UNIVERSIDADE FEDERAL DO AMAPÁ
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

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AVALIAÇÃO DO PERFIL METABOLÔMICO E DOS NÍVEIS PLASMÁTICOS DE LOSARTANA E EXP3174 E A SUA CORRELAÇÃO COM O CONTROLE PRESSÓRICO EM PACIENTES HIPERTENSOS E RENAIS CRÔNICOS

Macapá
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapápara obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Francisco Fábio Oliveira de Sousa
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Macapá
2022

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca Central da Universidade Federal do Amapá
Jamile da Conceição da Silva – CRB-2/1010

Santos, Ingrid Souza Reis.

S237a Avaliação do perfil metabolômico e dos níveis plasmáticos de losartana e exp3174 e a sua correlação com o controle pressórico em pacientes hipertensos e renais crônicos / Ingrid Souza Reis Santos. - 2021.
1 recurso eletrônico. 88 folhas.

Dissertação (Mestrado em Ciências Farmacêuticas) – Campus Marco Zero, Universidade Federal do Amapá, Coordenação do Programa de Pós-Graduação em Ciências Farmacêuticas, Macapá, 2021.
Orientador: Professor Doutor Francisco Fábio Oliveira de Sousa
Coorientadora: Professora Doutora Lílian Grace da Silva Solon

Modo de acesso: World Wide Web.
Formato de arquivo: Portable Document Format (PDF).

Inclui referências, anexos e apêndices.

1. Rim. 2. Hipertensão arterial. 3. Hipertensão – Tratamento. 4. Farmacologia. I. Sousa, Francisco Fábio Oliveira de, orientador. II. Solon, Lílian Grace da Silva, coorientadora. III. Título.

Classificação Decimal de Dewey, 22. Edição, 615.7

SANTOS, Ingrid Souza Reis. **Avaliação do perfil metabolômico e dos níveis plasmáticos de losartana e exp3174 e a sua correlação com o controle pressórico em pacientes hipertensos e renais crônicos.** Orientador: Francisco Fábio Oliveira de Sousa. Coorientadora: Lílian Grace da Silva Solon. 2021. 88 f. Dissertação (Mestrado em Ciências Farmacêuticas) – Campus Marco Zero, Universidade Federal do Amapá, Coordenação do Programa de Pós-Graduação em Ciências Farmacêuticas, Macapá, 2021.

Programa de Pós-Graduação em Ciências Farmacêuticas
da Universidade Federal do Amapá

BANCA EXAMINADORA

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Data: 28/01/2022

Dedico este trabalho a minha família, com todo amor e gratidão, por todos os ensinamentos e esforços ao longo de nossas vidas.

AGRADECIMENTOS

Agradeço a todos aqueles que tornaram possível a realização deste trabalho:

Ao professor Francisco Fábio e professora Lílian, expresso o meu imenso agradecimento pela orientação e apoio na realização deste trabalho. Pela partilha do conhecimento científico, pelos conselhos e amizade e pelo profissionalismo e disponibilidade que sempre demonstraram.

A todos os meus amigos e colegas da turma de mestrado, pelo apoio, incentivo e grandes momentos de alegria e crescimento ao longo de nossa jornada que ficarão marcados. Ao Programa de Pós-Graduação em Ciências Farmacêuticas, pela oportunidade de ingressar na pós-graduação. À equipe do LCBM, por todo conhecimento, suporte técnico e científico. Agradeço ao Centro de Apoio Científico-Tecnológico da Universidade de Santiago de Compostela -Espanha pelo apoio técnico durante as análises e à Fundação de Amparo à Pesquisa no Amapá e CAPES pelo apoio financeiro concedido.

Aos profissionais do Programa de saúde da Família da equipe 005 da Unidade de Saúde Unifap e da nefrologia do Hospital Alberto Lima onde coletei os dados da pesquisa. E em especial, a todos os participantes da pesquisa que dispuseram uma parcela do seu tempo para participar desta caminhada. Obrigada pela confiança e incentivo para seguir em frente sempre em minha trajetória.

À toda minha família, meu profundo agradecimento por acreditarem sempre em mim, pela certeza de minha competência e por todos os ensinamentos de vida.

