proteases (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride [AEBSF]) and specific inhibitors for renin and aminopeptidases A and N to a final concentration of 5% v/v (Attoquant Diagnostics, Vienna, Austria).

For the analysis of ex vivo equilibrium RAS peptide concentrations, blood was collected into standard lithium heparin tubes. Equilibrium angiotensin peptide levels were measured following 30 min of equilibration of conditioned Li-Heparin plasma at 37°C and subsequent stabilization of equilibrium peptide levels. For the analysis of drug and renin concentrations blood was collected into EDTA tubes. All tubes were centrifuged within 30 min at 1500 g for 10 min at 4 °C and plasma was stored at -80°C until analysis.

Blood pressure and heart rate were recorded from the subjects in supine position for at least 10 min using an automatic oscillometric device. Measurements were done on the dominant arm at the time points of blood sample collection. At each time point, blood pressure and heart rate were measured three consecutive times with an interval of at least one minute between measurements and the average of the three readings was recorded. If two blood pressure readings differed by more than 5 mmHg, an additional measurement was done and the outlier was excluded for the calculation of the average.

No concomitant medication was allowed, except for the treatment of AEs. Consumption of alcoholic beverages was not allowed. All subjects were requested to follow a diet recommendation to prevent dietary salt excess for 72 h prior to each treatment period until after the collection of the last blood sample of each treatment period. Urine sodium excretion was determined in 24 h urine samples collected on day 1 and day 8 of each treatment period.

Study Drugs

Enalapril (Reniten®, MSD Merck Sharp & Dohme AG), losartan (Cosaar®, MSD Merck Sharp & Dohme AG), and aliskiren (Rasilez®, Novartis Pharma Schweiz AG) were purchased through the hospital pharmacy of the University Hospital Basel.

Materials and Reagents

Losartan, losartan-d4, losartan carboxylic acid (E-3174), enalaprilat, enalaprilat-d5, aliskiren, and aliskiren-d6 were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Formic acid, high-performance liquid chromatography (HPLC)-grade methanol and water were purchased from Merck KGaA (Darmstadt, Germany).

Stable isotope labeled internal standards for angiotensin metabolites and MS grade formic acid were purchased from Sigma-Aldrich (Vienna, Austria). LC-MS-grade water, methanol and acetonitrile were purchased from Fisher Scientific (Vienna, Austria). The protease inhibitor cocktail containing EDTA, Pepstatin A, p-hydroxymercuribenzoic acid, AEBSF and specific inhibitors for renin and aminopeptidases A and N was provided by Attoquant Diagnostics (Vienna, Austria).

Measurement of Drug Concentrations

Concentrations of losartan, E-3174, enalaprilat, and alsikiren in plasma were determined by reversed-phase HPLC with tandem mass spectrometry (HPLC–MS/MS). Briefly, 50 µL aliquots of plasma were mixed with 150 µL of an internal standard solution containing deuterated analogues of the study drugs at a concentration of 10 ng/mL, vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Ten microlitres of deproteinized supernatant were injected directly into the HPLC–MS/MS system. Chromatographic separation was done on a Shimadzu HPLC system (Shimadzu AG, Reinach, Switzerland) coupled to a triple quadrupole tandem mass spectrometer (API4000, Applied Biosystems, Rotkreuz, Switzerland) operating in electrospray-ionization positive-ion mode. An Atlantis T3 column (50 x 2.1 mm, Waters, Baden-Dättwil, Switzerland) was used for the separation of the analytes. The total run time was 2.3 min. Samples were quantified using peak area ratios. The assays were linear in the concentration ranges 1-250 ng/mL for losartan, E-3174, and alsikiren, and 1-100 ng/mL for enalaprilat. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Quantification of RAS Peptide Concentrations

Following 30 min of equilibration of conditioned Li-heparin plasma at 37°C, stabilized samples for quantification of angiotensin metabolite concentrations were spiked with stable isotope-labeled internal standards for each angiotensin metabolite (Ang 1-10, Ang 1-9, Ang 1-8, Ang 1-7, Ang 1-5, Ang 2-10, Ang 2-8, Ang 2-7, Ang 3-8, and Ang 3-7) at a concentration of 100 pg/ml. Following C18-based solid-phase-extraction, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC® C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in MRM mode. Internal standards were used to correct for peptide recovery of the sample preparation procedure for each angiotensin metabolite in each individual sample. Angiotensin metabolite concentrations were calculated considering the corresponding response factors determined in appropriate calibration curves in original sample matrix, on condition that integrated signals exceeded a signal-to-noise ratio of 10. Lower limits of quantification (LLOQ) for the different angiotensin peptides were between 1 and 5 pg/ml (Supplementary Table 2).

Pharmacokinetic Analysis

Plasma concentration data were analyzed using noncompartmental methods. Peak plasma concentrations (C_{max}) and time to reach C_{max} (t_{max}) were directly obtained from observed concentration-time data. The terminal elimination rate constant (λz) was determined by log-linear regression using at least three data points. The area under the concentration–time curve (AUC) from zero to 24 h after dosing (AUC_{0-24}) was estimated using the linear trapezoidal method. The terminal elimination half-life was calculated using λz. Calculations were done using the PK Solver add-in (version 2.0) for Microsoft Excel.[27]

Statistical analysis

Changes of systolic and diastolic blood pressure and heart rate were evaluated by comparing area under the effect-time curves from time zero to 8 hours ($AUEC_{0-8h}$). Statistical

analysis was performed using GraphPad Prism, version 5.03 (GraphPad Sofware, Inc., USA). Normal distribution of the data was assessed using Bartlett's test. Parameters with a normal distribution were tested using one-way analysis of variance (ANOVA). Post-hoc analysis of significant overall results was done with Dunnett's test for multiple comparisons. Non-normally distributed parameters were analyzed by the non-parametric Friedman test. For post-hoc analysis of significant overall results Dunn's test for multiple comparisons was used.

Results

Clinical study

Twelve healthy, non-smoking male volunteers (median age 29 years, range 22 – 42 years, median body mass index 22.6 kg/m^2, range 18.4 – 26.8 kg/m^2) completed the study according to the protocol. Ten study subjects were of Caucasian ethnicity, one subject was of Indian and another subject of South-Amercian descent. They all had no history of relevant disease or drug abuse, normal findings on physical examination, normal blood pressure (median systolic blood pressure 129 mmHg, range 118-139 mmHg, median diastolic blood pressure 76 mmHg, range 68-88 mmHg), normal screening laboratory tests (including screening for drugs of abuse), and normal electrocardiograms. The mean (±sd) urinary sodium excretion was 173 (±60) mmol/24 hours (corresponding to 10.1±3.5 grams of dietary sodium chloride) without any statistical difference between the baseline assessment and the different treatment periods.

Plasma concentration-time profiles of aliskiren, enalaprilat and losartan carboxylic acid after single and repeated once daily dosing are shown in Figure 1 and pharmacokinetic parameters are listed in Supplementary Table 1. Drug exposure after repeated dosing was not significantly different compared to single dose administration and in line with published values.[28-30]

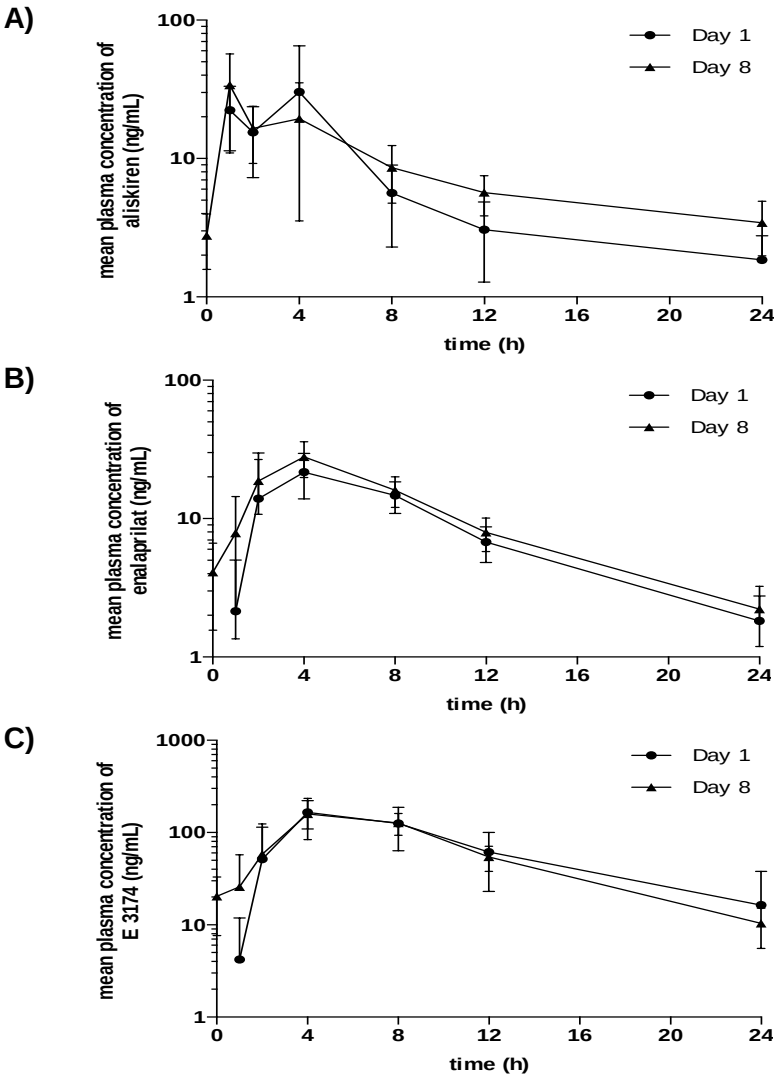

Figure 1. Mean (sd) plasma concentration–time profiles of aliskiren (A), enalaprilat (B), and losartan carboxylic acid (E3174, C) after single and repeated once-daily oral administration of aliskiren 150 mg, enalapril 10 mg, and losartan 50 mg to healthy subjects (n = 12).

Mean systolic blood pressure without treatment was 118 mmHg (range 103-145 mmHg) and mean heart rate was 58 beats per minute (range 43-77 bpm) without any significant changes during the 8 hour assessment period. The effects of single and multiple dose treatment with aliskiren, enalapril or losartan on blood pressure and heart rate are shown in Table 1. The largest effect was observed between 2 and 4 hours after single dose enalapril with a maximal decrease of systolic blood pressure of 7 mmHg and a maximal decrease of heart rate of 3 bpm. Single and multiple dose treatment with aliskiren or losartan had smaller effects on blood pressure and heart rate compared to enalapril.

Angiotensin peptide concentrations in untreated subjects

Without treatment, concentrations of circulating RAS peptides in healthy, normotensive, non-salt-depleted subjects were low. During the 8 hour baseline assessment, median concentrations of circulating Ang 1-10 and Ang 1-8 were between 12-16 pg/ml (range 6-100 pg/ml) and 9-12 pg/ml (range 1-68 pg/ml), respectively. Concentrations of Ang 1-9 were below the lower limit of quantification (LLOQ) in all subjects, and circulating concentrations of the remaining peptides (Ang 1-7, Ang 1-5, Ang 2-10, Ang 2-8, Ang 2-7, Ang 3-8, Ang 3-7) could only be quantified in some of the subjects (Table 2). There was no time-dependent change of quantifiable peptide concentrations during the 8 hour observation period. A representative bubble plot scheme of circulating RAS peptide concentrations in untreated healthy subjcets including enzymes involved in formation or degradation of the measured RAS peptides is shown in Figure 2.

In RAS equilibrium analysis, concentrations of Ang 1-8 were approx. five-fold higher (median 41-70 pg/ml) compared to circulating Ang 1-8 concentrations, whereas concentrations of all the other peptides in the equilibrium samples were comparable to circulating peptide concentrations (Table 3). Data of one subject had to be excluded due to pathologically increased renin concentrations.

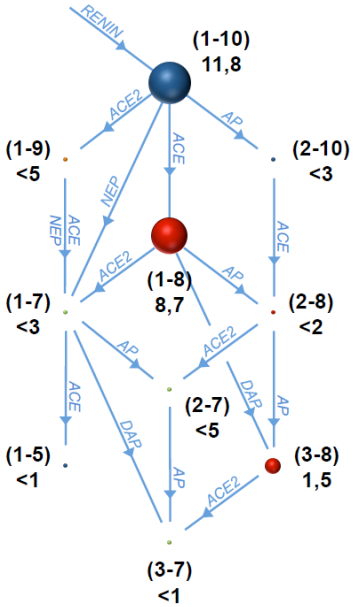

Figure 2. A representative bubble plot scheme of circulating angiotensin concentrations in untreated, normotensive, healthy subjects (n=12).

The diameter of the bubble corresponds to the median concentration (pg/ml), the numeric value of the median concentration is given below the peptide designation according to the number of amino acids in parenthesis. The arrows designate known degradation pathways with the responsible enzymes. ACE, angiotensin converting enzyme; AP, aminopeptidase A; DAP, dipeptidyl aminopeptidase; NEP, neutral endopeptidase.

Table 1. Maximum changes of blood pressure and heart rate without treatment (baseline) and after single and repeated dose treatment with aliskiren, enalapril and losartan.

	Baseline	**Aliskiren**		**Enalapril**		**Losartan**	
		Day 1	Day 8	Day 1	Day 8	Day 1	Day 8
Systolic blood pressure							
Maximum change (mmHg)	**1** (+13 / -16)	**1** (15 – -20)	**-4** (14 – -17)	**-7** (10 – -20)	**-3** (10 – -22)	**-2** (18 – -24)	**0** (15 – -13)
AUEC (mmHg x h)	**5** (57 – -93)	**-8** (60 – -116)	**-11** (46 – -136)	**-48** (35 – -109)	**-27** (46 – -135)	**-11** (32 – -157)	**-33** (58 – -159)
Diastolic blood pressure							
Maximum change (mmHg)	**-1** (9 – -12)	**-2** (14 – -16)	**-4** (11 – -14)	**-9** (8 – -17)	**-6** (4 – -18)	**-4** (7 – -18)	**-5** (6 – -15)
AUEC (mmHg x h)	**-6** (38 – -69)	**-11** (34 – -99)	**-27** (33 – -144)	**-64** (29 – -94)	**-42** (-8 – -106)	**-21** (20 – -122)	**-43** (21 – -129)
Heart rate							
Maximum change (beats/min)	**0** (24 – -14)	**1** (14 – -20)	**-2** (10 – -12)	**-3** (12 – -15)	**-3** (12 – -14)	**1** (13 – -7)	**-1** (14 – -20)
AUEC (beats/min x h)	**3** (138 – -76)	**5** (39 – -126)	**-23** (26 – -110)	**-4** (43 – -88)	**-7** (122 – -84)	**1** (70 – -29)	**18** (85 – -57)

Data are given as median (range). AUEC, area under effect curve from 0 to 8 hours.

Table 2. Circulating angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Baseline

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
0	**<7** (<5 – 87) [6]	**<4**	**9** (<1 – 68) [11]	**<3** (<3 – 13) [2]	**<1** (<1 – 3) [3]	**<3** (<3 – 2) [1]	**<2**	**<4**	**2** (<1 – 6) [7]	**<1** (<1 – 1) [1]
2	**14** (<5 – 45) [8]	**<4**	**10** (2 – 38)	**<3**	**<1** (<1 – 3) [1]	**<3** (<3 – 8) [2]	**<2**	**<4**	**2** (<1 – 5) [7]	**<1**
4	**<8** (<5 – 100) [6]	**<4**	**12** (<1 – 40) [10]	**<3** (<3 – 8) [3]	**<1** (<1 – 3) [3]	**<3** (<3 – 4) [2]	**<2**	**<4**	**<2** (<1 – 5) [6]	**<1** (<1 – 2) [2]
8	**<5** (<5 – 28) [5]	**<4**	**9** (2 – 32)	**<3** (<3 – 7) [2]	**<1** (<1 – 3) [1]	**<3** (<3 – 8) [2]	**<2**	**<4**	**1** (<1 – 4) [9]	**<1**

Aliskiren

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**13** (<5 – 105) [8]	**<4**	**8** (2 – 48)	**<3** (<3 – 3) [2]	**<1** (<1 – 2) [3]	**<3** (<3 – 5) [2]	**<2**	**<4** (<4 – 6) [1]	**2** (<1 – 4) [8]	**<1** (<1 – 1) [1]
2	**<5** (<5 – 5) [1]	**<4**	**4** (<1 – 20) [11]	**<3** (<3 – 9) [3]	**<1** (<1 – 2) [2]	**<3**	**<2**	**<4**	**2** (<1 – 2) [7]	**<1** (<1 – 2) [1]
4	**<5**	**<4**	**6** (<1 – 29) [9]	**<3** (<3 – 4) [1]	**<1** (<1 – 3) [2]	**<3** (<3 – 3) [3]	**<2**	**<4**	**<1** (<1 – 3) [4]	**<1** (<1 – 4) [1]
8	**<5** (<5 – 24) [2]	**<4**	**3** (<1 – 24) [11]	**<3** (<3 – 8) [2]	**<1** (<1 – 2) [1]	**<3**	**<2**	**<4**	**<1** (<1 – 3) [5]	**<1** (<1 – 1) [1]
24	**17** (<5 – 39) [8]	**<4**	**9** (3 – 29)	**<3**	**<1** (<1 – 3) [4]	**<3** (<3 – 7) [3]	**<2**	**<4**	**1** (<1 – 2) [7]	**<1**
168	**12** (<5 – 17) [6]	**<4**	**8** (<1 – 29) [11]	**<3** (<3 – 15) [2]	**<1** (<1 – 2) [2]	**<3**	**<2**	**<4**	**<1** (<1 – 7) [5]	**<1**
170	**<5**	**<4**	**6** (2 – 15)	**<3**	**<1** (<1 – 3) [3]	**<3** (<3 – 6) [3]	**<2**	**<4**	**<1** (<1 – 4) [6]	**<1** (<1 – 1) [1]
172	**<5** (<5 – 25) [2]	**<4**	**4** (<1 – 17) [11]	**<3** (<3 – 4) [1]	**<1** (<1 – 4) [2]	**<3** (<3 – 3) [3]	**<2**	**<4** (<4 – 9) [1]	**<1** (<1 – 3) [6]	**<1**
176	**<5** (<5 – 15) [5]	**<4**	**7** (<1 – 22) [11]	**<3** (<3 – 8) [2]	**<1** (<1 – 2) [4]	**<3** (<3 – 4) [1]	**<2**	**<4** (<4 – 8) [2]	**<1** (<1 – 4) [6]	**<1**
192	**10** (<5 – 18) [9]	**<4**	**7** (1 – 29)	**<3** (<3 – 3) [1]	**<1** (<1 – 4) [2]	**<3** (<3 – 6) [2]	**<2**	**<4**	**1** (<1 – 4) [8]	**<1**

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Table 2 cont. Circulating angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Enalapril

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**14** (<5 – 64) [7)]	**<4**	**7** (<1 – 38) [11)]	**<3**	**<1** (<1 – 2) [2)]	**<3** (<3 – 12) [4)]	**<2**	**<4** (<4 – 6) [1)]	**<1** (<1 – 4) [6)]	**<1**
2	**35** (<5 – 312) [11)]	**<4**	**4** (<1 – 20) [11)]	**<3** (<3 – 10) [4)]	**<1** (<1 – 3) [2)]	**<3** (<3 – 14) [5)]	**<2**	**<4**	**2** (<1 – 3) [7)]	**<1** (<1 – 1) [1)]
4	**82** (<5 – 299) [11)]	**<4**	**3** (1 – 22)	**7** (<3 – 16) [9)]	**<1** (<1 – 4) [2)]	**4** (<3 – 18) [8)]	**<2**	**<4**	**<1** (<1 – 2) [5)]	**<1** (<1 – 3) [3)]
8	**88** (8 – 287)	**<4** (<4 – 5) [1)]	**4** (<1 – 17) [10)]	**7** (<3 – 13) [7)]	**<1** (<1 – 5) [2)]	**<3** (<3 – 7) [6)]	**<2**	**<4**	**<1** (<1 – 5) [5)]	**<1** (<1 – 2) [1)]
24	**29** (11 – 164)	**<4** (<4 – 7) [1)]	**7** (<1 – 20) [11)]	**<3** (<3 – 4) [2)]	**<1** (<1 – 5) [3)]	**<3** (<3 – 12) [3)]	**<2**	**<4**	**2** (<1 – 2) [7)]	**<1** (<1 – 4) [1)]
168	**46** (18 – 194)	**<4**	**10** (<1 – 15) [11)]	**<3** (<3 – 9) [2)]	**<1** (<1 – 4) [5)]	**<3** (<3 – 19) [6)]	**<2**	**<4**	**2** (<1 – 3) [7)]	**<1** (<1 – 2) [2)]
170	**219** (9 – 1002)	**<4**	**4** (<1 – 17) [11)]	**10** (<3 – 43) [9)]	**<1** (<1 – 4) [2)]	**5** (<3 – 14) [9)]	**<2**	**<4** (<4 – 6) [3)]	**<1** (<1 – 3) [6)]	**<1** (<1 – 5) [4)]
172	**192** (36 –1711)	**<4**	**5** (<1 – 31) [11)]	**12** (<3 – 97) [9)]	**<1** (<1 – 2) [3)]	**5** (<3 – 11) [8)]	**<2**	**<4** (<4 – 6) [1)]	**<1** (<1 – 3) [5)]	**<1** (<1 – 8) [5)]
176	**179** (14 – 603)	**<4**	**6** (<1 – 33) [11)]	**11** (<3 – 20) [9)]	**<1** (<1 – 3) [1)]	**<3** (<3 – 8) [5)]	**<2**	**<4** (<4 – 5) [2)]	**2** (<1 – 3) [7)]	**<1** (<1 – 6) [2)]
192	**41** (20 – 187)	**<4**	**11** (6 – 22)	**<3**	**<1**	**<3** (<3 – 6) [5)]	**<2**	**<4** (<4 – 4) [1)]	**<1** (<1 – 4) [5)]	**<1** (<1 – 2) [2)]

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Table 2 cont. Circulating angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Losartan

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**13** (<5 – 39) [7)]	**<4**	**7** (1 – 45)	**<3** (<3 – 4) [1)]	**<1** (<1 – 4) [2)]	**<3** (<3 – 11) [3)]	**<2**	**<4** (<4 – 5) [1)]	**<1** (<1 – 3) [6)]	**<1** (<1 – 3) [2)]
2	**24** (<5 – 171) [8)]	**<4**	**14** (4 – 89)	**<3** (<3 – 9) [3)]	**<1** (<1 – 2) [4)]	**<3** (<3 – 6) [4)]	**<2**	**<4**	**2** (<1 – 4) [8)]	**<1** (<1 – 2) [1)]
4	**71** (<5 – 169) [8)]	**<4**	**36** (3 – 187)	**<3** (<3 – 5) [1)]	**<1** (<1 – 4) [5)]	**<3** (<3 – 4) [4)]	**<2**	**<4**	**2** (<1 – 6) [8)]	**<1** (<1 – 1) [2)]
8	**38** (<5 – 346) [10)]	**<4**	**23** (3 – 217)	**<3** (<3 – 9) [2)]	**2** (<1 – 6) [7)]	**<3** (<3 – 9) [3)]	**<2**	**<4**	**2** (<1 – 8) [7)]	**<1**
24	**32** (6 – 87)	**<4**	**18** (8 – 45)	BLQ	**<1** (<1 – 2) [4)]	**<3** (<3 – 6) [2)]	**<2**	**<4**	**<1** (<1 – 3) [6)]	**<1** (<1 – 2) [1)]
168	**31** (8 – 122)	**<4**	**22** (9 – 144)	**<3** (<3 – 10) [2)]	**<1** (<1 – 4) [5)]	**<3** (<3 – 3) [3)]	**<2**	**<4**	**2** (<1 – 5) [8)]	**<1**
170	**39** (15 – 251)	**<4**	**26** (11 – 149)	**<3** (<3 – 7) [3)]	**<1** (<1 – 4) [6)]	**<3** (<3 – 4) [1)]	**<2**	**<4**	**3** (<1 – 6) [8)]	**<1**
172	**73** (22 – 305)	**<4**	**45** (20 – 231)	**<3** (<3 – 13) [3)]	**2** (<1 – 9) [10)]	**<3**	**<2** (<2 – 2) [1)]	**<4**	**2** (<1 – 7) [8)]	**<1**
176	**78** (16 – 241)	**<4**	**64** (19 – 203)	**<3** (<3 – 4) [2)]	**2** (<1 – 7) [10)]	**<3** (<3 – 4) [2)]	**<2** (<2 – 2) [1)]	**<4**	**3** (<1 – 7) [10)]	**<1** (<1 – 2) [2)]
192	**46** (16 – 115)	**<4**	**22** (12 – 59)	**<3** (<3 – 5) [3)]	**2** (<1 – 3) [7)]	**<3** (<3 – 5) [1)]	**<2**	**<4**	**2** (<1 – 4) [9)]	**<1**

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Table 3. Equilibrium angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Baseline

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
0	**16** (<5 – 209) [10]	**<4** (<4 – 8) [2]	**66** (18 – 647)	**<3** (<3 – 15) [3]	**2** (<1 – 7) [8]	**<3** (<3 – 5) [4]	**<2** (<2 – 16) [6]	**<5**	**<1** (<1 – 25) [6]	**<1** (<1 – 3) [6]
2	**16** (<5 – 218) [9]	**<4** (<4 – 12) [2]	**70** (11 – 561)	**<3** (<3 – 17) [4]	**1** (<1 – 4) [9]	**<3** (<3 – 5) [5]	**<2** (<2 – 14) [6]	**<5**	**2** (<1 – 14) [9]	**<1** (<1 – 2) [5]
4	**16** (<5 – 241) [10]	**<4**	**45** (<2 – 207) [11]	**<3** (<3 – 4) [2]	**<1** (<1 – 8) [3]	**<3** (<3 – 8) [4]	**<2** (<2 – 5) [3]	**<5**	**2** (<1 – 3) [8]	**<1** (<1 – 3) [4]
8	**17** (<5 – 148) [11]	**<4** (<4 – 5) [1]	**41** (3 – 526)	**<3** (<3 – 12) [4]	**2** (<1 – 11) [10]	**<3** (<3 – 8) [5]	**<2** (<2 – 13) [5]	**<5** (<5 – 6) [2]	**2** (<1 – 14) [8]	**<1** (<1 – 1) [4]

Aliskiren

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**19** (<5 – 97) [11]	**<4** (<4 – 5) [2]	**89** (6 – 400)	**<3** (<3 – 6) [5]	**2** (<1 – 4) [9]	**<3** (<3 – 3) [5]	**<2** (<2 – 13) [4]	**<5** (<5 – 5) [4]	**3** (<1 – 16) [8]	**<1** (<1 – 3) [4]
2	**<5** (<5 – 29) [2]	**<4** (<4 – 9) [3]	**11** (<2 – 80) [11]	**<3** (<3 – 9) [5]	**<1** (<1 – 2) [5]	**<3** (<3 – 17) [5]	**<2** (<2 – 5) [1]	**<5** (<5 – 5) [2]	**<1** (<1 – 3) [4]	**<1** (<1 – 2) [6]
4	**<5** (<5 – 9) [2]	**<4** (<4 – 11) [2]	**8** (<2 – 93) [11]	**<3** (<3 – 4) [2]	**<1** (<1 – 2) [6]	**<3** (<3 – 11) [5]	**<2** (<2 – 9) [1]	**<5** (<5 – 6) [2]	**<1** (<1 – 4) [3]	**<1** (<1 – 2) [3]
8	**<5** (<5 – 50) [6]	**<4** (<4 – 10) [2]	**33** (<2 – 188) [11]	**<3** (<3 – 10) [3]	**<1** (<1 – 3) [5]	**<3** (<3 – 10) [4]	**<2** (<2 – 8) [5]	**<5** (<5 – 5) [1]	**1** (<1 – 8) [7]	**<1** (<1 – 2) [4]
24	**9** (<5 – 54) [10]	**<4** (<4 – 10) [3]	**64** (26 – 264)	**<3** (<3 – 8) [4]	**2** (<1 – 3) [8]	**<3** (<3 – 13) [5]	**<2** (<2 – 6) [5]	**<5**	**2** (<1 – 8) [9]	**<1** (<1 – 2) [3]
168	**16** (<5 – 38) [10]	**<4** (<4 – 4) [1]	**48** (6 – 117)	**<3** (<3 – 14) [3]	**2** (<1 – 7) [7]	**<3** (<3 – 7) [5]	**<2** (<2 – 5) [3]	**<5** (<5 – 5) [2]	**2** (<1 – 4) [10]	**<1** (<1 – 2) [3]
170	**9** (<5 – 26) [7]	**<4** (<4 – 7) [2]	**22** (5 – 63)	**<3** (<3 – 13) [4]	**<1** (<1 – 3) [4]	**<3** (<3 – 11) [5]	**<2** (<2 – 12) [4]	**<5** (<5 – 5) [2]	**<1** (<1 – 2) [6]	**<1** (<1 – 2) [5]
172	**<5** (<5 – 17) [5]	**<4** (<4 – 5) [2]	**21** (5 – 51)	**<3** (<3 – 12) [3]	**<1** (<1 – 2) [4]	**<3** (<3 – 13) [4]	**<2** (<2 – 5) [4]	**<5**	**<1** (<1 – 3) [5]	**<1** (<0 – 4) [4]
176	**15** (<5 – 41) [7]	**<4** (<4 – 8) [3]	**33** (9 – 106)	**<3** (<3 – 9) [4]	**1** (<1 – 3) [7]	**7** (<3 – 13) [8]	**<2** (<2 – 9) [3]	**<5** (<5 – 5) [2]	**<1** (<1 – 9) [5]	**<1** (<1 – 2) [3]
192	**19** (<5 – 52) [10]	**<4**	**56** (10 – 186)	**<3** (<3 – 31) [4]	**2** (<1 – 4) [7]	**<3** (<3 – 17) [5]	**<2** (<2 – 6) [4]	**<5** (<5 – 5) [3]	**2** (<1 – 4) [7]	**<1** (<1 – 2) [3]

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Table 3 cont. Equilibrium angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Enalapril

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**16** (<5 – 49) [11)]	**<4** (<4 – 7) [2)]	**55** (5 – 259)	**<5** (<3 – 6) [6)]	**2** (<1 – 4) [8)]	**<3** (<3 – 9) [5)]	**<2** (<2 – 8) [6)]	**<5** (<5 – 5) [3)]	**3** (<1 – 6) [8)]	**<1** (<1 – 2) [2)]
2	**177** (33 – 1131)	**<4** (<4 – 4) [1)]	**10** (2 – 96)	**<6** (<3 – 43) [6)]	**<1** (<1 – 1) [6)]	**14** (<3 – 133) [9)]	**<2** (<2 – 5) [2)]	**<5**	**<1** (<1 – 5) [3)]	**<1** (<1 – 6) [6)]
4	**424** (23 – 1826)	**<4** (<4 – 5) [2)]	**16** (<2 – 138) [11)]	**27** (<3 – 58) [9)]	**<1** (<1 – 4) [6)]	**27** (<3 – 106) [10)]	**<2** (<2 – 4) [5)]	**<5** (<5 – 8) [4)]	**<1** (<1 – 5) [2)]	**1** (<1 – 3) [8)]
8	**624** (41 – 3001)	**<4** (<4 – 3) [2)]	**16** (3 – 219)	**27** (<3 – 60) [10)]	**<1** (<1 – 4) [6)]	**36** (<3 – 131) [11)]	**<2** (<2 – 9) [2)]	**<5** (<5 – 6) [1)]	**<1** (<1 – 9) [6)]	**1** (<1 – 3) [7)]
24	**164** (25 – 339)	**<4** (<4 – 7) [1)]	**62** (16 – 260)	**6** (<3 – 13) [7)]	**2** (<1 – 4) [9)]	**10** (<3 – 20) [8)]	**<3** (<2 – 7) [6)]	**<5**	**2** (<1 – 8) [10)]	**2** (<1 – 3) [4)]
168	**260** (64 – 1545)	**<4**	**80** (23 – 190)	**9** (<3 – 74) [10)]	**2** (<1 – 5) [9)]	**12** (3 – 87)	**4** (<2 – 6) [7)]	**<5** (<5 – 5) [3)]	**3** (<1 – 7) [10)]	**1** (<1 – 3) [6)]
170	**960** (116 – 3229)	**<4** (<4 – 22) [3)]	**38** (8 – 135)	**38** (<3 – 134) [10)]	**1** (<1 – 5) [7)]	**88** (9 – 126)	**5** (<2 – 6) [4)]	**<5**	**2** (<1 – 4) [8)]	**2** (<1 – 7) [7)]
172	**916** (267 – 4288)	**<4** (<4 – 10) [3)]	**16** (7 – 112)	**47** (<3 – 107) [11)]	**1** (<1 – 4) [8)]	**75** (<3 – 176) [11)]	**<2** (<2 – 7) [2)]	**<5** (<5 – 6) [4)]	**<1** (<1 – 5) [5)]	**2** (<1 – 8) [8)]
176	**979** (102 – 4502)	**<4** (<4 – 5) [2)]	**51** (2 – 303)	**50** (<3 – 153) [10)]	**<1** (<1 – 3) [5)]	**62** (9 – 248)	**<2** (<2 – 10) [4)]	**<5** (<5 – 7) [4)]	**2** (<1 – 13) [7)]	**2** (<1 – 8) [9)]
192	**158** (112 – 807)	**<4**	**123** (37 – 361)	**13** (<3 – 46) [8)]	**2** (<1 – 6) [11)]	**12** (<3 – 31) [11)]	**6** (<2 – 18) [8)]	**<5**	**4** (<1 – 12) [11)]	**2** (<1 – 4) [5)]

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Table 3 cont. Equilibrium angiotensin concentrations (pg/ml) before (baseline) and after single and multiple doses of aliskiren, enalapril or losartan.

Losartan

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7
Predose	**14** (<5 – 80) [11)]	**<4**	**50** (24 – 371)	**<3** (<3 – 8) [6)]	**1** (<1 – 5) [9)]	**<3** (<3 – 9) [5)]	**<2** (<2 – 10) [5)]	**<5**	**2** (<1 – 11) [9)]	**<1** (<1 – 2) [4)]
2	**33** (6 – 184)	**<4** (<4 – 7) [2)]	**151** (43 – 997)	**<3** (<3 – 7) [4)]	**2** (<1 – 15) [7)]	**<3** (<3 – 12) [4)]	**6** (<2 – 30) [8)]	**<5** (<5 – 5) [3)]	**4** (<1 – 35) [9)]	**<1** (<1 – 2) [2)]
4	**108** (<5 – 357) [11)]	**<4**	**283** (42 – 2084)	**<3** (<3 – 19) [5)]	**3** (<1 – 38) [11)]	**6** (<3 – 15) [8)]	**9** (<2 – 71) [9)]	**<5** (<5 – 6) [2)]	**7** (<1 – 73) [11)]	**<1** (<1 – 1) [3)]
8	**111** (13 – 841)	**<4** (<4 – 4) [3)]	**261** (79 – 2941)	**6** (<3 – 23) [7)]	**4** (<1 – 38) [11)]	**6** (<3 – 13) [9)]	**7** (<2 – 110) [11)]	**<5** (<5 – 5) [2)]	**7** (2 – 114)	**<1** (<1 – 1) [5)]
24	**73** (13 – 208)	**<4** (<4 – 5) [3)]	**276** (84 – 708)	**<3** (<3 – 12) [3)]	**4** (<1 – 12) [11)]	**4** (<3 – 28) [8)]	**8** (<2 – 22) [11)]	**<5**	**7** (2 – 37)	**<1** (<1 – 2) [3)]
168	**91** (11 – 333)	**<4** (<4 – 7) [3)]	**278** (7 – 1298)	**11** (<3 – 16) [6)]	**4** (<1 – 16) [11)]	**9** (<3 – 16) [7)]	**9** (<2 – 40) [9)]	**<5** (<5 – 6) [3)]	**7** (1 – 41)	**<1** (<1 – 2) [3)]
170	**102** (50 – 421)	**<4**	**310** (66 – 1749)	**<3** (<3 – 13) [4)]	**4** (1 – 23)	**6** (<3 – 14) [7)]	**8** (<2 – 48) [10)]	**<5** (<5 – 10) [2)]	**8** (2 – 52)	**<1** (<1 – 2) [3)]
172	**165** (59 – 769)	**<4**	**416** (184 – 2425)	**5** (<3 – 26) [6)]	**6** (1 – 27)	**9** (<3 – 25) [9)]	**13** (5 – 86)	**<5** (<5 – 6) [3)]	**13** (6 – 74)	**<1** (<1 – 1) [2)]
176	**230** (68 – 1210)	**<4**	**695** (229 – 3484)	**11** (<3 – 26) [10)]	**8** (2 – 48)	**14** (<3 – 26) [8)]	**22** (3 – 92)	**<5** (<5 – 7) [5)]	**20** (7 – 119)	**<1** (<1 – 3) [3)]
192	**124** (41 – 321)	**<4** (<4 – 8) [3)]	**422** (102 – 1103)	**6** (<3 – 14) [8)]	**5** (2 – 13)	**10** (<3 – 19) [6)]	**8** (<2 – 21) [11)]	**<5**	**11** (5 – 24)	**<1** (<1 – 2) [5)]

Data are given as median (range). Data after multiple dose treatment (168-192h) shaded in grey. Number of footnote denotes number of subjects with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 subject, 2) quantifiable concentrations in 2 subjects, etc.

Angiotensin peptide concentrations after single and multiple dose treatment

After a single dose of the renin inhibitor aliskiren, concentrations of circulating Ang 1-10 decreased to levels below the limit of quantification for most of the subjects and returned to predose concentrations after 24 hours. Despite the long half-life of aliskiren, this was also observed after multiple dosing (Figure 3A). Concentrations of circulating Ang 1-8 decreased, but remained above the LLOQ for most subjects and returned to baseline concentrations 24 hours after dosing. Concentrations of the remaining analyzed peptides were mostly at or below the LLOQ (Table 2). Similar observations were made in RAS equilibrium analysis (Figure 4A) while equilibrium levels were more often detectable.

Four and eight hours after 10 mg enalapril, median concentrations of circulating Ang 1-10 increased approx. 5-fold compared to baseline and did not completely return to baseline 24 hours after dosing. After multiple dosing a more pronounced increase (approx. 15-fold) of Ang 1-10 concentrations was observed (Figure 3B). Concentrations of circulating Ang 1-8 decreased below baseline concentrations 2 hours after dosing and returned to baseline 24 hours after dosing. After repeated enalapril doses, circulating concentrations of the ACE substrate Ang 1-7 increased to quantifiable concentrations (median 10-12 pg/ml, range 5-97 pg/ml) between 2 hours and 8 hours after intake of the ACE inhibitor in 75% of the subjects (Table 2). In RAS equilibrium analysis, concentrations of Ang 1-10, Ang 1-8 and Ang 1-7 were higher, but showed a similar pattern of changes compared to circulating peptide concentrations (Figure 4B). In addition, Ang 2-10 concentrations increased between 2 hours and 8 hours after single and multiple enalapril doses (Table 3). For the remaining peptides, no relevant changes of circulating or equilibrium concentrations were observed.

Single and multiple dose treatment with the angiotensin receptor antagonist losartan led to a significant increase of circulating Ang 1-10 and Ang 1-8 concentrations without return to baseline values 24 hours after dosing (Table 2). RAS equilibrium analysis showed a similar pattern at a higher concentration level. After repeated dosing also a small increase of Ang 2-8 and Ang 3-8 concentrations was observed (Figure 4C, Table 3), with these

metabolites being more frequently above the LLOQ in contrast to circulating angiotensin levels.

Ang 1-8 / Ang 1-10 ratios as a marker for ACE activity are shown in Figure 5. Without drug treatment, Ang 1-8 / Ang 1-10 ratios based on circulating peptide concentrations were 1.1 (0.7) and did not show relevant variation during the 8-hour observation period. After single and multiple dose treatment with the ACE inhibitor enalapril, Ang 1-8 / Ang 1-10 ratios decreased significantly compared to predose ratios and did not return to baseline levels at the end of the 24 hour dose interval (Figure 5 B). The renin inhibitor and the angiotensin receptor antagonist had no relevant effect on the ratio (Figure 5A and 5C). Ang 1-8 / Ang 1-10 ratios based on equilibrium angiotensin levels were 2.0 (1.4) but showed comparable changes after ACE inhibition as the circulating peptide ratios (Figure 5D-5E).

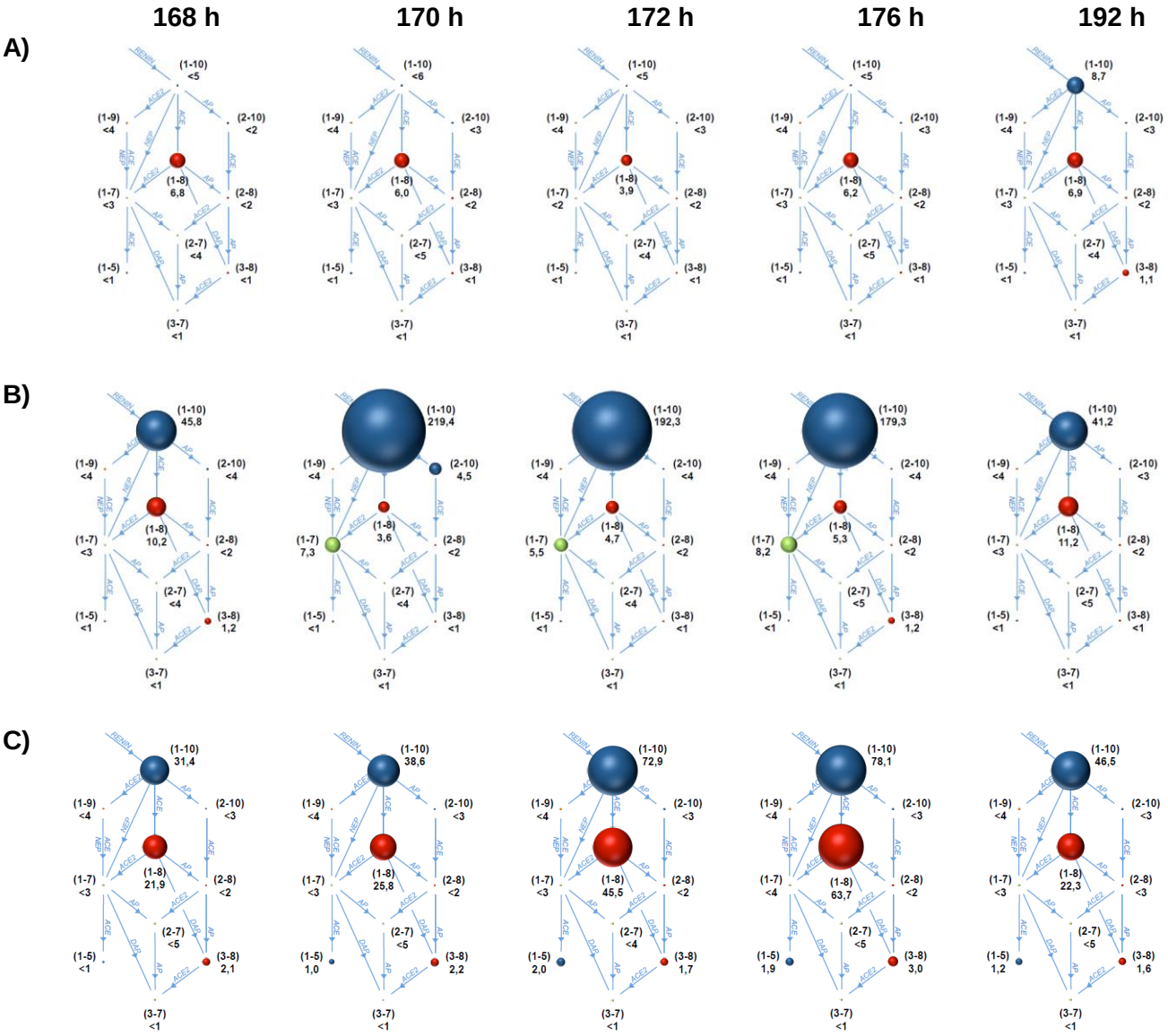

Figure 3. RAS-Fingerprint graphs of circulating angiotensin concentrations after 8 days of once-daily treatment with aliskiren 150 mg (A), enalapril 10 mg (B), and losartan 50 mg (C) before dosing (168h) and 2 hours (170h), 4 hours (172h), 8 hours (176h) and 24 hours (192h) after the last dose.