E agradeço principalmente e grandemente a Deus que conduziu todos os meus passos e que está presente em todos os momentos de minha trajetória.

Espero que esta etapa possa de alguma forma, retribuir e compensar todo o carinho, apoio e dedicação que, constantemente me proporcionaram sentir.

SUMÁRIO

RESUMO

Introdução: Em muitos pacientes hipertensos o controle pressórico não é alcançando, mesmo com o tratamento realizado adequadamente, podendo ser influenciado por diversos fatores. A variação interindividual no metabolismo de fármacos é bastante comum e pouco explorada frente aos casos de resistência terapêutica. O losartana é um dos fármacos mais utilizados para controle da pressão arterial, contudo tanto este como o seu metabólito ativo (EXP3174), apresentam um elevado grau de diferenças interindividuais em seus níveis plasmáticos, os quais podem afetar tanto a eficácia como o surgimento de efeitos adversos. Assim, a farmacometabolômica tem sido aplicada na predição da resposta terapêutica a partir da análise do fenótipo metabolômico, relacionando o uso de fármacos às alterações nos metabólitos em um determinado espaço de tempo. **Objetivo**: Revisar os aspectos mais relevantes da losartana utilizada na terapia anti-hipertensiva, determinar os aspectos socioeconômicos e a percepção associada ao tratamento em hipertensos e correlacionar o perfil metabolômicoe os níveis plasmáticos de losartana e EXP3174 ao controle pressórico em pacientes hipertensos e renais crônicos. **Metodologia**: Inicialmente realizou-se uma revisão de literatura sobre o objeto de estudo, seguido de entrevistas semi-estruturadaspara obtenção dos dados sociodemográficos e clínico de pacientes hipertensos e renais crônicos. Por último, procedeu-se a realização de um estudo observacional tipo caso-controle com pacientes hipertensos, renais crônicos e grupo controle (normotensos), através do monitoramento das concentrações plasmáticas de losartana e EXP3174 e análise farmacometabolômica mediante Cromatografia Líquida de Ultra Eficiência acoplada a Espectroscopia de Massas (CLUE-EM/EM) e Ressonância Magnética Nuclear (RMN), respectivamente, para correlação dos dados. **Resultados e discussão**: A variabilidade interindividual pode ser analisada sob diferentes contextos e influenciar diretamente a farmacocinética de anti-hipertensivos, sua resposta clínica e segurança. No presente estudo, a maior parte dos hipertensos entrevistados não soube relacionar a importância do cumprimento do seu tratamento com losartana. Em paralelo, a análise metabolômica por ^{1}H RMN identificou vários biomarcadores plasmáticos capazes de distinguir entre os grupos estudados. Notavelmente, níveis mais elevados de trigonelina, uréia e ácido fumárico foram considerados marcadores característicos de insuficiência renal. Por outro lado, para o grupo de hipertensos, os níveis de ureia encontrados poderiam indicar o ínicio de injúria renal, quando associados ao descontrole pressórico encontrado. Os achados permitiram ainda determinar a efetividade clínica do tratamento com losartana e avaliar a correlação do perfil metabolômico com os níveis plasmáticos e com o controle pressórico alcançado nestes pacientes. Paralelamente a idade avançada, o baixo nivel de compreensão de muitos pacientes e a característica silenciosa da doença, o não recebimento de instrução adequada e não aferir regularmente a pressão arterial, podem comprometer os tratamentos anti-hiprtensivos, os quais podem ser fácilmente descumpridos, impactando na percepção negativa associada ao tratamento. As diferenças encontradas foram compatíveis com os níveis pressóricos alcançados e oferecem dados relevantes sobre o perfil farmacometabolômico associado ao uso de losartana e a diferenciação entre os pacientes hipertensos e renais crônicos. **Conclusão:** Protocolos para monitoramento clínico de losartana podem ser elaborados a partir dos achados do presente trabalho, para dar apoio aos tratamentos dos pacientes hipertensos e também aos pacientes renais crônicos.

Palavras-Chave:Metabolômica; Hipertensão; Doença Renal Crônica; Losartana; EXP3174; Monitoramento Plasmático.