The diameter of the bubble corresponds to the median concentration (pg/ml), the numeric value of the median concentration is given below the peptide designation according to the number of amino acids in parenthesis. The arrows designate known degradation pathways with the responsible enzymes. ACE, angiotensin converting enzyme; AP, aminopeptidase A; DAP, dipeptidyl aminopeptidase; NEP, neutral endopeptidase.

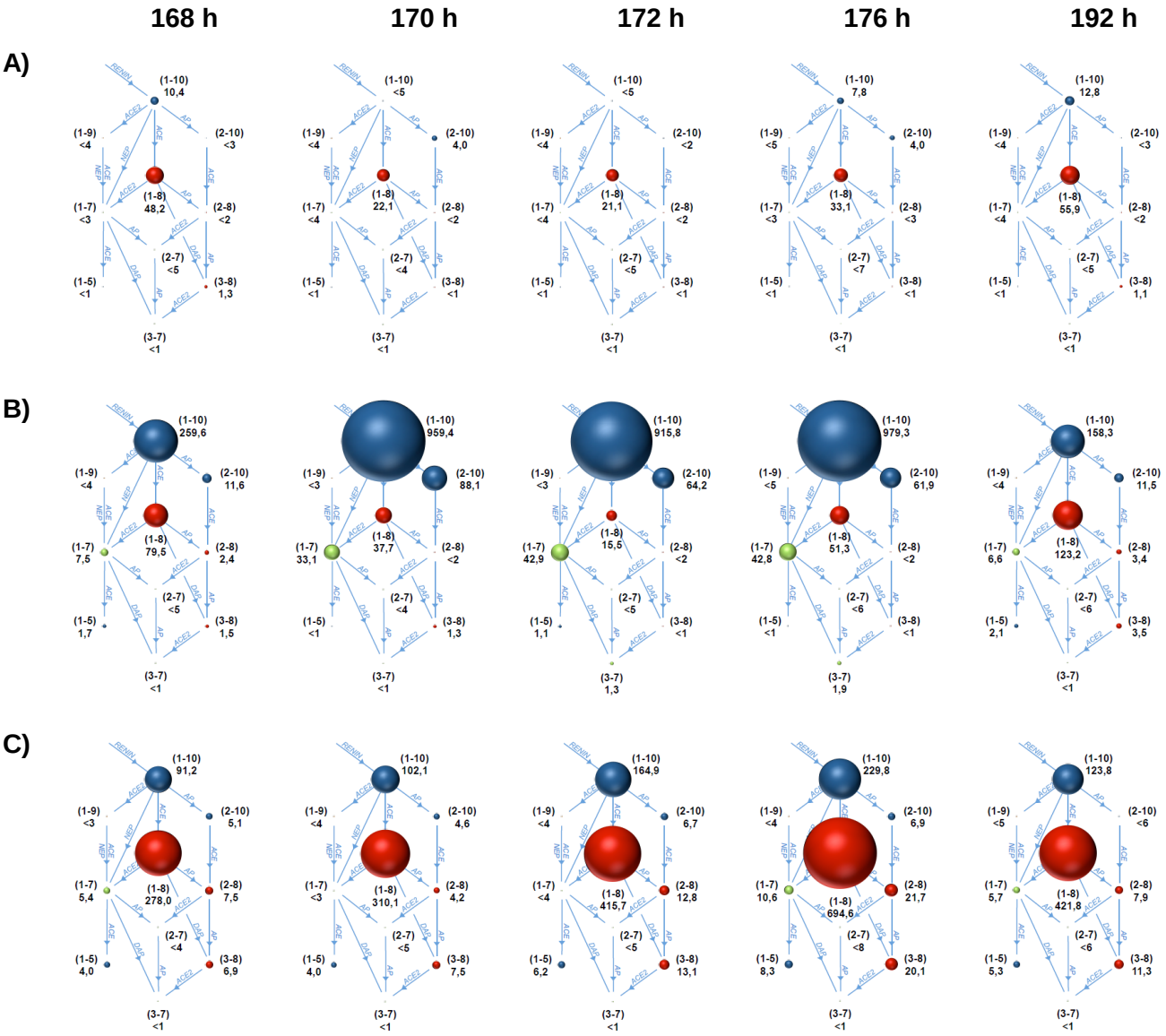

Figure 4. RAS-Fingerprint graphs of equilibrium RAS peptide concentrations after 8 days of once-daily treatment with aliskiren 150 mg (A), enalapril 10 mg (B), and losartan 50 mg (C) before dosing (168h) and 2 hours (170h), 4 hours (172h), 8 hours (176h) and 24 hours (192h) after the last dose.

The diameter of the bubble corresponds to the median concentration (pg/ml), the numeric value of the median concentration is given below the peptide designation according to the number of amino acids in parenthesis. The arrows designate known degradation pathways with the responsible enzymes. ACE, angiotensin converting enzyme; AP, aminopeptidase A; DAP, dipeptidyl aminopeptidase; NEP, neutral endopeptidase.

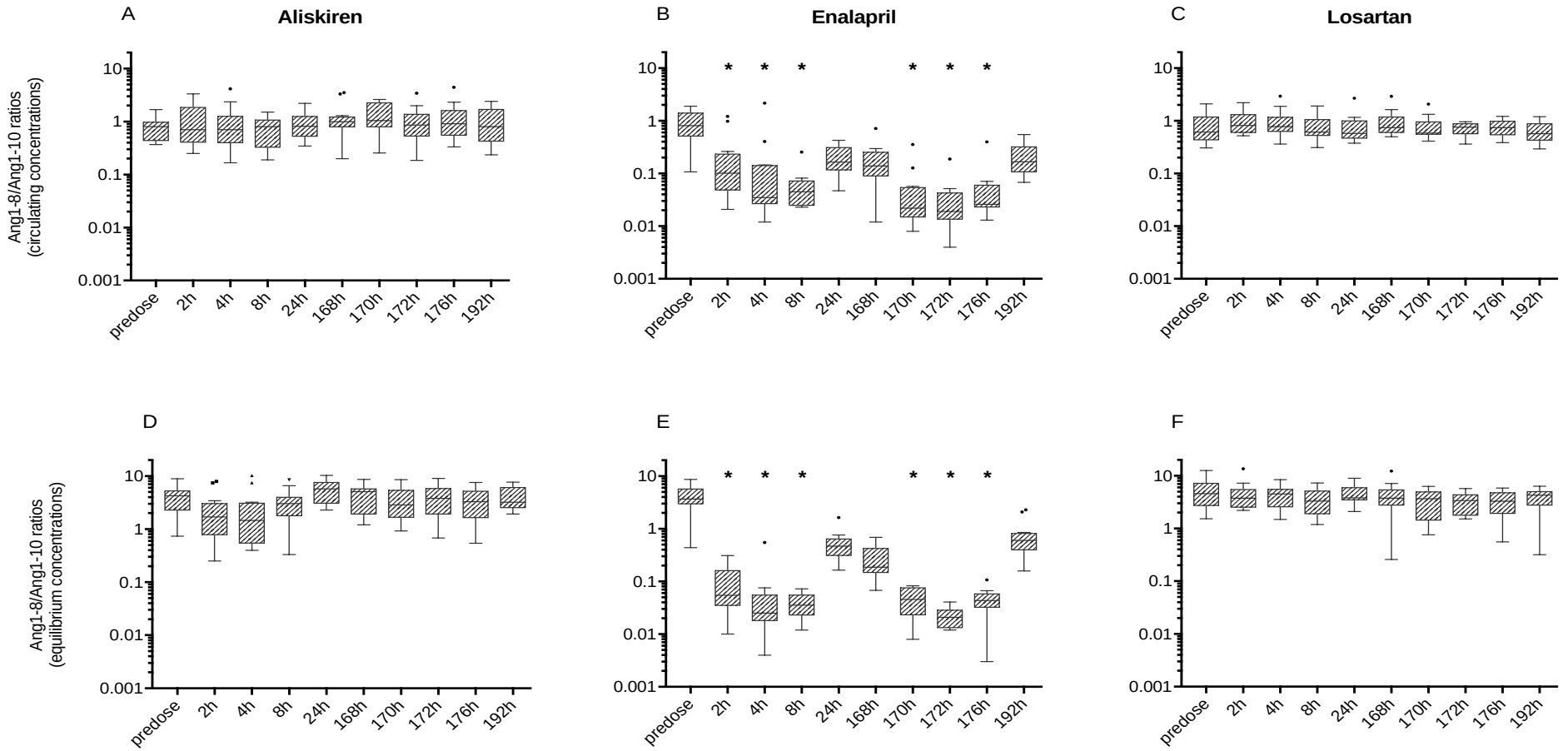

Figure 5. Ratios of the ACE product Ang 1-8 and the ACE substrate Ang 1-10 after single and multiple dose treatment with the renin inhibitor aliskiren, the ACE inhibitor enalapril and the angiotensin receptor antagonist losartan. The Ang 1-8 / Ang 1-10 ratio reflects ACE activity and is significantly reduced after ACE inhibitor treatment while renin inhibition or angiotensin receptor blockade have no relevant effect. Ratios are based on circulating angiotensin concentrations (A-C) or equilibrium angiotensin concentrations (D-F). * $p<0.001$

Discussion

In this study we quantified circulating and equilibrium concentrations of different renin-angiotensin system peptides in normotensive, non-salt-depleted healthy subjects. Concentrations were measured before and after single and multiple dose treatment with three drugs that inhibit the RAS cascade on different levels.

Without treatment, concentrations of circulating Ang 1-10 and Ang 1-8 could be quantified in all study participants. The concentrations were in the low picomolar range and did not show time-dependent changes during the 8-hour baseline observation period. Concentrations of the remaining analyzed circulating angiotensin peptides were either below the limit of quantification or could only be quantified in a few study subjects. These observations are in line with the expected low activity of the blood pressure maintaining RAS system in normotensive subjects on a non-sodium-restricted diet. For Ang 1-10, Ang 1-8, Ang 2-10, Ang 2-8 and Ang 3-8 comparably low concentrations have been reported in healthy untreated subjects.[20, 22, 24, 31-33] For Ang 1-7 and Ang 1-9 reported values were either comparable with [34] or higher [35, 36] than the concentrations we found in our study, which could be explained by an overestimation of values usually observed in antibody-based quantification methods. Ang 1-5 was previously only detected in equilibrium analysis in hemodialysis patients treated with ARBs [37] and was proposed as a surrogate marker for alternative RAS activation in the absence of ACE inhibition [38]. Ang 3-7 levels have not been published yet.

Single doses of the renin inhibitor aliskiren, the ACE inhibitor enalapril and the angiotensin receptor antagonist losartan lead to characteristic and time-dependent changes of RAS peptide concentrations. Aliskiren caused a decrease of Ang 1-10 and Ang 1-8 while the already low concentrations of downstream angiotensin metabolites were not affected. The decrease of Ang 1-10 and Ang 1-8 did not last over the whole 24 hour dosing interval. Interestingly, this was not even the case under pharmacokinetic steady state conditions where AUC and Cmax of aliskiren increase approx. two-fold.[28] Comparable

dose-dependent effects of renin inhibitors on Ang 1-10 and Ang 1-8 concentrations have been reported in healthy subjects.[31, 39] After single and multiple dose treatment with aliskiren Ang 1-10 and Ang 1-8 concentrations decreased to approx. 25% and started to return to baseline levels between 6 hours and 10 hours after dosing.[31]

In contrast to aliskiren, inhibition of the RAS cascade with an ACE inhibitor one step further downstream caused a completely different RAS peptide profile. Concentrations of the two ACE substrates Ang 1-10 and Ang 1-7 increased while Ang 1-8 decreased. Concentrations of the remaining angiotensin peptides showed no quantifiable changes. A qualitatively similar but more pronounced pattern was observed after multiple dose treatment. The ACE inhibitor treatment was the only one that caused an increase in circulating Ang 1-7 concentrations, with even more prominent effects in RAS equilibrium analysis. After single or multiple dose treatment with 20 mg enalapril comparable changes of Ang 1-10, Ang 1-8 [19, 31] and Ang 1-7 [34] concentrations have been described previously. No significant changes were found for Ang 2-8 and Ang 3-8, while for Ang 2-10 approx. 10-fold higher concentrations were found in equilibrium analysis, indicating that like Ang 1-10, Ang 2-10 is a substrate for C-terminal conversion via ACE.[19]

Blockade of the angiotensin receptor led to an angiotensin peptide pattern which was distinct from the patterns observed after renin or ACE inhibition. Already after the first dose, Ang 1-10 and Ang 1-8 concentrations increased and did not return to baseline within 24 hours. Again, these changes were more pronounced after multiple dose treatment and they correspond well with published changes of Ang 1-10 and Ang 1-8 concentrations after treatment with losartan.[40]

The observed changes of circulating and equilibrium angiotensin peptide concentrations were specific for the three different RAS inhibitors and correlated with drug concentrations in plasma. Maximal effects were observed 4 to 8 hours after dosing, corresponding to maximal drug concentrations in plasma. Twenty-four hours after a single dose, drug concentrations in plasma had decreased to levels of 10 ng/ml or below and RAS peptide concentrations had returned to baseline (aliskiren) or close to baseline levels (enalapril, losartan). After 8 days of

repeated daily dosing the characteristic changes of the angiotensin peptide profiles observed after single dose treatment were preserved or even more pronounced. This could be explained by enhanced renin feedback regulation resulting in increased angiotensin levels and a larger impact of pharmacological interference with the RAS.

The ratio of the ACE product Ang 1-8 and the ACE substrate Ang 1-10 allows direct assessment of ACE activity. While renin inhibition or angiotensin receptor blockade had no relevant effect on the Ang 1-8 / Ang 1-10 ratio, the ACE inhibitor enalapril significantly decreased the ratio, indicating efficient blockade of ACE activity. This was also the case after multiple dose treatment and low Ang 1-8 / Ang 1-10 ratios thus could be used as a marker for ACE inhibitor treatment. This of course raises the question whether RAS inhibitor induced changes of angiotensin peptide concentrations are maintained over a longer period of time. Data from a small number of patients with essential hypertension continuously treated with ramipril suggest that the characteristic ACE inhibitor induced changes of Ang 1-10 and Ang 1-8 concentrations will persist for two years and possibly even longer.[41] However, whether the ratio also can be used to assess patients under long-term ACE inhibitor treatment will have to be tested in subsequent studies.

After single and multiple dose treatment, the RAS inhibitor specific changes observed for circulating angiotensin peptides is reflected in equilibrium analysis. Compared to circulating concentrations, equilibrium angiotensin levels were several-fold higher and additional peptides such as Ang 2-10 (after ACE inhibition) or Ang 2-8 and Ang 3-8 (after angiotensin receptor blockade) reached quantifiable concentrations. Thus, although circulating angiotensin peptide concentrations may intuitively be thought to better reflect the intravascular situation, equilibrium peptide profiles surprisingly deliver the same qualitative information on the circulating RAS, while having the advantage of easier sampling and quantification combined with additional information related to RAS blocker pharmacology.

A strength of our study is that it not only provides simultaneous and accurate quantification of angiotensin peptides with known biological functions such as Ang 1-8 and Ang 1-7, but also

of smaller less well characterized downstream peptides. For those angiotensin peptides, where published concentration ranges in normotensive subjects are available, we found good agreement between concentrations determined in our study and established values obtained by quantification approaches including HPLC fractionation. The short observation period and the healthy normotensive study population are limitations of our study that do not allow extrapolation of the results to patients with arterial hypertension with or without established RAS inhibitor treatment.

In conclusion we have shown that in normotensive subjects single dose treatment with a renin inhibitor, an ACE inhibitor or an angiotensin receptor antagonist causes characteristic changes of circulating angiotensin peptide profiles which directly reflect the mechanism of action of the different RAS inhibitors. The changes are preserved after multiple dose treatment for one week. The drug-specific pattern of changes in circulating angiotensin peptide concentrations is also reflected in RAS equilibrium analysis. Compared to circulating RAS peptides equilibrium peptide levels are higher and show additional drug-specific changes in small downstream angiotensin metabolites. The present data suggest that RAS equilibrium analysis could be of value for the assessment of patients under RAS inhibitor treatment. Their potential as possible biomarkers in such patients has to be assessed in subsequent studies.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding this work.

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Supplementary

Supplementary Table 1: Pharmacokinetic parameters of aliskiren, enalaprilat, and losartan carboxylic acid (E-3174) in plasma after single and multiple dose treatment.

	Aliskiren		***Enalaprilat***		***E-3174***	
	single dose	*multiple dose*	*single dose*	*multiple dose*	*single dose*	*multiple dose*
C_{max} *(ng/ml)*	40.8 ± 29.3	39.3 ± 22.4	24.6 ± 12.8	28.1 ± 8.2	182.3 ± 69.0	174.8 ± 58.8
t_{max} *(h)*	1 (1 – 4)	1 (1 – 4)	4 (2 – 8)	4 (2 – 4)	4 (4 – 8)	4 (4 – 8)
AUC_{0-24} *(ng/ml*h)*	194.4±127.0	218.5±63.9	212.0±41.4	262.3±51.3	1684.0± 672.3	1603.1± 410.8
$t_{½}$ *(h)*	15.4 ± 9.9	17.4 ± 15.0	5.5 ± 1.5	5.7 ± 1.3	5.1 ± 1.4	4.5 ± 1.0

Data are presented as mean±SD (n = 12), data for t_{max} are presented as median and range. C_{max}, maximal plasma concentration; t_{max}, time to reach maximal plasma concentration; AUC_{0-24}, area under effect curve from time 0 to 24 hours; $t_{1/2}$, half-life; E-3174, losartan carboxylic acid.

Supplementary Table 2: Lower limits of quantification (LLOQ) for different angiotensin peptides

	Protease inhibitor analysis (pg/ml)	Equilibrium analysis (pg/ml)
Ang 1-10	5	5
Ang 1-9	4	4
Ang 1-8	1	2
Ang 1-7	3	3
Ang 1-5	1	1
Ang 2-10	3	3
Ang 2-8	2	2
Ang 2-7	4	5
Ang 3-8	1	1
Ang 3-7	1	1

3.2 RAS peptide profiles in patients with primary arterial hypertension

Angiotensin peptide profiles in patients with arterial hypertension before and after treatment initiation

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Keywords:

Renin-angiotensin system, angiotensin peptides, ACE inhibitor, angiotensin receptor antagonist, calcium channel antagonist, hydrochlorothiazide, arterial hypertension

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Abstract

Background Important effectors of the renin-angiotensin system (RAS) are small bioactive peptides like angiotensin II (Ang 1-8) or Ang 1-7. Ang 1-8 as the main effector of the RAS is involved in the development of arterial hypertension and progression of vascular disease. Some antihypertensive drugs that are recommended as first line treatment, such as ACE inhibitors or angiotensin receptor antagonists act on key enzymes or receptors of the RAS, and are expected to cause drug-type specific changes of RAS peptide concentrations. The objective of this study was to assess equilibrium angiotensin profiles in patients with treatment naïve primary arterial hypertension before and after 4 weeks of treatment with intermediate and high doses of the ACE inhibitor perindopril, the angiotensin receptor antagonist olmesartan, the calcium channel antagonist amlodipine, the thiazide diuretic hydrochlorothiazide.

Methods In a single-center, open-label, randomized, parallel group study in 80 patients with arterial hypertension treatment was initiated with either a daily intermediate dose monotherapy of perindopril, olmesartan, amlodipine, or hydrochlorothiazide for at least 4 weeks and, if the blood pressure target was not reached within that period, with a high dose monotherapy for another 4 weeks. Concentrations of antihypertensive drugs and angiotensin peptides were determined from standard lithium heparin aliquots using a liquid chromatography-tandem mass spectrometry method. Hemodynamic parameters were assessed noninvasively using thoracic electrical bioimpedance (HOTMAN® System).

Results In untreated patients with arterial hypertension, plasma angiotensin concentrations were in the mid picomolar range. Multiple dose treatment caused drug-specific changes of angiotensin peptide profiles which directly reflect the mechanism of action of the different RAS inhibitors. The ACE inhibitor perindopril increased concentrations of the ACE substrate angiotensin I (Ang 1-10). The angiotensin receptor antagonist olmesartan, the calcium channel antagonist amlodipine and the diuretic hydrochlorothiazide increased concentrations

of Ang 1-10 and Ang 1-8. None of the treatments led to a significant change in the evaluated hemodynamic parameters (CI and SSVRI).

Conclusion Equilibrium angiotensin profiles show drug-specific changes which directly reflect the mechanism of action of the different RAS inhibitors perindopril and olmesartan. Amlodipine and hydrochlorothiazide, which do not interfere directly with the RAS cascade, show a pattern of RAS peptide profiles similar to olmesartan. The present data suggest that RAS equilibrium analysis could be of value for the assessment of patients under RAS inhibitor therapy. Their potential as possible biomarkers in patients will have to be assessed in additional studies.

Introduction

Angiotensin II (Ang 1-8) is the main effector of the renin-angiotensin aldosterone system (RAS) and is involved in the development of arterial hypertension and progression of cardiovascular disease. Ang 1-8 acts mainly through angiotensin II type 1 receptors (AT1). The AT1 is ubiquitously expressed in the cardiovascular system mediating vasoconstriction, stimulation of aldosterone and antidiuretic hormone release, inflammation, cardiac hypertrophy, vascular proliferation, and oxidative stress.[1, 2] On the other hand, Ang 1-7, with its counter-regulatory effects, is assumed to play a beneficial role on endothelial function and thus cardiovascular performance. Ang 1-7 binds specifically to the G-protein coupled receptor MAS and thereby opposes the AT1 mediated effects of Ang 1-8.[2, 3] The opposing cardiovascular effects of Ang 1-7 include vasodilation, vascular protection and anti-proliferation. Also anti-fibrinogenic, anti-thrombogenic and anti-arrythmogenic effects are described to be mediated by activation of receptor MAS1.[4-6] Similar counter-regulatory effects are mediated by binding of Ang 1-8 to the angiotensin II type 2 receptor (AT2) showing low expression in healthy adults but can be up-regulated under pathologic conditions such as hypertension.[1]

In recent years, several mass spectrometry based methods have been developed for the quantification of angiotensin peptides in human plasma.[7-11] According to their different mechanisms of action, ACE inhibitors and angiotensin receptor antagonists cause characteristic concentration changes of Ang 1-10 and Ang 1-8. Simultaneous quantification of multiple RAS peptides may provide drug-type specific RAS peptide profiles that could be useful for the assessment of patients under RAS inhibitor treatment.