Agradecimentos: CAPES, FAPEAP/CNPq/SESA-AP (PPSUS), USC, Spain, PPGCF-UNIFAP

Introduction: In many hypertensive patients, blood pressure control is not achieved, even with the treatment performed properly, and may be influenced by several factors. Inter-individual variation in drug metabolism is quite common and little explored in cases of therapeutic resistance. Losartan is one of the most used drugs for blood pressure control, however, both this drug and its active metabolite (EXP 3174) present a high degree of inter-individual differences in their plasma levels, which can affect both the efficacy and the emergence of effects. adverse. Thus, pharmacometabolomics has been applied to predict the therapeutic response from the analysis of the metabolomic phenotype, relating the use of drugs to changes in metabolites in each period. **Objective:** To review the most relevant aspects of losartan used in antihypertensive therapy, determine the socioeconomic aspects and perception associated with the treatment in hypertensive patients, and correlate the metabolomic profile and plasma levels of losartan and EXP 3174 to blood pressure control in hypertensive and chronic renal patients. **Methodology:** Initially, a literature review was carried out on the object of study, followed by semi-structured interviews to obtain sociodemographic and clinical data from hypertensive and chronic renal patients. Finally, a case-control observational study was carried out with hypertensive patients, with chronic kidney disease and a control group (normotensive), through the monitoring of plasma concentrations of losartan and EXP 3174 and pharmacometabolomic analysis by means of Ultra Efficiency Liquid Chromatography coupled to Mass Spectroscopy (UHPLC-MS/MS) and Nuclear Magnetic Resonance (NMR), respectively, for data correlation. **Results and discussion:** Inter-individual variability can be analyzed under different contexts and directly influence the pharmacokinetics of antihypertensives, their clinical response and safety. In the present study, most of the hypertensive patients interviewed did not know how to relate the importance of complying with their treatment with losartan. In parallel, the ^{1}H NMR metabolomic analysis identified several plasma biomarkers capable of distinguishing between the studied groups. Notably, higher levels of trigonelline, urea, and fumaric acid were found to be characteristic markers of renal failure. On the other hand, for the hypertensive group, the urea levels found could indicate the beginning of kidney injury, when associated with the lack of blood pressure found. The findings also made it possible to determine the clinical effectiveness of the treatment with losartan and to assess the correlation of the metabolomic profile with plasma levels and with the blood pressure control achieved in these patients. In parallel with advanced age, the low level of understanding of many patients and the silent characteristic of the disease, not receiving adequate instruction and not regularly measuring blood pressure, can compromise antihypertensive treatments, which can be easily disregarded, impacting in the negative perception associated with the treatment. The differences found were compatible with the blood pressure levels achieved and provide relevant data on the pharmacometabolomic profile associated with the use of losartan and the differentiation between hypertensive and chronic renal patients. **Conclusion:** Protocols for clinical monitoring of losartan can be developed based on the findings of the present study, to support the treatment of hypertensive patients and also for patients with chronic kidney disease.

Keywords: Metabolomic Profile; Hypertension; Chronic Kidney Disease; Losartan; EXP3174; Plasma Monitoring.
Acknowledgements: CAPES, FAPEAP/CNPq, SESA-AP (PPSUS), USC-Spain, PPGCF-UNIFAP

APRESENTAÇÃO

Esta dissertação de mestrado faz um apanhado sobre os aspectos sociodemográficos, a percepção e a resposta terapêutica do uso de losartana, contemplando pacientes hipertensos, dentre os quais aqueles com doença renal crônica. Na **introdução** encontra-se o **artigo 1** que se trata de uma revisão bibliográfica e constitui o embasamento deste estudo, apresentando os principais conceitos e estudos pertinentes sobre a temática. Em seguida, são apresentados os **objetivos** do presente estudo. A **metodologia**, juntamente com os **resultados e discussão**, apresentam-se divididos em **dois artigos,** ambos formatados de acordo com as normas das respectivas revistas e de acordo com a descrição a seguir: O **artigo 2** aborda a identificação e relação do perfil socioeconômicocom a hipertensão arterial e a percepção de pacientes em relação à sua farmacoterapia com o antihipertensivado losartana. O **artigo 3** contempla a identificação do perfil metabolômico e sua interrelação com o nível plasmático e a resposta clínica ao tratamento anti-hipertensivo com o losartanaavaliando três grupos: pacientes hipertensos, hipertensos e renais crônicos e grupo controle. Por último, são apresentados as **considerações finais** e os **anexos**.

1 ARTIGO 1

A ser submetido ao JournalClinicalTherapeutics

Losartan and personalized medicine: optimaldosage, drugmonitoring, pharmacometabolomicandhypertensioncontrol

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Abstract

Systemic arterial hypertension is characterized by high and sustained levels of blood pressure (BP) of 130/80 mm Hg or higher. It is estimated that about 50% of hypertensive patients undergoing treatment achieve adequate control of blood pressure levels. One of the most accurate ways to assess the pharmacological response is through the therapeutic drug monitoring (TDM), correlating the plasma levels of drugs and active metabolites to the clinical response. This study aimed to review the recent aspects of losartan therapeutic monitoring by accessing the published literature in the last 15 years (2006-2021). Among the drugs prescribed, hydrochlorothiazide, followed by losartan, captopril and enalapril, represent 81% of the antihypertensives. Despite the type, the use of drugs results in differences in therapeutic efficacy, toxicity and metabolism in view of their pharmacokinetics, resulting from interindividual variations, such as pathophysiological conditions, diet, environment and genetic component, which affect the metabolism and distribution of drugs. The therapeutic response, when found to be related to individual variations, whether good or inadequate, allows the therapeutic individualization based, for instance on the genotype and the metabolomic phenotypeof each patientor group. The application of metabolomics in healthcare is rising, as it is a non-invasive technique, in which biological fluids are used for diagnostic or to predict the efficacy and toxicity of drugs. Hence, it allows improving the treatment changes in behavior and better quality of life for hypertensive individuals through personalized medicine.

Keywords: “Losartan”, “Hypertension”, “Plasmatic”, “Pharmacokinetic”, “Precision Medicine”

Introduction

Cardiovascular diseases are responsible for over 17 million deaths worldwide, [1] mostly resulting from complications related to systemic arterial hypertension (SAH) [2]. SAH is characterized by high and sustained levels of blood pressure (BP) ≥ 130/80 mm Hg [3]. The angiotensin II receptor antagonist losartan is widely prescribed in the treatment of SAH and congestive heart failure [4,5]. Its active metabolite EXP3174 makes it more effective, by increasing the half-life and potentiating the blockade of smooth muscle wall of blood vessels induced by angiotensin II [6].

It is estimated that about half of the hypertensive patients undergoing treatment achieve adequate control of blood pressure [7]. This aspect must be investigated in order to improve the clinical response.

According to the patient's body composition, the increase in body fat and lean mass directly influences the distribution and effect of medicines, creating a complex and dynamic pattern specific to each patient [8,9].

One of the most accurate ways to assess the pharmacological response is the therapeutic drug monitoring (TDM), correlating the plasma levels of drugs and active metabolites to the clinical response. This analysis allows distinguishing, for instance, a non-adherent patient from a patient with no response due to changes in their genetic or idiopatic characteristics [10].

In this sense, the prediction of individual metabolic variations, through pharmacometabolomics, has shown to be a novel approach to individualize the pharmacotherapy, making possible to build a metabolic fingerprint to identify and act in the therapeutic adjustment of doses against individual differences in metabolism.

Materials and Methods

In order to access the aspects related to losartan therapeutic monitoring to the current date, a literature review over the theme has beenperformed.

The bibliographic survey was carried out in the following databases: Medical Literature Analysis and Retrieval System online (MEDLINE) and Latin American and Caribbean literature in Health Sciences (LILACS), limited to publications from the last fifteen years (2006 -2021). In addition, very relevant studies related to the proposed topic outside the period above were exceptionally included.

The following descriptors were used in combination: “Losartan”, “Hypertension”, “Plasma monitoring”, “Pharmacokinetic profile” and “losartan plasmatic” “Losartan and pharmacokinetic”.

The following inclusion criteria were used: manuscripts published in English, Portuguese or Spanish; full papers that addressed the topic available and articles published and indexed in these databases over the last fifteen years. Incomplete texts, duplicates, abstracts, and articles that were not related to the topic were excluded.