In clinical practice there is no uniform agreement which of the antihypertensive drugs that are recommended as first line treatment by European Society of Hypertension should be given for initial therapy. Relative efficacy of antihypertensive drugs in lowering blood pressure is 30 to 50 percent, however, wide interpatient variability is observed and there are few clinical parameters that reliably can predict success of individual responses to the different drugs including ACE inhibitors, angiotensin receptor antagonists, calcium channel antagonists, and thiazide diuretics.[12]

As a first step, we therefore characterized equilibrium angiotensin profiles in patients with treatment naïve primary arterial hypertension before and after 4 weeks of treatment with intermediate and high doses of the ACE inhibitor perindopril, the angiotensin receptor antagonist olmesartan, the calcium channel antagonist amlodipine, the thiazide diuretic hydrochlorothiazide.

Methods

Clinical Study

A single-center, randomized, open-label, parallel group study (ClinicalTrials.gov ID: NCT02449811) was performed at the Medical Outpatient Clinic at the University Hospital Basel, Switzerland. The study was approved by the local Ethics Committee (Ethikkommission Zentral- und Nordwestschweiz, EKNZ) and conducted in accordance with the principles of the Declaration of Helsinki. Patients with treatment-naïve grade I and grade II primary hypertension were randomized to one of four standard first-line

treatments issued by the European Society of Hypertension.[13] According to clinical practice, the first treatment period was a daily intermediate dose monotherapy of either 5 mg perindopril, 20 mg olmesartan, 5 mg amlodipine, or 25 mg hydrochlorothiazide for at least 4 weeks. Before initiation of antihypertensive drug therapy, blood samples for the assessment of baseline peptide profiles were collected and non-invasive hemodynamic measurements were performed. After at least 4 weeks, blood sampling for angiotensin profiles and drug concentrations, and non-invasive hemodynamic measurements were done before drug intake and 4 hours after observed drug intake. All patients who did not reach blood pressure targets after 4 weeks according to the 2013 ESH guidelines for the management of arterial hypertension continued in the second treatment period with doubled monotherapy drug dose (10 mg perindopril, 40 mg olmesartan, 10 mg amlodipine, or 50 mg hydrochlorothiazide) for another 4 weeks. On study days the patients were fasted (including consumption of alcoholic beverages) for 10 h before observed drug intake. A light breakfast was allowed after drug intake. No concomitant medication was allowed, except for the treatment of AEs.

Study Measurements

Venous blood samples were obtained by direct puncture before and 4 h after observed drug intake on day 28 (+ 7 days) of each treatment period. For RAS equilibrium analysis, blood was collected into standard lithium heparin tubes. Equilibrium angiotensin peptide levels were measured following 30 min of equilibration of conditioned Li-heparin plasma at 37°C and subsequent stabilization of equilibrium peptide levels. For the analysis of drug and renin concentrations blood was collected into EDTA tubes. All tubes were centrifuged within 20 min at 1500 g for 10 min at 4°C and plasma was stored at -80°C until analysis.

Hemodynamic parameters (volemia, inotropy and vasoactivity) were non-invasively assessed from the patients in supine position using the HOTMAN® System (Hemo Sapiens Medical Inc., Sedona, AZ, USA). After 5 min of rest, 3 consecutive measurements spaced 2 min

apart were recorded together with blood pressure measurements at the time points of blood sample collection.

Blood pressure and heart rate were recorded from the patients using a Mobil-O-Graph 24 h PWA Monitor (I.E.M. GmbH Stolberg Germany). Blood pressure was measured every 20 min during the day and evening (from 6:00 to 22:00) and every 30 min at night (from 22:00 to 06:00). Presence of at least 70 percent of measurements was considered as an acceptable 24 h blood pressure monitoring recording for our study. The mean systolic blood pressure, diastolic blood pressure and heart rate were calculated for 24 h.

Materials and Reagents

Amlodipine, amlodipine-d4, hydrochlorothiazide, hydrochlorothiazide-13C15N2d2 olmesartan, olmesartan-d6, perindoprilat and perindoprilat-d4 were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Formic acid, acetic acid, high-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and water were purchased from Merck KGaA (Darmstadt, Germany).

Stable isotope labeled internal standards for angiotensin metabolites and MS grade formic acid were purchased from Sigma-Aldrich (Vienna, Austria). LC-MS-grade water, methanol and acetonitrile were purchased from Fisher Scientific (Vienna, Austria).

Measurement of Drug Concentrations

Concentrations of amlodipine, hydrochlorothiazide, olmesartan, and perindoprilat, in plasma were determined by reversed-phase HPLC with tandem mass spectrometry (HPLC–MS/MS). Chromatographic separation was done on a Shimadzu HPLC system (Shimadzu AG, Reinach, Switzerland) coupled to a triple quadrupole tandem mass spectrometer (API4000 Qtrap, ABSciex, Massachusetts, USA). Three different methods were developed to analyze the analytes.

Analysis of perindoprilat

Fifty microliters aliquots of plasma were mixed with 150 µL of an internal standard solution (Methanol:acetonitril (1:1 v/v)) containing perindoprilat-d4 at a concentration of 50 ng/mL, vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Fifty microliters deproteinized supernatant was injected directly into the HPLC–MS/MS system. Samples were concentrated and purified on a trap cartridge (EXP HALO C18, 4.6 x 5 mm, Optimize technologies, Oregon, USA) at a flow rate of 5 mL/min water supplemented with 1% formic acid. Perindoprilat was eluted on a Kinetex^{TM} XB-C18 analytical column (2.6 µm, 100 Å, 50 x 2.1 mm) using a gradient of acetonitrile (mobile phase B) and water plus 1% formic acid (mobile phase A). The total run time was 3.3 min. Samples were quantified using peak area ratios. Perindoprilat and perindoprilat-d4 were analyzed using electrospray ionization with multiple reaction monitoring (MRM) in negative polarity mode. The applied mass transitions and retention times are illustrated in Table 1. The method was linear over a concentration range of 0.25-250 ng/mL. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Analysis of olmesartan and hydrochlorothiazide

Fifty microliter aliquots of plasma were mixed with 150 µL of an internal standard solution (methanol plus 0.01% acetic acid) containing hydrochlorothiazide-13C15N2d2 (100 ng/mL), and olmesartan-d6 (25 ng/mL), vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Ten microliters deproteinized supernatant was injected directly into the HPLC–MS/MS system. The analytes were separated on an Atlantis dC18 analytical column (3µm, 100 x 2.1 mm, Waters, Massachusetts, USA) using a gradient of water (mobile phase A) and acetonitrile (mobile phase A) both supplemented with 0.01% acetic acid. The total run time was 3 min. Samples were quantified using peak area ratios. Olmesartan, olmesartan-d6, hydrochlorothiazide, and hydrochlorothiazide-13C15N2d2 were analyzed using electrospray ionization with multiple reaction monitoring (MRM) in negative polarity mode. The applied mass transitions and retention times are illustrated in Table 1. The

method was linear over a concentration range of 1-500 ng/mL. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Analysis of amlodipine

Fifty microliter aliquots of plasma were mixed with 150 µL of an internal standard solution (methanol plus 0.01% acetic acid) containing amlodipine-d4 at a final concentration of 10 ng/mL, vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Fifty microliters deproteinized supernatant was injected directly into the HPLC–MS/MS system. Samples were concentrated and purified on a trap cartridge (EXP HALO C18, 4.6 x 5 mm, Optimizetechnologies, Oregon, USA) at a flow rate of 3 mL/min acetonitrile and water (80:20 v/v) supplemented 0.01% acetic acid. The analytes were separated on an Atlantis dC18 analytical column (3 µm, 100 x 2.1 mm, Waters, Massachusetts, USA) using a gradient of water (mobile phase A) and acetonitrile (mobile phase A) both supplemented with 0.01% acetic acid. The total run time was 3.5 min. Samples were quantified using peak area ratios. The analytes were analyzed using electrospray ionization with multiple reaction monitoring (MRM) in positive polarity mode. The applied mass transitions and retention times are illustrated in Table 1. The method was linear over a concentration range 1-500 ng/mL for amlodipine. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Table 1

Analyt	MRM [m/z]	Retention time [min]
Perindoprilat	339.1 → 167.8	1.04
Perindoprilat-d4	343.1 → 167.8	
Hydrochlorothiazide	295.9 → 204.7	1.01
Hydrochlorothiazide-13C15N2d2	301.1 → 207.7	
Olmesartan	445.2 → 148.9	1.34
Olmesartan-d6	451.2 → 153.9	
Amlodipine	409.1 → 294.0	1.53
Amlodipine-d4	413.1 → 297.9	

Quantification of RAS Peptide Concentrations

Following 30 min of equilibration of conditioned Li-heparin plasma at 37°C, stabilized samples for quantification of angiotensin metabolite concentrations were spiked with stable isotope-labeled internal standards for each angiotensin metabolite (Ang 1-10, Ang 1-9, Ang 1-8, Ang 1-7, Ang 1-5, Ang 2-10, Ang 2-8, Ang 2-7, Ang 3-8, and Ang 3-7) at a concentration of 100 pg/ml. Following C18-based solid-phase-extraction, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC® C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in MRM mode. Internal standards were used to correct for peptide recovery of the sample preparation procedure for each angiotensin metabolite in each individual sample. Angiotensin metabolite concentrations were calculated considering the corresponding response factors determined in appropriate calibration curves in original sample matrix, on condition that integrated signals exceeded a signal-to-noise ratio of 10. Lower limits of quantification (LLOQ) for the different angiotensin peptides were between 1 and 5 pg/ml (Supplementary Table 1).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 7.01 (GraphPad Sofware, Inc., USA). Normal distribution of the data was assessed using D'Agostino-Pearson omnibus test. Parameters with a normal distribution were tested using one-way analysis of variance (ANOVA). Post-hoc analysis of significant overall results was done with Tukey-Kramer's test for multiple comparisons. Non-normally distributed parameters were analyzed by the non-parametric Kruskal-Wallis test and Friedman test, respectively, in case of paired groups. For post-hoc analysis of significant overall results Dunn's test for multiple comparisons was used.

Results

Patients

Table 2. Demographics and patients characteristics (N = 44).

Sex, % (n)	
Male	29 (66%)
Female	15 (34%)
Age, years	50 (24 – 76)
BMI, kg/m^2	27 (19 – 35)
Ethnic race	
Caucasian	41 (93%)
Black	1 (2.3%)
American	1 (2.3%)
Asian	1 (2.3%)
Ambulatory Daytime BP	
SBP, mmHg	141 (128 – 168)
DBP, mmHg	89 (72 – 103)
Heart rate, bmp	78 (52 – 102)

Data are presented as number (percentage) for categorical variables and median (range) for continuous variables.

A subgroup of 44 patients with treatment-naive primary arterial hypertension recruited from the Medical Outpatient Clinic at the University Hospital Basel, Switzerland between April 2015 and October 2016 was evaluated. In all patients diagnosis of arterial hypertension was confirmed by a 24 hour ambulatory blood pressure measurement. Demographic data of the study patients are given in Table 2. Forty-one patients were of Caucasian ethnicity, one patient was of African, one of American and another patient of Asian descent. They all had no history of relevant disease or drug abuse and no clinically relevant findings on laboratory tests (including renal and hepatic function) or electrocardiograms. Mean systolic daytime

blood pressure on ambulatory 24 hour blood pressure monitoring was 141 mmHg and mean diastolic daytime blood pressure was 89 mmHg.

Clinical Study

Patients with a new diagnosis of arterial hypertension were screened. One patient dropped out after randomization but before treatment was started. Twelve patients were randomized to perindopril, eleven to olmesartan, nine to amlodipine and eleven to hydrochlorothiazide. Therapeutic success according to the 2013 ESH guidelines for management of arterial hypertension [13] was defined as 24 h blood pressure measurements below the criteria of hypertension, i.e. mean SBP/DBP ≤ 130/80 mmHg on average, ≤ 135/85 during the day, OR ≤ 120/70 during the night. After the first treatment period 18 patients (42%) had reached the blood pressure goal on an intermediate dose of perindopril, olmesartan, amlodipine, or hydrochlorothiazide. Of the remaining 25 patients, only 4 patients (16%) reached blood pressure goal after the second treatment period despite being treated with a two-fold higher dose compared to the first treatment period. In the perindopril group, 7 (58%) patients had reached the blood pressure goal after treatment period 1 (Table 3). Of the 5 remaining patients, 2 patients reached blood pressure goal after second treatment period, 1 patient did not reach blood pressure goal and 2 patients had to stop drug treatment due to adverse events (dizziness, swollen hands).

In the olmesartan group, 7 (64%) patients had reached blood pressure goal after treatment period 1. Of the 4 remaining patients, 3 patients did not reach blood pressure goal and one patient had to stop drug treatment due to adverse effects (dizziness). In the amlodipine group, only 2 (22%) patients reached blood pressure goal after treatment period 1. Of the 7 remaining patients, 2 patient reached blood pressure goal after second treatment period, 3 patients did not reach blood pressure goal and 2 patients had to stop drug treatment due to adverse events (dizziness, exanthema). In the hydrochlorothiazide group, also only 2 (18%) patients reached blood pressure goal after treatment period 1. Of the 9 remaining patients,

none of the patients reached blood pressure goal after the second treatment period and 2 patients had to stop drug treatment due to dizziness.

Table 3. Proportion of patients reaching blood pressure goal.

	BP goal reached		**Drop outs**
	YES	NO	
Treatment period 1 (N = 43)			
overall	18 (42%)	25 (58%)	-
Perindopril, 5 mg	7 (58%)	5 (42%)	-
Olmesartan, 20 mg	7 (64%)	4 (36%)	-
Amlodipine, 5 mg	2 (22%)	7 (78%)	-
Hydrochlorothiazide, 25 mg	2 (18%)	9 (82%)	-
Treatment period 2 (N = 25)			
overall	4 (16%)	14 (56%)	7 (28%)
Perindopril, 10 mg	2 (40%)	1 (20%)	2 (40%)
Olmesartan, 40 mg	0	3 (75%)	1 (25%)
Amlodipine, 10 mg	2 (29%)	3 (42%)	2 (29%)
Hydrochlorothiazide, 50 mg	0	4 (78%)	2 (22%)

Data are presented as number (percentage).

Blood pressure changes

Average decrease of systolic blood pressure of patients reaching the blood pressure goal after treatment period 1 was 21, 18, 14, 11 mmHg for perindopril, olmesartan, amlodipine, hydrochlorothiazide, respectively (Figure 1). In the perindopril group, of the 3 patients who did not reach blood pressure goal 1 patient showed an increase of mean SBP and DBP of 3 mmHg and 5 mmHg, respectively, after dose increase in treatment period 2. This patient already showed a decrease of mean SBP/DBP of only 2/1 mmHg in treatment period 1. The patient who had to stop drug treatment due to dizziness after the dose was doubled showed a predominantly diastolic decrease of average SBP/DBP of 1/11 in treatment period 1. The patient, who had to stop drug treatment due to swollen hands, did respond to intermediate

dose treatment (decrease of SBP/DBP of 25/11 mmHg). In the olmesartan group, of the 4 patients who did not reach blood pressure goal, 2 patients did respond to intermediate dose treatment with a decrease of mean SBP/DBP of 16/6 mmHg and 21/9 mmHg, respectively, and on high dose treatment with an additional decrease of mean SBP/DBP of 4/3 mmHg and 11/4 mmHg. The patient who had to stop drug treatment due to dizziness after the dose was doubled showed a decrease of SBP/DBP of 22/8 mmHg on average, of 26/10 mmHg at daytime and of 14/5 mmHg at nighttime while on intermediate dose treatment. One patient erroneously received high dose treatment in treatment period 1. No treatment period 1 data are available for this patient and he was excluded from the analysis Figure 1.

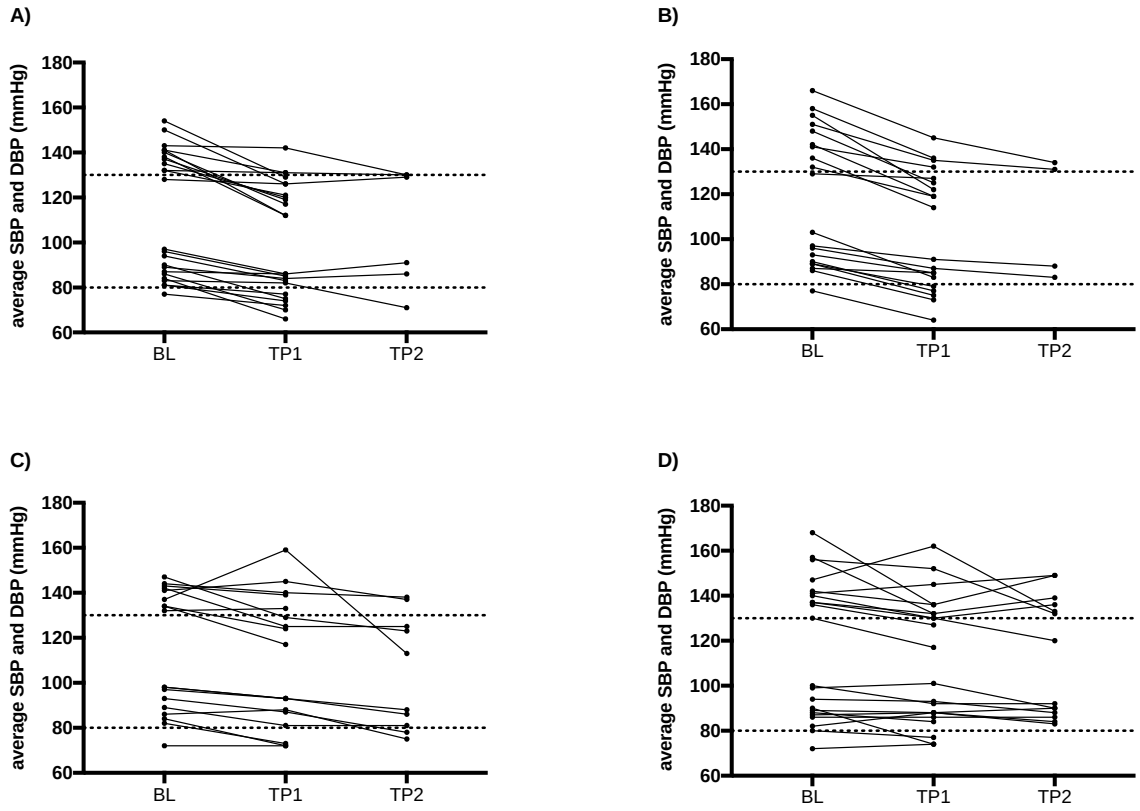

Figure 1. Average 24 h systolic (SBP) and diastolic (DBP) blood pressure of patients at baseline (BL) and after treatment with a daily intermediate dose monotherapy of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide (HCT) for at least 4 weeks (treatment period 1, TP1) and, if necessary, with a high dose monotherapy for another 4 weeks (TP2). Note: For therapeutic success according to the 2013 ESH guidelines for

management of arterial hypertension patients also had to fulfil criteria for daytime (SBP/DBP ≤ 130/80 mmHg) and nighttime (≤ 120/70) blood pressure.

In the amlodipine group, of the 5 patients who did not reach blood pressure goal, 2 patients responded to intermediate dose treatment with a decrease of average SBP/DBP of 18/5 mmHg and to high dose treatment with a further decrease of mean SBP/DBP of 6/5 mmHg. One patient responded to intermediate dose treatment with an increase in SBP (4 mmHg) and a decrease in DBP (5 mmHg) and to high dose treatment with a decrease of average SBP/DBP of 8/7 mmHg. The patient who had to stop drug treatment due to dizziness after the dose was doubled showed a decrease of SBP/DBP of 4/4 mmHg on average. The patient who had to stop drug treatment due to exanthema after the dose was doubled showed no change of SBP/DBP on average. One patient responded to intermediate dose treatment with an increase of average SBP/DBP of 22/2 mmHg and to high dose treatment with a decrease of average SBP/DBP of 46/13 mmHg. In the hydrochlorothiazide group, none of the 9 patients reached blood pressure goal after the second treatment period and 2 patients had to stop drug treatment due to dizziness.

Antihypertensive drug concentrations

Plasma concentrations after once daily intermediate or high dose monotherapy with perindopril (5/10 mg), olmesartan (20/40 mg), amlodipine (5/10 mg) and hydrochlorothiazide (25/50 mg) are shown in Table 4 and Figure 2.

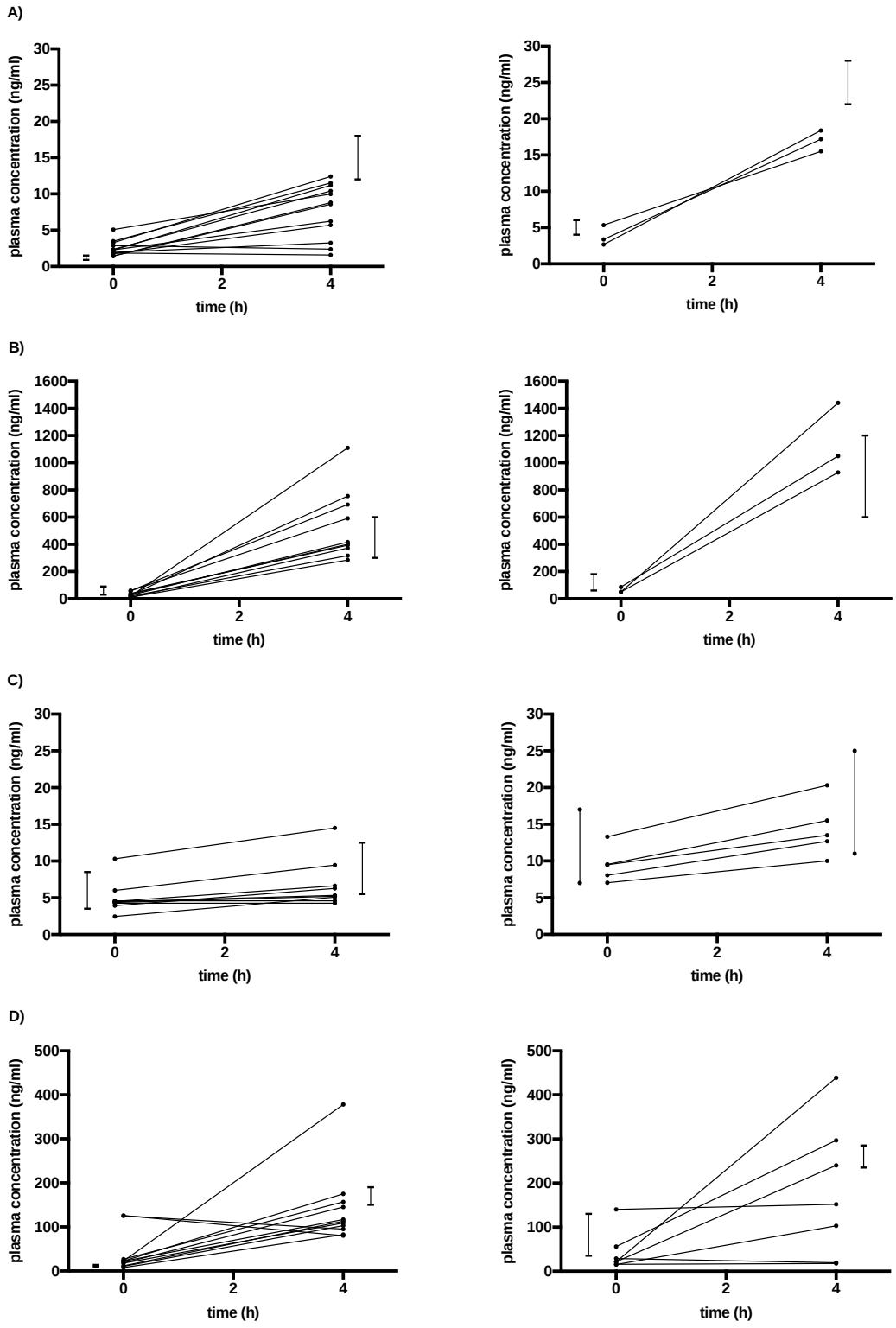

Figure 2. Plasma concentration of A) perindoprilat (5 mg / 10 mg), B) olmesartan (20 mg / 40 mg), C) amlodipine (5 mg / 10 mg), D) hydrochlorothiazide (25 mg / 50 mg) after once daily treatment for 4 weeks. Ranges of published steady-state trough and 4 hour post dose drug concentrations are given next to the individual drug concentrations.

Table 4. Plasma concentrations of perindoprilat, olmesartan, amlodipine and hydrochlorothiazide after 4 weeks repeated once daily dosing before (C_{trough}) and 4 hours after observed drug intake.

Antihypertensive Drug Treatment	**Plasma concentration (ng/ml)**	
	$C_{through}$	4 h
Perindoprilat, 5 mg	2.3 (1.4 – 5.1)	9.4 (3.4 – 12.4)
Perindoprilat, 10 mg	3.4 (2.7 – 5.3)	17.2 (15.5 – 18.4)
Olmesartan, 20 mg	27 (10 – 60)	409 (285 – 1110)
Olmesartan, 40 mg	51 (50 – 86)	1050 (928 – 1440)
Amlodipine, 5 mg	4.5 (2.5 – 10.3)	6.3 (5.1 – 14.5)
Amlodipine, 10 mg	9.5 (7.0 – 13.3)	13.5 (10.0 – 20.3)
Hydrochlorothiazide, 25 mg	18.3 (7.7 – 26.9)	21.5 (15.4 – 55.9)
Hydrochlorothiazide, 50 mg	117.0 (82.8 – 378.0)	268.5 (103.0 – 439.0)

Data are presented as median (range).

In 2 patients there was no increase in perindoprilat plasma concentration after observed drug intake. The values were confirmed by re-analysis of the samples. However, treatment lead to a decrease in average SBP/DBP from 154/96 mmHg and 150/81 mmHg to 129/85 mmHg and 126/74 mmHg, respectively. In 2 patients there was no increase in measured amlodipine plasma concentration after observed drug intake; however, both patients did reach BP goals after intermediate dose monotherapy. In 4 patients there was no increase in measured hydrochlorothiazide plasma concentrations after observed drug intake. Furthermore, in 2 of these patients there was no increase after one treatment period but an increase after the other. One patient showed no increase after both treatment periods.

Angiotensin peptide concentrations in treatment-naïve primary arterial hypertension

Before start of treatment, concentrations of equilibrium angiotensins as well as renin and aldosterone concentrations in patients with primary arterial hypertension were stable over 2 weeks and in the same range as concentrations measured in normotensive healthy subjects (NCT01771783) and 10 sex and age matched healthy control subjects. At

pre-baseline assessment (conducted two weeks before baseline assessment in a subgroup of patients), median equilibrium concentrations of Ang 1-10 and Ang 1-8 were 30 pg/ml (range 2-81 pg/ml) and 67 pg/ml (range range 7-169 pg/ml), respectively, in the morning and 42 pg/ml (range 3-97 pg/ml) and 97 pg/ml (range 17-267 pg/ml), respectively, at noon. At baseline assessment, median concentrations of Ang 1-10 and Ang 1-8 were 37 pg/ml (range 2-145 pg/ml) and 60 pg/ml (range 9-330 pg/ml), respectively, in the morning and 41 pg/ml (range 3-219 pg/ml) and 92 pg/ml (range 7-404 pg/ml), respectively, at noon. At baseline, concentrations of Ang 1-9, Ang 2-7 and Ang 3-7 were below the lower limit of quantification (LLOQ) in all patients, and concentrations of the remaining peptides (Ang 1-7, Ang 1-5, Ang 2-10, Ang 2-8, Ang 3-8) could only be quantified in a few subjects. There was a significant increase in Ang 1-10 and Ang 1-8 concentration at noon compared to values obtained in the morning in paired samples, while without pairing the concentration did not differ significantly.

Angiotensin peptide concentrations after single and multiple dose treatment

Multiple dose treatment with an intermediate dose of the ACE inhibitor perindopril led to a significant increase of Ang 1-10 concentrations without return to baseline values 24 hours after dosing (Figure 3). Median concentrations of Ang 1-8 decreased significantly below baseline concentrations 4 hours after dosing and returned to baseline 24 hours after dosing. Concentrations of the ACE substrate Ang 1-7 increased to quantifiable concentrations (median 14 pg/ml, range 4-90 pg/ml) 4 hours after intake of the ACE inhibitor in 54% (7/13) of the patients (Supplementary Table 2). In addition, Ang 2-10 concentrations increased 4 hours after observed drug intake. For the remaining peptides, either no relevant changes of concentrations were observed or their values remained below the LLOQ. Multiple dose treatment with an intermediate dose of the angiotensin receptor antagonist olmesartan and the calcium channel antagonist amlodipine led to a significant increase of Ang 1-10 and Ang 1-8 concentrations without return to baseline values 24 hours after dosing for olmesartan (Figure 3). As a consequence of the very high Ang 1-8 levels, the concentration

of downstream peptides Ang 3-8, Ang 2-8 and Ang 1-5 were also elevated without returning to baseline values throughout 24 hours after dosing. Multiple dose treatment with an intermediate dose of the hydrochlorothiazide led to an increase of Ang 1-10 which was significant only 24 hours after dosing. Median concentrations of Ang 1-8 increased significantly above baseline concentrations 4 hours after dosing and returned to baseline 24 hours after dosing (Figure 3). Ang 1-8 concentrations after multiple dose treatment with hydrochlorothiazide are higher than with amlodipine and significant higher compared to baseline values, but after drug intake the increase in Ang 1-8 concentrations is less for hydrochlorothiazide compared to amlodipine and compared to baseline values, not statistically significant. Multiple dose treatment with an intermediate dose of the angiotensin receptor antagonist olmesartan led to a significant increase of Ang 1-10 and Ang 1-8 concentrations without return to baseline values 24 hours after dosing (Figure 3). This effect was much more pronounced in patients who reached blood pressure targets compared to patients who did not reach blood pressure targets. Such a difference between patients who reached blood pressure targets compared to patients who did not reach blood pressure targets was not present in the effects observed after multiple dose treatment with perindopril, amlodipine or hydrochlorothiazide.

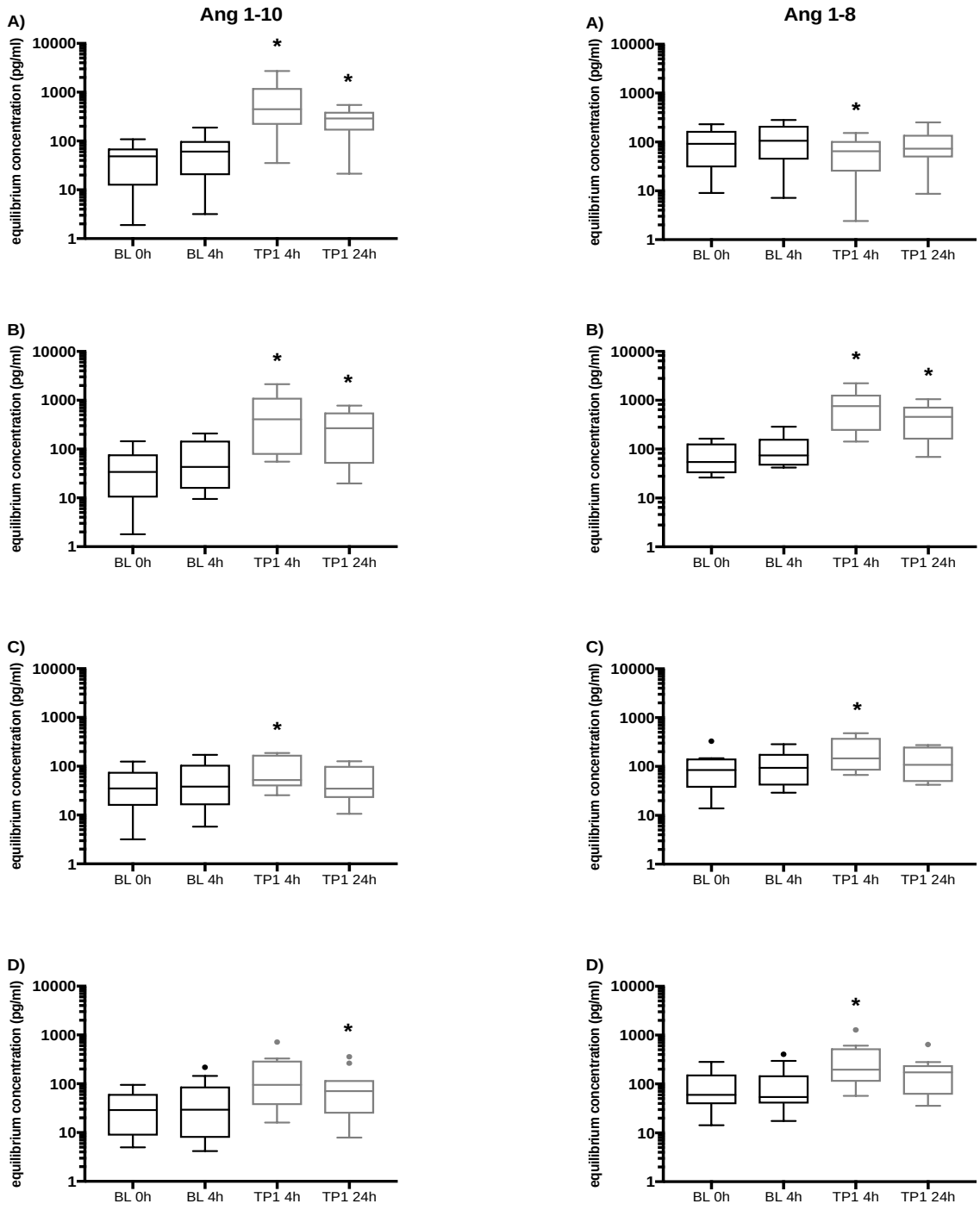

Figure 3. Ang 1-10 and Ang 1-8 peptide concentrations without treatment (BL) and after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 hours (TP1 4h) and 24 hours (TP1 24h) after dosing.

Representative RAS-Fingerprint graphs in patients with arterial hypertension without treatment (baseline) including enzymes involved in formation or degradation of the measured RAS peptides is shown in Figure 4.

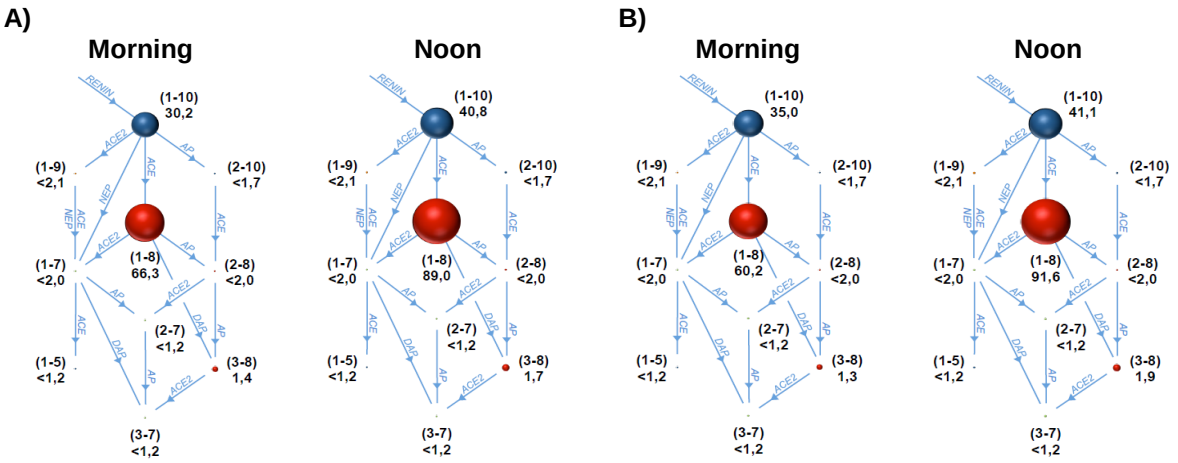

Figure 4. Angiotensin peptide concentrations in the morning and at noon at A) pre-baseline and B) baseline assessment.

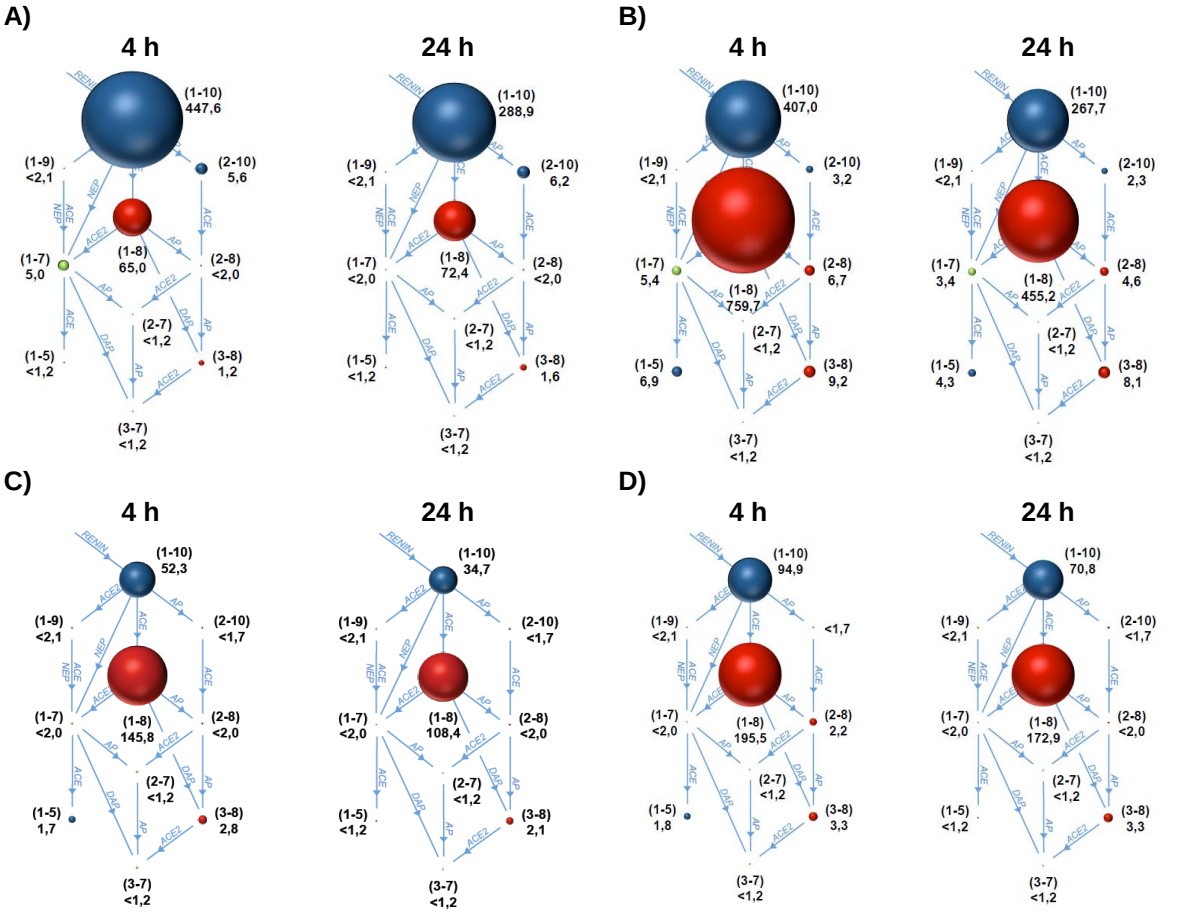

Figure 5. Angiotensin peptide concentrations after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 and 24 hours after dosing.

In the subgroup of patients where a pre-baseline assessment was done (n = 20) no relevant change in RAS peptide profiles assessed two weeks apart was observed. Bubble plots after

treatment with either perindopril (10 mg), olmesartan (20 mg), amlodipine (5 mg), or hydrochlorothiazide (25mg) are shown in Figure 5.

Renin and aldosterone concentrations

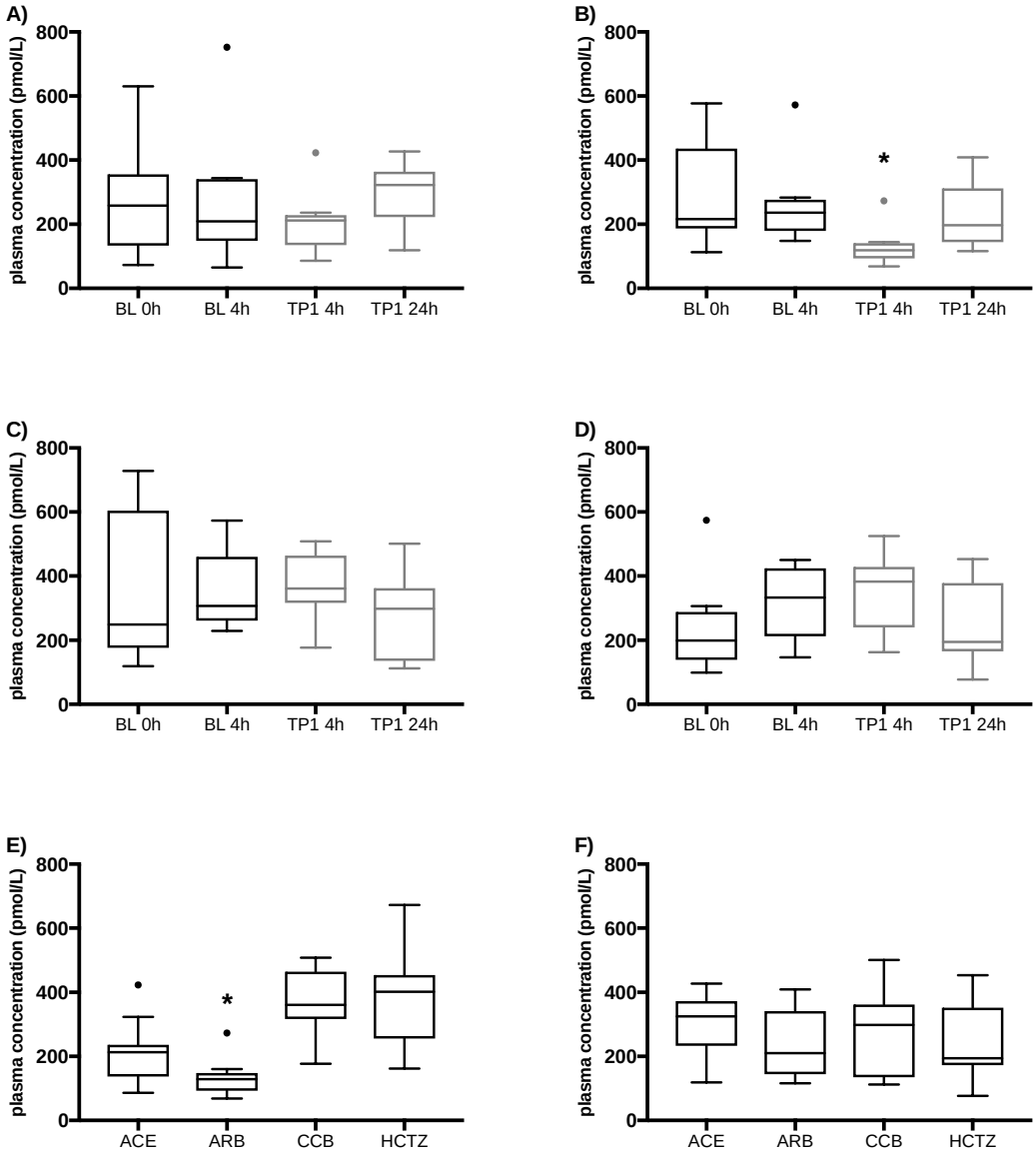

Figure 6. Aldosterone plasma concentrations at baseline (BL) and after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 and 24 hours after dosing. Aldosterone plasma concentrations in direct comparison E) 4 hours and F) 24 hours after dosing.

The renin feedback induced by ACE inhibitors and angiotensin receptor antagonists was observed for perindopril and olmesartan, respectively. A slight renin feedback was observed

for amlodipine and hydrochlorothiazide. In the perindopril and olmesartan group plasma aldosterone concentrations decreased 4 hours after dosing and returned to baseline values 24 hours after dosing (Figure 6A-B). In the amlodipine and hydrochlorothiazide groups plasma aldosterone concentrations slightly increased 4 hours after dosing and returned to baseline values 24 hours after dosing (Figure 6C-D). As shown in Figure 7, there was no correlation of equilibrium Ang 1-8 plasma concentration and plasma aldosterone concentration.

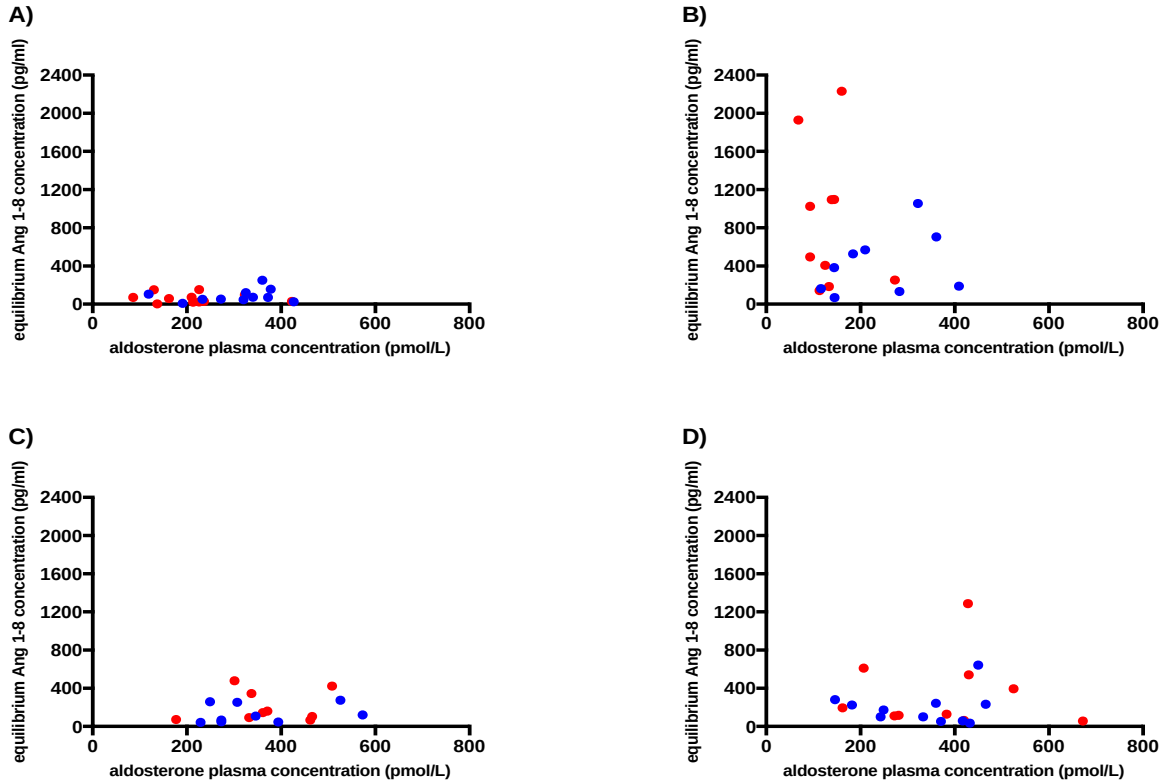

Figure 7. Aldosterone plasma concentration versus equilibrium Ang 1-8 concentrations after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 hours (red dots) and 24 hours (blue dots) after dosing.

The Ang 1-8 / Ang 1-10 ratio as a marker for ACE activity is shown in Figure 8. Without drug treatment, mean Ang 1-8 / Ang 1-10 ratios were 2.8 (sd 2.2) and 2.5 (sd 1.5) when assessed in the morning or at noon, respectively. After multiple dose treatment with the ACE inhibitor perindopril, the Ang 1-8 / Ang 1-10 ratio decreased significantly compared to baseline and

did not return to baseline levels at the end of the 24 hour dose interval (Figure 8). The angiotensin receptor antagonist, the calcium channel antagonist and hydrochlorothiazide on the other hand had no relevant effects on the Ang 1-8 / Ang 1-10 ratio (Figure 8).

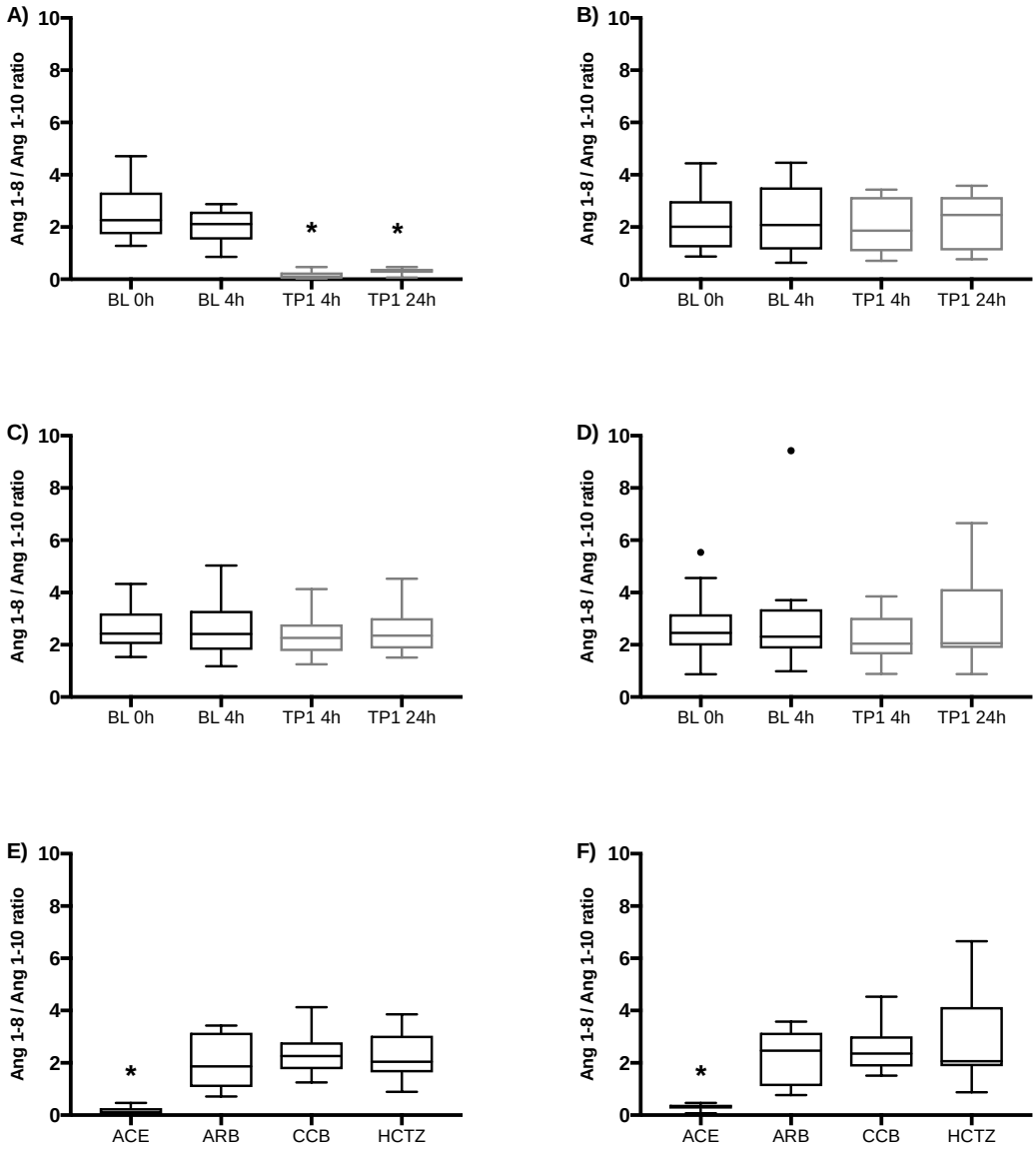

Figure 8. Ang 1-8 / Ang 1-10 at baseline (BL) and after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 and 24 hours after dosing. Ang 1-8 / Ang 1-10 ratio in direct comparison at E) 4 hours and F) 24 hours after dosing.

Cardiac Index and SSVRI

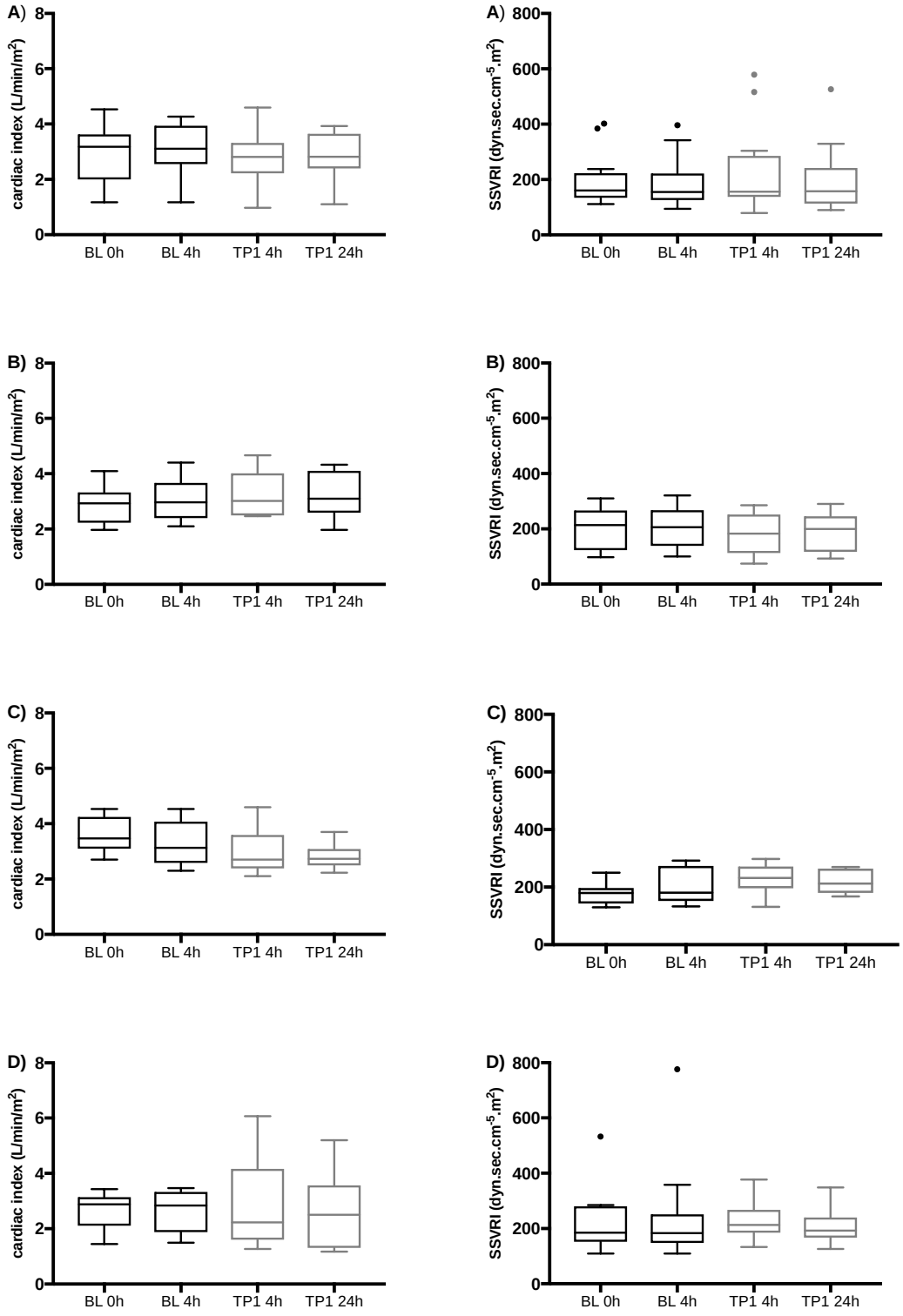

Figure 9. Cardiac index and SSVRI without treatment (BL) and after treatment with a daily intermediate dose monotherapy (TP1) of A) perindopril, B) olmesartan, C) amlodipine, D) hydrochlorothiazide for at least 4 weeks 4 hours and 24 hours after dosing.

Organ perfusion is determined by the arterial blood pressure. In general, the arterial blood pressure is determined by the product of cardiac output (CO) and systemic vascular resistance (SVR). The cardiac index (CI) is the cardiac output indexed to patient's body surface area. The systemic vascular resistance indexed to patient's body surface area and measured per beat leads to stroke systemic vascular resistance (SSVRI) which is an index of vasoactivity. Comparison of measured CI and SSVRI showed stability during the day as well as stability over 2 weeks. None of the treatments led to a significant change in CI or SSVRI (Figure 9).

Discussion

It is known that only 40-50% of patients reach blood pressure targets on hydrochlorothiazide monotherapy and therapy is more successful in elderly patients and patients from African descent.[14, 15] Neutral or negative results for hydrochlorothiazide in the management of hypertension were reported by clinical trials before.[16] Nevertheless, ESH recommends thiazide diuretics as a first-line drug for the initiation of antihypertensive drug treatment based on data derived from multiple randomized clinical trials or meta-analyses. A systematic review by Musini et al. 2014 [17] revealed a mean systolic/diastolic blood pressure-lowering effect of 4/2 mmHg, 6/3 mmHg, 8/3 mmHg and 11/5 mmHg for hydrochlorothiazide doses of 6.25 mg, 12.5 mg, 25 mg and 50 mg/day, respectively. Furthermore, thiazide diuretics were effective in the primary prevention of cardiovascular events. A further blood pressure decrease would be achieved by combination with a second antihypertensive agent.

Drug exposure after repeated dosing was in line with published values, except for olmesartan.[18-23] Four hours after observed drug intake measured values were slightly higher than reported in literature.[19, 20] The lack of increase in some pharmacokinetic samples remained unclear. In case of concomitant medication drug-drug interactions were excluded. According to the case report forms all respective patients had been fasting. This is

relevant especially for perindopril as food has been shown to significantly reduce relative bioavailability of perindoprilat up to 35% +/- 42%.[24]

In this study we quantified equilibrium concentrations of different angiotensin peptides in patients with primary arterial hypertension. Concentrations were measured before start of treatment and 4 or 8 weeks after multiple dose treatment with one of four drugs used as first line treatment. Without treatment, concentrations of Ang 1-10 and Ang 1-8 could be quantified in all study participants. Equilibrium angiotensin concentrations were in the mid picomolar range and Ang 1-10 and Ang 1-8 showed significant circadian changes. Concentrations of the remaining analyzed RAS peptides were either below the limit of quantification or could only be quantified in a few study participants. A significant increase of Ang 1-10 and Ang 1-8 at noon was not observed in normotensive healthy subjects (NCT01771783), but in this study the area under the effect curve from 0 to 24 hours of Ang 1-10 and Ang 1-8 at baseline was calculated and intra-individual changes in each peptide from morning to noon were not compared. If measured Ang 1-10 and Ang 1-8 concentration between morning and noon measurement are compared regardless of paired values, the increase is no longer significant as the changes are small. Median values and range of Ang 1-10 and Ang 1-8 of patients with primary arterial hypertension are slightly higher than values measured in normotensive healthy subjects (NCT01771783). Although the renin-angiotensin system plays an important role in blood pressure regulation, there are additional primary factors determining blood pressure including the sympathetic nervous system, or the plasma volume. Furthermore, plasma levels of Ang 1-10 and Ang 1-8 may not correlate directly with tissue levels.[25]

Multiple doses of the ACE inhibitor perindopril and the angiotensin receptor antagonist olmesartan, both inhibiting the RAS cascade on different levels, lead to the sane characteristic changes of RAS peptide concentrations observed previously in normotensive healthy subjects (NCT01771783). After multiple dose treatment with an intermediate dose of perindopril for 4 weeks, the ACE inhibitor produced a significant increase of Ang 1-10. In

normotensive healthy subjects the ACE inhibitor also caused an increase in the ACE substrate Ang 1-7. This was also observed in patients with arterial hypertension. After multiple dose treatment with an intermediate dose of olmesartan for 4 weeks, the angiotensin receptor antagonist produced a significant increase of both Ang 1-10 and Ang 1-8. The changes in Ang 1-10 and Ang 1-8 equilibrium concentrations were maintained to a significant extent over the whole 24 hour dosing interval for perindopril and olmesartan.

The multiple dose treatment with an intermediate dose of amlodipine, a calcium channel antagonist, for 4 weeks led to a significant increase in Ang 1-10 and Ang 1-8 as observed after treatment with olmesartan and the changes of Ang 1-10 and Ang 1-8 equilibrium concentrations were maintained over the whole 24 hour dosing interval as well. However, the observed changes were statistically not significant. After multiple dose treatment with an intermediate dose of hydrochlorothiazide for 4 weeks, also the diuretic produced an increase in Ang 1-10 and Ang 1-8, but only the increase in Ang 1-8 was statistically significant. While the changes of Ang 1-8 were maintained over the whole 24 hour dosing interval (but not to a significant extent), the changes of Ang 1-10 were only significant at the end of the 24 hour dosing interval. This might reflect the slow mechanism of action of hydrochlorothiazide.

Olmesartan treatment resulted in a significant reduction in plasma aldosterone levels, which is in line with previous findings reported by Nakamura et al. 2009.[26] However, for none of the four drugs a direct relationship between Ang 1-8 and aldosterone concentration was observed. Besides Ang 1-8, also potassium is a main regulator of aldosterone.[27] High plasma concentrations of Ang 1-8 or potassium increase aldosterone production. But a low blood volume or low sodium levels, in turn, regulate Ang 1-8 concentrations. In addition, there are other factors such as adrenocorticotrophin (ACTH) and a local renin-angiotensin system in the zona glomerulosa of the adrenal glands that also influence aldosterone production.[27, 28]

The ratio of the ACE product Ang 1-8 and the ACE substrate Ang 1-10 can be used as a marker of ACE activity. While angiotensin receptor and calcium channel blockade or hydrochlorothiazide had no relevant effect on the Ang 1-8 / Ang 1-10 ratio, it was significantly

decreased by the ACE inhibitor perindopril. This was also observed after multiple dose treatment with enalapril in healthy normotensive subjects (NCT01771783) and low Ang 1-8 / Ang 1-10 ratios thus could be used as a marker for ACE inhibitor treatment.
RAS inhibitor induced changes of angiotensin peptide concentrations are maintained over a period of up to 8 weeks. Data from a small number of patients with essential hypertension continuously treated with ramipril suggest that the characteristic ACE inhibitor induced changes of Ang 1-10 and Ang 1-8 concentrations may persist for up to two years and possibly even longer.[29] However, whether the ratio can be used to assess patients under long-term ACE inhibitor treatment will have to be tested in subsequent studies.

A strength of our study is that it systematically quantifies equilibrium angiotensin peptide concentrations in patients with arterial hypertension before and after start of monotherapy treatment with first-line antihypertensive drugs with different mechanisms of action. The study provides essential baseline data which is required for the interpretation of drug-induced changes of RAS peptide concentrations in more complex patients. The short observation period and the absence of comorbidities in our study population are limitations of our study that do not allow extrapolation of the results to patients with arterial hypertension on long-term treatment and / or with comorbidities.

In conclusion we have shown that characteristic changes of angiotensin peptide concentrations in serum observed after multiple dose treatment with an ACE inhibitor or an angiotensin receptor antagonist directly reflect the mechanism of action of these RAS inhibitors and that these changes are maintained after multiple dose treatment in patients with arterial hypertension. Furthermore we also showed that non-RAS inhibitors antihypertensive drugs which do not directly interfere with the RAS cause changes in equilibrium angiotensin concentrations with a pattern similar to the one observed for the angiotensin receptor antagonist. These changes are preserved after multiple dose treatment for up to eight weeks. The present data suggest that RAS peptide profiles may be useful

biomarkers to assess drug treatment efficacy in patients with arterial hypertension. RAS peptide profiles now need to be further characterized in subsequent studies in patients with arterial hypertension under combination drug treatment as well as in patients with different comorbidities.

Acknowledgements

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding this work.

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Supplementary

Supplementary Table 1: Lower limits of quantification (LLOQ) for different angiotensin peptides

	Equilibrium analysis (pg/ml)
Ang 1-10	2
Ang 1-9	2
Ang 1-8	1
Ang 1-7	2
Ang 1-5	1
Ang 2-10	1
Ang 2-8	2
Ang 2-7	1
Ang 3-8	1
Ang 3-7	1

Supplementary Table 2: RAS peptide concentrations (pg/ml) in equilibrium samples before (baseline) and after multiple doses of perindopril, olmesartan, amlodipine, hydrochlorothiazide.

Pre-Baseline (n = 20)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**30** (2 - 81)	**<2**	**67** (7 - 169)	**<1**	**<1** (<1 - 2) [2)]	**<1** (<1 - 4) [1)]	**<2** (<2 - 1) [1)]	**<1**	**2** (<1 - 3) [15)]	**<1**	**8** (2 - 19)	**214** (56 - 480)
4	**42** (<2 - 97) [18)]	**<2**	**99** (<1 - 267) [18)]	**<2**	**<1** (<1 - 2) [5)]	**<1**	**<2** (<2 - 2) [1)]	**<1**	**2** (<1 - 4) [15)]	**<1**	**10** (1 - 45)	**199** (30 - 540)

Baseline (n = 43)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**37** (<2 - 145) [42)]	**<2**	**60** (9 - 330)	**<2**	**<1** (<1 - 3) [14)]	**<1** (<1 - 7) [7)]	**<2** (<2 - 3) [2)]	**<1**	**2** (<1 - 5) [30)]	**<1**	**7** (1 - 32)	**232** (73 - 728)
4	**41** (3 - 219)	**<2**	**92** (7 - 404)	**<2** (<2 - 6) [7)]	**<1** (<1 - 6) [14)]	**<1** (<1 - 8) [7)]	**<2** (<2 - 8) [9)]	**<1**	**2** (<1 - 11) [32)]	**<1**	**9** (1 - 66)	**222** (65 - 752)

Perindopril, 5 mg (n = 12)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**289** (21 - 546)	**<2**	**72** (9 - 252)	**<3** (<2 - 20) [6)]	**<1** (<1 - 5) [5)]	**10** (<1 - 38) [10)]	**<2** (<2 - 3) [2)]	**<1**	**2** (<1 - 5) [10)]	**<1**	**21** (1 - 63)	**325** (119 - 427)
4	**448** (35 - 2718)	**<2**	**65** (2 - 153)	**14** (<2 - 90) [7)]	**<1** (<1 – 4) [5)]	**12** (<1 - 238) [10)]	**<2**	**<1**	**1** (<1 - 5) [9)]	**<1**	**37** (1 - 127)	**213** (86 - 423)

Perindopril, 10 mg (n = 3)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**480** (289 - 756)	**<2**	**95** (71 - 129)	**15** (<2 – 21) [2)]	**4** (<1 - 4) [2)]	**19** (<1 - 19) [2)]	**<2**	**<1**	**2** (<1 - 2) [2)]	**<1**	**29** (21 - 68)	**467** (208 - 555)
4	**1104** (1081 - 1954)	**<2**	**38** (27 - 99)	**62** (<2 - 91) [2)]	**<1** (<1 - 5) [1)]	**43** (17 - 46)	**<2**	**<1**	**1** (<1 - 2) [2)]	**<1**	**60** (48 - 174)	**198** (156 - 274)

Data are given as median (range). Number of footnote denotes number of patients with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 patient, 2) quantifiable concentrations in 2 patient, etc.

Supplementary Table 2 cont.: RAS peptide concentrations (pg/ml) in equilibrium samples before (baseline) and after multiple doses of perindopril, olmesartan, amlodipine, hydrochlorothiazide.

Olmesartan, 20 mg (n = 10)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**268** (20 - 778)	**<2**	**455** (70 - 1055)	**10** (<2 - 25) [6]	**5** (<1 – 15) [8]	**<2** (<1 - 28) [5]	**9** (<2 - 17) [6]	**<1**	**9** (<1 - 24) [9]	**<1**	**51** (5 - 155)	**210** (116 - 409)
4	**407** (55 - 2125)	**<2**	**760** (143 - 2230)	**<8** (<2 – 38) [5]	**11** (<1 – 26) [9]	**19** (<1 - 49) [6]	**21** (<2 - 32) [6]	**<1**	**9** (3 - 56)	**<1**	**84** (8 - 764)	**129** (68 - 273)

Olmesartan, 40 mg (n = 3)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**128.4** (43 - 242)	**<2**	**256** (138 - 352)	**<2**	**3** (<1 – 3) [2]	**<1** (<1 - 4) [1]	**<2**	**<1**	**9** (2 - 9)	**<1**	**22** (8 - 36)	**212** (156 - 357)
4	**348** (56 - 556)	**<2**	**612** (203 - 924)	**7** (<2 - 10) [2]	**7** (<1 - 7) [2]	**<1** (<1 - 7) [1]	**7** (<2 - 9) [2]	**<1**	**19** (3 - 21)	**<1**	**41** (10 - 72)	**89** (79 - 189)

Amlodipine, 5 mg (n = 9)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**35** (11 - 126)	**<2**	**108** (42 - 274)	**<2**	**<1** (<1 - 4) [4]	**<1** (<1 - 2) [1]	**<2** (<2 - 4) [2]	**<1**	**3** (<1 - 6) [8]	**<1**	**11** (4 - 24)	**307** (229 - 573)
4	**52** (26 - 187)	**<2**	**146** (67 - 479)	**<2** (<2 - 4) [2]	**3** (<1 - 6) [6]	**<1** (<1 - 3) [3]	**<2** (<2 - 5) [3]	**<1**	**4** (<1 - 9) [8]	**<1**	**12** (5 - 33)	**361** (177 - 508)

Amlodipine, 10 mg (n = 5)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**59** (18 - 153)	**<2**	**105** (41 - 353)	**<2**	**1** (<1 - 5) [3]	**<1** (<1 - 1) [1]	**<2** (<2 - 3) [2]	**<1**	**3** (<1 - 6) [4]	**<1**	**13** (8 - 26)	**361** (219 - 500)
4	**96** (52 - 173)	**<2**	**309** (91 - 436)	**<2**	**3** (<1 – 4) [4]	**<1** (<1 - 3) [1]	**<2** (<2 - 3) [2]	**<1**	**4** (<1 - 6) [4]	**<1**	**26** (22 - 32)	**250** (214 - 488)

Data are given as median (range). Number of footnote denotes number of patients with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 patient, 2) quantifiable concentrations in 2 patient, etc.

Supplementary Table 2 cont.: RAS peptide concentrations (pg/ml) in equilibrium samples before (baseline) and after multiple doses of perindopril, olmesartan, amlodipine, hydrochlorothiazide.

Hydrochlorothiazide, 25 mg (n = 11)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**71** (8 - 359)	**<2**	**173** (36 - 642)	**<2** (<2 - 13) [2)]	**<1** (<1 - 9) [5)]	**<1** (<1 - 8) [3)]	**<2** (<2 - 9) [3)]	**<1**	**4** (<1 - 14) [8)]	**<1**	**11** (4 - 60)	**360** (146 - 466)
4	**95** (16 - 714)	**<2**	**195** (58 - 1287)	**<2** (<2 - 20) [2)]	**3** (<1 - 17) [6)]	**<1** (<1 - 8) [2)]	**2** (<2 - 17) [6)]	**<1**	**6** (<1 - 31) [9)]	**<1**	**14** (5 - 102)	**402** (162 - 672)

Hydrochlorothiazide, 50 mg (n = 7)

Time (h)	Ang 1-10	Ang 1-9	Ang 1-8	Ang 1-7	Ang 1-5	Ang 2-10	Ang 2-8	Ang 2-7	Ang 3-8	Ang 3-7	Renin (ng/L)	Aldosterone (pmol/L)
0	**100** (47 - 157)	**<2**	**155** (98 - 424)	**<2**	**2** (<1 - 4) [5)]	**<1** (<1 - 6) [3)]	**<2** (<2 - 3) [3)]	**<1**	**4** (2 - 7)	**<1**	**13** (6 - 26)	**361** (273 - 556)
4	**133** (58 - 443)	**<2**	**267** (85 - 600)	**<2** (<2 - 4) [2)]	**2** (<1 - 4) [6)]	**4** (>1 - 12) [5)]	**3** (<2 - 3) [5)]	**<1**	**5** (2 - 12)	**<1**	**25** (10 - 31)	**305** (199 - 694)

Data are given as median (range). Number of footnote denotes number of patients with quantifiable peptide concentrations, i.e: 1) quantifiable concentrations in 1 patient, 2) quantifiable concentrations in 2 patient, etc.

3.3 RAS peptide profiles in patients with uncontrolled hypertension

Angiotensin peptide profiles in patients with uncontrolled hypertension

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Renin-angiotensin system, angiotensin peptides, uncontrolled hypertension

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Abstract

Background Ang 1-8 is the main effector of the renin angiotensin system (RAS) and involved in the development of arterial hypertension and progression of vascular disease. Usually, patients with uncontrolled hypertension do have an antihypertensive drug in their drug combination acting on a key enzymes or receptors of the RAS, which cause drug-type specific changes of RAS peptide concentrations. The objective of this study was to assess equilibrium angiotensin profiles in patients with uncontrolled hypertension at a random time point and before and 4 hours after observed drug intake.

Methods In an observational single-center study 20 patients with uncontrolled hypertension concentrations of antihypertensive drugs and RAS peptides were determined using a liquid chromatography-tandem mass spectrometry method on a random time point, and before and 4 hours after observed drug intake of the patients current antihypertensive drugs. Equilibrium angiotensin concentrations were determined from standard lithium heparin aliquots.

Results In patients with uncontrolled hypertension drug-specific changes of angiotensin peptide profiles caused by different RAS inhibitors were present despite combined drug treatment with at minimum a calcium channel antagonist and a diuretic. Drug combinations including an ACE inhibitor increased concentrations of the ACE substrate angiotensin I (Ang 1-10) while reducing Ang 1-8 concentrations, resulting in a profound decrease of the Ang 1-8 / Ang 1-10-Ratio. Drug combinations including an angiotensin receptor antagonist increased concentrations of both, Ang 1-10 and Ang 1-8.

Conclusion Equilibrium RAS peptide profiles show drug-specific changes, which directly reflect the mechanism of action of angiotensin receptor antagonists and ACE inhibitors. The present data suggest that RAS peptide profiles could be of value for the assessment of patients under RAS inhibitor therapy. Their potential as possible biomarkers in patients will have to be assessed in additional studies.

Introduction

Resistant hypertension is defined as blood pressure that remains above goal in spite of concurrent use of 3 antihypertensive agents (including a diuretic) at optimal doses or if blood pressure control requires four or more medications.[1] In patients with apparent resistant hypertension differentiation between true resistant and pseudo-resistant hypertension is difficult. Reasons for a pseudo-resistant hypertension are white-coat hypertension, BP elevating co-medications, poor adherence, insufficient drug therapy, or secondary causes such as obstructive sleep apnea or primary aldosteronism. Several studies indicate that a considerable number of patients do not reach blood pressure goals and thus uncontrolled hypertension is a major clinical problem.[1-4] Patients with uncontrolled arterial hypertension are at greater risk of stroke, myocardial infarction, heart failure, and/or chronic kidney disease and the benefits of successful treatment are substantial.[5, 6] Moreover, literature indicates that non-adherence is one of the major causes of uncontrolled hypertension and therefore treatment failure.[7, 8] Detection and treatment of uncontrolled hypertension due to non-adherence is important to improve the management of these patients but few tools exist to accurately and routinely detect it.

Recent studies confirmed reliable quantification of angiotensin peptides in human plasma and showed characteristic drug-type specific concentration changes of Ang 1-10 and Ang 1-8 after treatment with ACE inhibitors and angiotensin receptor antagonists (NCT01771783 and NCT02449811). In this study, we characterized equilibrium RAS peptide profiles in patients with uncontrolled arterial hypertension. In this setting RAS peptide profiles may add valuable information, which in combination with measured drug concentrations could help to differentiate between true drug resistance and non-adherence.

Methods

Study Design

An observational, exploratory single-center pilot study (ClinicalTrials.gov ID: NCT02962778) was performed at the Medical Outpatient and Hypertension Clinic, University Hospital Basel, Switzerland. The study was approved by the local Ethics Committee (Ethikkommission Nordwest- und Zentralschweiz) and conducted in accordance with the principles of the Declaration of Helsinki.

In patients with uncontrolled hypertension referred to the Medical Outpatient and Hypertension Clinic blood samples for the assessment of RAS peptide profiles and antihypertensive drug concentrations were collected. Blood samples were collected once together with routine blood sampling at a clinical check up visit (random time point) as well as before and 4 hours after a planned observed drug intake. On the day of observed drug intake the patients were fasted for 10 h before drug intake. A light breakfast was allowed after study drug intake. For the analysis of equilibrium angiotensin concentrations, blood was collected into standard lithium heparin tubes. Equilibrium angiotensin peptide levels were measured following 30 min of equilibration of conditioned Li-Heparin plasma at 37°C and subsequent stabilization of equilibrium peptide levels. For the analysis of drug concentrations blood was collected into EDTA tubes. All tubes were centrifuged within 30 min at 1500 g for 10 min at 4°C and plasma was stored at -80°C until analysis.

Blood pressure and heart rate were recorded from the patients in sitting position for at least 10 min using an automatic oscillometric device. Measurements were done at the time points of blood sample collection. At each time point, blood pressure and heart rate were measured three consecutive times with an interval of at least one minute between measurements and the average of the three readings was recorded. If two blood pressure readings differed by more than 5 mmHg the outlier was excluded for the calculation of the average.

Patient Population

Patients aged ≥ 18 years of either gender referred to the Medical Outpatient and Hypertension Clinic of the University Hospital Basel, Switzerland were included. Uncontrolled hypertension was defined according to ESH guidelines as office blood pressure ≥ 140/90 mmHg and mean 24 hour blood pressure > 130/80 mmHg on average, or > 135/85 mmHg during the day, or > 120/70 mmHg during the night, in spite of concurrent use of 3 antihypertensive agents at optimal doses, including a diuretic OR office blood pressure and 24 hour mean blood pressure controlled with four or more medications.[9] Patients had to qualify by 24 hour automatic blood pressure measurement. Prior to the qualifying ambulatory blood pressure measurement, drug treatment was unchanged for four weeks. Main exclusion criteria were a known secondary arterial hypertension at time of inclusion, history of, or clinically relevant or uncontrolled cardiovascular disease, and diagnosis of secondary hypertension after study inclusion.

Materials and Reagents

Amlodipine, amlodipine-d4, hydrochlorothiazide, hydrochlorothiazide-13C15N2d2 olmesartan, olmesartan-d6, perindoprilat, perindoprilat-d4, candesartan, candesartan-d5, metoprolol, metoprolol-d6, bisoprolol, bisoprolol-d5, lercanidipine, lercanidipine-d3, nebivolol, and nevivolol-d4 were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Formic acid, acetic acid, high-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and water were purchased from Merck KGaA (Darmstadt, Germany).

Stable isotope labeled internal standards for angiotensin metabolites and MS grade formic acid were purchased from Sigma-Aldrich (Vienna, Austria). LC-MS-grade water, methanol and acetonitrile were purchased from Fisher Scientific (Vienna, Austria).

Measurement of Drug Concentrations

Concentrations of amlodipine, hydrochlorothiazide, olmesartan, perindoprilat, candesartan, metoprolol, bisoprolol, lercanidipine, and nebivolol in plasma were determined by reversed-phase HPLC with tandem mass spectrometry (HPLC–MS/MS). Chromatographic separation

was done on a Shimadzu HPLC system (Shimadzu AG, Reinach, Switzerland) coupled to a triple quadrupole tandem mass spectrometer (API4000 Qtrap, ABSciex, Massachusetts, USA). Three different were developed to analyze the analytes.

Perindoprilat, olmesartan, amlodipine and hydrochlorothiazide were analyzed as described in chapter 3.2..

Analysis of candesartan

Fifty microliter aliquots of plasma were mixed with 150 µL of an internal standard solution (methanol plus 0.01% acetic acid) containing hydrochlorothiazide-13C15N2d2 (100 ng/mL), olmesartan-d6 (25 ng/mL), and candesartan-d5 (50 ng/mL), vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Ten microliters deproteinized supernatant was injected directly into the HPLC–MS/MS system. The analytes were separated on an Atlantis dC18 analytical column (3 µm, 100 x 2.1 mm, Waters, Massachusetts, USA) using a gradient of water (mobile phase A) and acetonitrile (mobile phase A) both supplemented with 0.01% acetic acid. The total run time was 3 min. Samples were quantified using peak area ratios. Olmesartan, olmesartan-d6, candesartan, candesartan-d5, hydrochlorothiazide, and hydrochlorothiazide-13C15N2d2 were analyzed using electrospray ionization with multiple reaction monitoring (MRM) in negative polarity mode. The applied mass transitions and retention times are illustrated in Table 1. The method was linear over a concentration range of 1-500 ng/mL. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Analysis of amlodipine, metoprolol, bisoprolol, lercanidipine, and nebivolol

Fifty microliter aliquots of plasma were mixed with 150 µL of an internal standard solution (methanol plus 0.01% acetic acid) containing amlodipine-d4, metoprolol-d6, bisoprolol-d5, lercanidipine-d3, and nebivolol-d4 at a final concentration of 10 ng/mL, vortex mixed for 30 seconds and centrifuged (3,220g for 30 min at 10°C). Fifty microliters deproteinized

supernatant was injected directly into the HPLC–MS/MS system. Samples were concentrated and purified on a trap cartridge (EXP HALO C18, 4.6 x 5 mm, Optimizetechnologies, Oregon, USA) at a flow rate of 3 mL/min acetonitrile and water (80:20 v/v) supplemented 0.01% acetic acid. The analytes were separated on an Atlantis dC18 analytical column (3 µm, 100 x 2.1 mm, Waters, Massachusetts, USA) using a gradient of water (mobile phase A) and acetonitrile (mobile phase A) both supplemented with 0.01% acetic acid. The total run time was 3.5 min. Samples were quantified using peak area ratios. The analytes were analyzed using electrospray ionization with multiple reaction monitoring (MRM) in positive polarity mode. The applied mass transitions and retention times are illustrated in Table 1. The method was linear over a concentration range of 0.1-50 ng/mL for nebivolol and lercanidipine, and over 1-500 ng/mL for metoprolol, bisoprolol, and amlodipine. Concentrations were calculated by interpolation from a calibration curve. Quality control samples were analyzed throughout the sample analysis.

Table 1

Analyt	MRM [m/z]	Retention time [min]
Perindoprilat	339.1 → 167.8	1.04
Perindoprilat-d4	343.1 → 167.8	
Candesartan	439.2 → 308.8	1.85
Candesartan-d5	444.2 → 309.1	
Hydrochlorothiazide	295.9 → 204.7	1.01
Hydrochlorothiazide-13C15N2d2	301.1 → 207.7	
Olmesartan	445.2 → 148.9	1.34
Olmesartan-d6	451.2 → 153.9	
Metoprolol	268.3 → 116.1	1.28
Metoprolol-d6	274.3 → 122.0	
Bisoprolol	326.4 → 116.0	1.41
Bisoprolol-d5	331.4 → 121.1	
Amlodipine	409.1 → 294.0	1.53
Amlodipine-d4	413.1 → 297.9	
Nebivolol	406.1 → 150.9, 103.1, 123.1	1.55
Nebivolol-d4	410.2 → 151.0	
Lercanidipine	612.4 → 100.3, 280.0	1.84
Lercanidipine-d3	615.4 → 283.2	

Quantification of RAS Peptide Concentrations

RAS peptide concentrations were quantified as described in chapter 3.2..

Statistical analysis

No statistical analysis was performed. Data were described descriptively.

Results

Patients

A subgroup of 10 patients with uncontrolled hypertension recruited between October 2016 and December 2016 was evaluated.

Table 2. Demographics and patient characteristics (N = 10).

Sex	
Male	8 (80%)
Female	2 (20%)
Age, years	56 (28 – 82)
BMI, kg/m^2	31 (28 – 38)
Ethnicity	
Caucasian	9 (90%)
Black	1 (10%)
Ambulatory Daytime BP	
SBP, mmHg	144 (130 – 162)
DBP, mmHg	79 (71 – 92)
Heart rate, bmp	84 (52 – 98)
Antihypertensive drugs (n)	4 (3 – 5)
Diuretic	90% (90%)
ACE inhibitor / Angiotensin receptor antagonist	40% (40%) / 60% (60%)
Calcium channel antagonist	10 (100%)
Beta-receptor antagonist	6 (60%)
Alpha-receptor antagonist	2 (20%)
Co-Medication	
Proton pump inhibitor	5 (50%)
Antidiabetic	5 (50%)
Lipid lowering agent	5 (50%)
Platelet aggregation inhibitor	6 (60%)

Data are presented as number (percentage) for categorical variables and median (range) for continuous variables.

All patients presented with a documented history of uncontrolled hypertension under their respective antihypertensive therapy. Patients included in the subgroup were 8 men and 2 women from 28 to 82 years, and their body mass index ranged between 28 and 38 kg/m^2. Nine patients were of Caucasian ethnicity and one subject was of African descent. Ambulatory blood pressure monitoring revealed an average systolic blood pressure of 144 mmHg and a mean diastolic blood pressure of 79 mmHg. Patients used tolerated doses of 4 antihypertensive drugs on average (range 3 to 5), including a diuretic in every patient. One patient was excluded from evaluation because of a lack of fulfilling the inclusion criteria of inclusion of a diuretic in antihypertensive drug treatment. Detailed patient characteristics at time of inclusion are presented in Table 2.

Plasma drug concentrations and blood pressure

Ambulatory blood pressure at first visit (random time after drug intake) as well as before and 4 hours after observed drug intake (n = 9). Antihypertensive drugs of evaluated patients are listed in Table 3.

Table 3. Antihypertensive treatment.

Subject	Diuretic	ARB	ACE	CCB	B-Blocker
TRHT-001	Hydrochlorothiazide*	Candesartan*	-	Amlodipine	-
TRHT-002	Indapamide*	-	Perindopril*	Amlodipine*	-
TRHT-003	Indapamide*	-	Perindopril*	Lercanidipine	Bisoprolol
TRHT-004	Hydrochlorothiazide*	Olmesartan*	-	Amlodipine*	-
TRHT-006	Indapamide*	-	Perindopril*	Amlodipine*	Nebivolol
TRHT-007	Hydrochlorothiazide*	Olmesartan*	-	Amlodipine*	Metoprolol
TRHT-008	Hydrochlorothiazide*	Candesartan*	-	Lercanidipine	Bisoprolol
TRHT-009	Chlortalidon*, Torasemide	Azilsartan*		Lercanidipine	Metoprolol
TRHT-010	Hydrochlorothiazide*	Olmesartan*	-	Amlodipine*	Metoprolol

* given as fixed drug-combination

Plasma concentrations of perindoprilat, olmesartan, candesartan, amlodipine and hydrochlorothiazide at a random time point as well as before and after observed drug intake are shown in Figure 1.

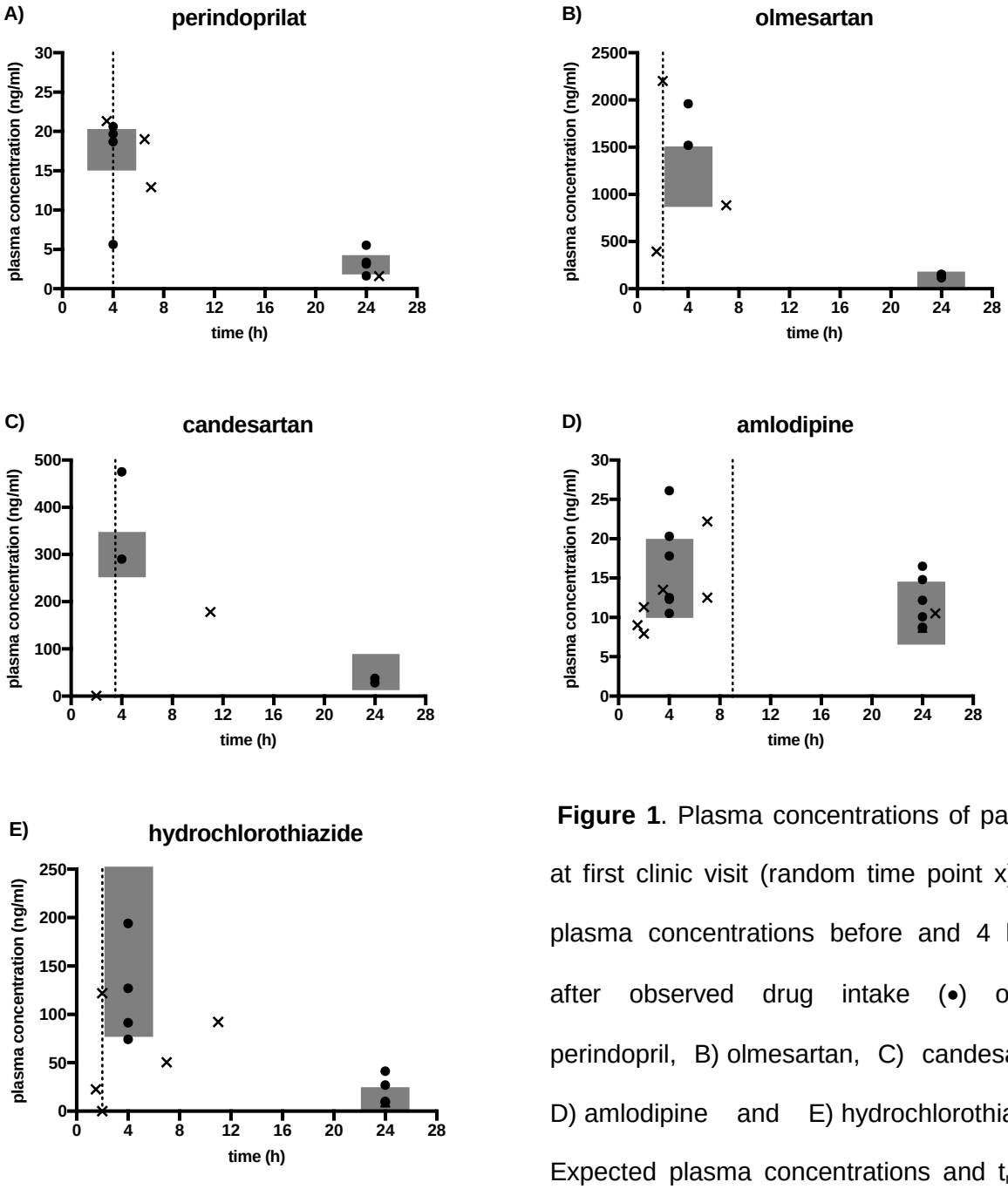

Figure 1. Plasma concentrations of patients at first clinic visit (random time point x) and plasma concentrations before and 4 hours after observed drug intake (•) of A) perindopril, B) olmesartan, C) candesartan, D) amlodipine and E) hydrochlorothiazide. Expected plasma concentrations and t_{max} of respective drugs are indicated as grey boxes and a vertical dotted line, respectively.

One patient showed at random time point a partial non-adherence for combined preparation of hydrochlorothiazide and candesartan and possibly also amlodipine. Increased plasma

concentration and a decrease of blood pressure from 137/99 mmHg to 121/90 mmHg on day of observed drug intake justify a more regular intake of drugs. Blood pressure decreased again 4 hour after observed drug intake to 114/82 mmHg. In 1 patient with a combined preparation of indapamide, perindopril and amlodipine plasma concentrations of indapamide and amlodipine were in line with published values, but plasma concentrations of perindopril were low. Assuming that the patient was compliant, a drug interaction with food could explain this observation. Plasma concentration measurements of the remaining 8 patients confirmed adherence to their respective drug treatment. Systolic and diastolic blood pressure decreased in all patients 4 hour after observed drug intake, but systolic blood pressure remained ≥ 145 mmHg in 5 of the patients. In 2 patients blood pressure decreased to 129/68 mmHg and 118/69 mmHg, respectively. Both of these patients were on a four drug combination. Ambulatory blood pressure at first visit (random time after drug intake) as well as before and 4 hours after observed drug intake are given in Table 4.

Table 4. Ambulatory blood pressure at first visit (random time after drug intake) as well as before and 4 hours after observed drug intake (n = 9).

	Random time point	**Before observed drug intake**	**4 h after observed drug intake**
SBP (mmHg)	144 (121 – 181)	150 (121 – 186)	147 (118 – 165)
DBP (mmHg)	83 (68 – 90)	81 (62 – 100)	78 (68 – 90)
Heart rate (bmp)	69 (53 – 94)	68 (50 – 102)	71 (45 – 98)

Data are presented as median (range).

Angiotensin peptide concentrations in patients with uncontrolled hypertension

All patients who had measurable plasma concentrations of an angiotensin receptor antagonist showed strongly increased equilibrium Ang 1-10 and Ang 1-8 concentrations (TRHT-001, TRHT-004, TRHT-008, and TRHT-009). Peak concentrations of Ang 1-10 and Ang 1-8 were ≥ 800 pg/ml and ≥ 2000 pg/ml, respectively, comparable to concentrations observed in patients

with primary arterial hypertension on olmesartan monotherapy (NCT02449811). Only the patient with azilsartan (TRHT-009) showed a smaller increase of Ang 1-8 (1414 pg/ml) but a comparable increase of Ang 1-10 (956 pg/ml). In addition, the increase in Ang 2-8, Ang 1-5 and Ang 3-8 observed in normotensive healthy subjects (NCT01771783) and patients with primary arterial hypertension (NCT02449811) was observed in patients with uncontrolled hypertension as well. Despite almost two-fold olmesartan plasma compared to literature values one patient (TRHT-007) did not show characteristic increases of Ang 1-10 or Ang 1-8 concentrations observed after angiotensin receptor blockade. Ang 1-10 or Ang 1-8 concentrations were 52 pg/ml and 123 pg/ml 4 hours after dosing and also blood pressure remained elevated with 165/74 mmHg. The patient with no measurable plasma concentration of hydrochlorothiazide and very low plasma concentration of candesartan at random visit, showed baseline concentrations of Ang 1-10 and Ang 1-8 of 185 pg/ml and 189 pg/ml, respectively, and characteristic changes produced by an angiotensin receptor antagonist with Ang 1-10 and Ang 1-8 concentrations of 3260 pg/ml and 6188 pg/ml 4 hours after observed drug intake (TRHT-001).

Of the 3 patients with perindopril in their drug combination, only 1 patient (TRHT-002) showed the characteristic increase of Ang 1-10 concentrations observed after ACE inhibition in normotensive healthy subjects (NCT01771783) and patients with newly diagnosed arterial hypertension (NCT02449811). Despite measurable plasma concentrations, in the remaining 2 patients (TRHT-003 and TRHT-006), Ang 1-10 concentrations were not increased at all, neither in the random samples nor on the days after observed drug intake. However, the ratio between Ang 1-8 and Ang 1-10 is clearly suppressed, indicating efficient blockade of ACE in these patients, while very low renin activity appears to be evident. Interestingly, the only difference in drug treatment of these patients compared do the other patient with perindopril treatment is a beta-receptor antagonist. Another observation is, that despite a low renin hypertension of these 2 patients, 1 patient (TRHT-006) has an aldosterone concentration of up to 372 pM, which could indicate primary aldosteronism. Despite a lower aldosterone concentration of patient TRHT-003 (147 pM), aldosterone mediated hypertension and resulting

suppression of renin cannot be excluded in this patient, as suggested by an elevated Aldosterone / Angiotensin II ratio. Bubble plot schemes of RAS peptide concentrations of every patient with uncontrolled hypertension before and 4 hours after observed drug intake are given in Figure 2.

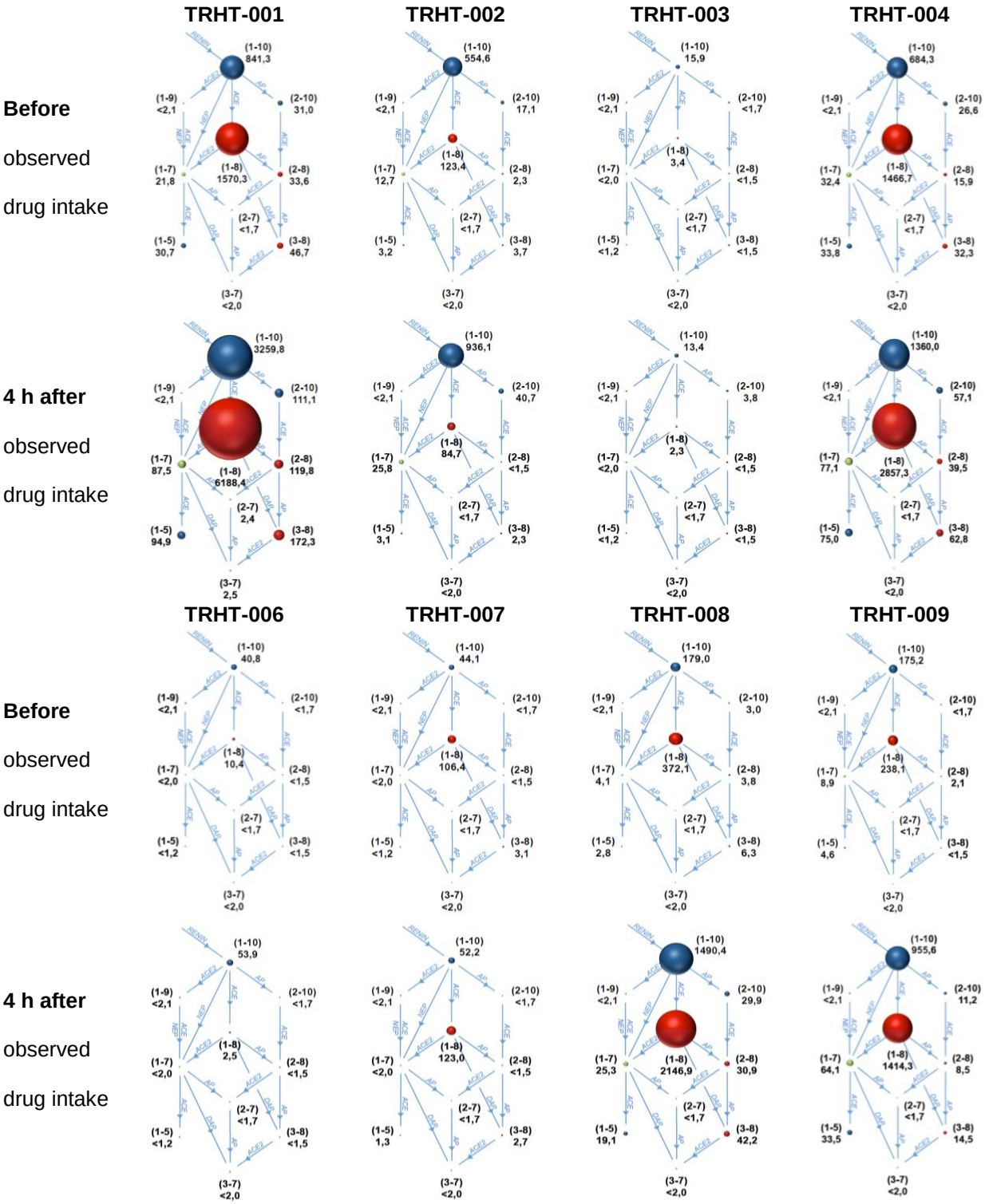

Figure 2. Bubble plot schemes of ex vivo equilibrium RAS peptide concentrations before and 4 hours after observed drug intake. The diameter of the bubble corresponds to the concentration

(pg/ml). The numeric value of the concentration is given below the peptide designation according to the number of amino acids in parenthesis. The arrows designate known degradation pathways with the responsible enzymes. ACE, angiotensin converting enzyme; AP, aminopeptidase A; DAP, dipeptidyl aminopeptidase; NEP, neutral endopeptidase.

Ang 1-8 / Ang 1-10 ratios as a marker for ACE activity are shown in Figure 3. Before observed drug intake, mean Ang 1-8 / Ang 1-10 ratios were 2.2 (sd 0.6) and 0.2 (sd 0.1) for the angiotensin receptor antagonists and the ACE inhibitor, respectively. After observed drug intake mean Ang 1-8 / Ang 1-10 ratios were 1.8 (sd 0.5) and 0.1 (sd 0.1) for the angiotensin receptor antagonists and the ACE inhibitor, respectively. Calculated with Ang 1-8 and Ang 1-10 concentrations at a random time point mean Ang 1-8 / Ang 1-10 ratios differed with 2.1 (sd 1.3) and 0.1 (sd 0.1) for the angiotensin receptor antagonists and the ACE inhibitor, respectively (Figure 3).

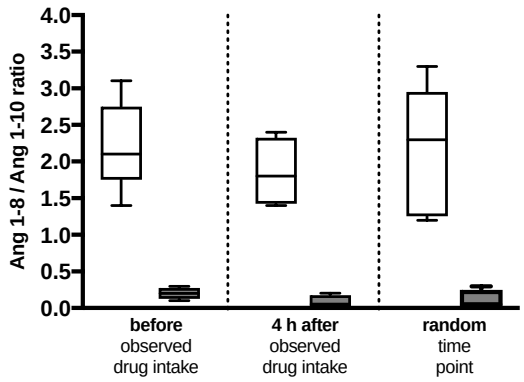

Figure 3. Ratios of equilibrium levels of the ACE product Ang 1-8 and the ACE substrate Ang 1-10 before and 4 hours after observed drug intake of a multiple antihypertensive drug treatment including an angiotensin receptor antagonist (white boxes) or an ACE inhibitor (grey boxes) and at a random time point.

Discussion

In this study we quantified equilibrium concentrations of different renin-angiotensin system peptides in patients with uncontrolled hypertension. Concentrations were measured once together with routine blood sampling at a clinical check-up visit (random time point) as well as before and 4 hours after a planned observed drug intake. Concentrations of Ang 1-10 and Ang 1-8 could be quantified in all study participants. Pharmacokinetic analyses showed

antihypertensive drug concentrations compatible with drug intake prior to the clinic visit in all but one patient, who did not have measurable concentrations of his antihypertensive combination drug (hydrochlorothiazide and candesartan), compatible with partial non-adherence to his triple drug regimen. Treatments including the ACE inhibitor perindopril or angiotensin receptor antagonists (olmesartan and candesartan) lead to characteristic changes of RAS peptide concentrations which were previously observed in normotensive healthy subjects (NCT01771783) and in patients with newly diagnosed arterial hypertension (NCT02449811) on monotherapy. Azilsartan showed a less pronounced effect on Ang 1-8 compared to olmesartan, although Kurtz and Kajiya (2012) [10] showed it to be more potent in inhibiting binding of angiotensin II to human angiotensin II type 1 receptor membrane preparations compared to olmesartan and candesartan. Quantification of RAS peptide concentrations after monotherapy drug treatment with azilsartan and candesartan might indicate whether differences in angiotensin receptor affinity lead to differences in Ang 1-10 and Ang 1-8 increase. However, it is questionable whether differences in receptor affinity really would be reflected in RAS concentrations in serum.

Two patients treated with a combination of an ACE inhibitor (perindopril) and a beta-receptor antagonist (bisoprolol / nebivolol) did not show an increase of Ang 1-10 concentrations typically seen after ACE-inhibitor monotherapy. This might reflect the effect of beta-receptor antagonists which cause a reduction of renin secretion [11], but might also be contributed to a state of primary aldosteronism, which is supported by elevated aldosterone levels and an elevated Aldosterone / Angiotensin II ratio. In contrast to bisoprolol, nebivolol also has vasodilatory properties presumably by NO-modulation, this however, did not lead to a detectable difference in RAS peptide profiles.

Ang 1-7 was quantifiable in all patients except in patients with low renin hypertension (TRHT-003, TRHT-006, and TRHT-007). An increase of Ang 1-7 after ACE inhibitor treatment was observed in normotensive healthy subjects (NCT01771783) and in patients with newly diagnosed arterial hypertension started on monotherapy (NCT02449811).

The ratio of the ACE product Ang 1-8 and the ACE substrate Ang 1-10 can be used as a marker of ACE activity. RAS peptide profiles could qualify as a surrogate marker of drug adherence to an angiotensin receptor antagonist and ACE inhibitor also in patients with drug combinations and based on the equilibrium Ang 1-8 / Ang 1-10 ratio, ACE inhibition can also be monitored if the characteristic renin feedback mediated increase of angiotensin concentrations is not observed.

Two patients underwent renal denervation after the end of study visit. Further assessments of RAS peptide profiles before and after renal denervation may provide more information about the impact of the local renal RAS system on measured RAS peptide concentration.

The strength of our study lies in the comprehensive assessment of angiotensin peptides using a mass spectrometry based approach, known to provide high quality angiotensin data. The simultaneous analysis of multiple angiotensin metabolites in a single sample provided additional information in terms of general activity of the RAS in individual patients and revealed drug treatment specific differences in enzyme activities (Ang 1-8 / Ang 1-10 ratio), which could be identified as a surrogate for ACE activity by using this multiplex approach.

The small sample size and considering the big variety in drug combinations are limitations of our study that do not allow a conclusion of unclear findings such as the effect of azilsartan compared to olmesartan and candesartan or the impact of a combination of an ACE inhibitor with a beta-receptor antagonist.

In conclusion we have shown that in patients with uncontrolled hypertension and combined drug treatment for a long period of time, the characteristic changes of angiotensin peptide profiles induced by an ACE inhibitor or an angiotensin receptor antagonist monotherapy, are maintained even in patients treated with antihypertensive combination drug regimens.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding this work.

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4 Discussion and Conclusions

The thesis describes RAS peptide concentrations in healthy subjects and in patients with a new diagnosis of primary arterial hypertension. Furthermore, the thesis presents changes in RAS peptide concentrations after multiple dose administration of different RAS inhibitors in these conditions and changes in RAS peptide concentrations after multiple dose administration of different non-RAS inhibitors used for the treatment of arterial hypertension. Finally, the thesis presents RAS peptide concentrations in patients with uncontrolled hypertension treated with a drug combination of RAS inhibitors and drugs without direct pharmacologic effects on the RAS.

Our studies show that different RAS inhibitors produce characteristic changes of RAS peptide profiles in normotensive healthy subjects as well as in patients with arterial hypertension. The changes directly reflect the mechanisms of action of the investigated RAS inhibitors. After multiple dose monotherapy we can distinguish between treatment with an angiotensin receptor antagonist and an ACE inhibitor by measurement of plasma Ang 1-10 and Ang 1-8 concentration. The characteristic changes of an angiotensin receptor antagonist and an ACE inhibitor are reflected in the Ang 1-8/Ang 1-10 ratio. After ACE inhibition the ratio is low (indicating low ACE activity), after angiotensin receptor blockade, the ratio is higher (indicating non-inhibited ACE activity) comparable to control samples. In addition, data from the third project provide evidence that it is even possible to detect the effects of an angiotensin receptor antagonist or an ACE inhibitor in patients with uncontrolled hypertension treated with a combination of antihypertensive drugs. Interestingly, RAS activity appeared to be very low in a portion of patients as reflected by low to undetectable angiotensin levels. Depending on the patient cohort analyzed, there are multiple possible explanations for low renin activity. Patient specific variations, certain anti-hypertensive drugs (e.g. beta-receptor antagonists) or disease states (e.g. primary aldosteronism) are known to result in renin suppression, however, higher patient numbers would be required to draw definitive conclusions in our patient cohorts. Calcium channel antagonists and hydrochlorothiazide produce changes of RAS peptides similar (but less pronounced) to the pattern observed after an angiotensin receptor antagonist. However, if they are combined with an ACE inhibitor, the low Ang 1-8 / Ang 1-10 ratio typical for ACE inhibitor treatment remains detectable.

Over all, data generated by these studies showed a correlation between RAS peptide concentrations and treatment with an ACE inhibitor or angiotensin receptor antagonist in normotensive healthy subjects and in patients with arterial hypertension, as well as in patients with uncontrolled hypertension treated with a combination drug regimen. Our data indicate that RAS peptide profiles merit further investigations as a biomarker for the assessment of adherence and (in combination with drug concentrations in plasma) possibly true drug resistance, and that they also may provide guidance for the treatment of patients with difficult to control arterial hypertension. Whether RAS peptide profiles will also be of use in patients with other cardiovascular diseases treated with RAS inhibitors (e.g. heart failure) remains to be shown in subsequent studies.

Outlook

The anticipated study end for the two ongoing research projects, the clinical trial investigating RAS peptides in patients with arterial hypertension and the study investigating RAS peptides in patients with uncontrolled hypertension, will be in the third or fourth quarter 2017. Final data evaluation will show whether the preliminary results can be confirmed. Investigation of RAS peptide profiles in patients with isolated diastolic hypertension will start even before the anticipated end of the two ongoing clinical studies mentioned above. In subsequent studies RAS peptide profiles will be assessed in other patient populations treated with the same drug groups as patients with arterial hypertension (e.g. patients with heart failure) where differentiation between “drug resistance” and adherence related problems is of similar interest. In addition, quantification of RAS peptide concentrations offers many additional interesting applications, e.g. assessment of hyperaldosteronism, where the use of the Aldosterone / Angiotensin II ratio instead of the classic Aldosterone / Renin ratio may offer advantages such as lower variability and reduced interference by concomitant drug treatment (e.g. ACE inhibitors).

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