Discussion

Hypertension

SAH is a very prevalent cardiovascular disease and considered the main risk factor for acute myocardial infarction (AMI), stroke (CVA) and chronic kidney disease (CKD), associated with high hospital and socioeconomic costs [11]. Other cardiovascular and kidney diseases can arise from blood pressure (BP) dysregulation or inadequate treatment, which can be classified as (over 18 years old): normal (<120 mmHg and <80 mmHg); high (120-129 mmHg and <80 mmHg); stage 1 hypertension (130-139 mmHg or 80-89 mmHg) and stage 2 hypertension (≥140 mmHg or ≥90 mmHg) [3].

Excessive use of sodium, sedentary lifestyle, alcohol and tobacco abuse, as well as obesity and genetic and environmental factorsalso aggravate the risks for cardiovascular diseases and contribute to the emergence and installation of SAH [12].

The treatment of SAH follows its staging. The main objective is to reduce cardiovascular morbimortality resulting from the increase and maintenance of high BP levels (systolic above 130 mmHg and/or diastolic above 80 mmHg) [3], for which pharmacological and non-pharmacological treatments are prescribed [11].

Healthy lifestyle modifications and the use of antihypertensive drugs are recommended for patients with stage 1 hypertension with clinical cardiovascular disease or with a calculated risk of 10% or more of developing atherosclerotic cardiovascular disease within 10 years. For stage 2, the recommendation is two drugs to reduce BP, in addition to lifestyle changes (Table 1). There are also specific measures according to populations, considering that black adults are more likely to

develop SAH than other groups. Therefore, the use of two or more antihypertensive drugs is recommended toattainBP ≤130/ 80 mm Hg in this group [3].

Table 1. Classification of arterial hypertension according to American College of Cardiology/ American Heart Association (2017).

Hypertension stages	Systolic pressure		Diastolic pressure	Treatment or follow-up
Normal	<120 mm Hg	and	<80 mm Hg	Evaluate annually to encourage healthy lifestyle to maintain normal BP.
High	120-129 mm Hg	and	<80 mm Hg	Recommend healthy lifestyle changes and recheck in 3-6 months.
Stage 1	130-139 mm Hg	or	80-89 mm Hg	Assess the 10-year risk for heart disease and stroke using the atherosclerotic cardiovascular disease risk calculator: • If risk < 10%, start with healthy lifestyle recommendations and reassess after 3-6 months. • If risk ≥10% or if the patient has clinical cardiovascular disease, diabetes mellitus, or CKD, recommend lifestyle changes and medication to lower BP (1 drug); reassess the effectiveness of drug therapy after 1 month. If goal is reached after 1 month, recheck in 3 to 6 months. If goal is not reached after 1 month, consider different medication or titration. Continue monthly follow-up until control is reached.
Stage 2	≥140mm Hg	or	≥90 mm Hg	Recommend healthy lifestyle changes and medication to reduce BP (2 drugs from different classes); recheck after 1 month for effectiveness. • If the goal is reached after 1 month, recheckafter 3 to 6 months;

	• If the goal is not reached after 1 month, consider different drugs or titrations; • Continue monthly follow-up until control is achieved.

Hydrochlorothiazide, followed by losartan, captopril and enalapril, represent 81% of the most used antihypertensives [13]. Despite the type, the use of drugs results in differences in efficacy, toxicity and metabolism, according to their pharmacokinetics, [14,15] resultant from interindividual variations, such as pathophysiological conditions, diet, environment and genetic component, which also affects directly the metabolism and distribution of drugs [16].

Adequate control is directly related to the patients´ life quality, as uncontrolled BP implies in anxiety and depression, which impact the psychosocial well-being [17]. The occurrence of long-term health problemscompromises the productivity and survival of hypertensive patients [18].

In this scenario, along with lifestyle changes, drugs play a fundamental role in hypertensive control. However, the variability of the pharmacological response between individuals makes pharmacotherapy challenging.

Losartan

As an important biological factor for arterial regulation, angiotensin II, as well as its AT_1 high affinity receptor, when “overactivated” is related to the development of SAH and consequently other cardiovascular and renal injuries [19].

In the late 1980s, angiotensin II receptor blockers (ARANG II) such as losartan (Figure 1a) were developed and are widely used until nowadays. By interfering with the renin-angiotensin-aldosterone system, ARANG II acts in an antagonistic, competitive and specific way solely on angiotensin II AT1 receptors [20].

a) b)

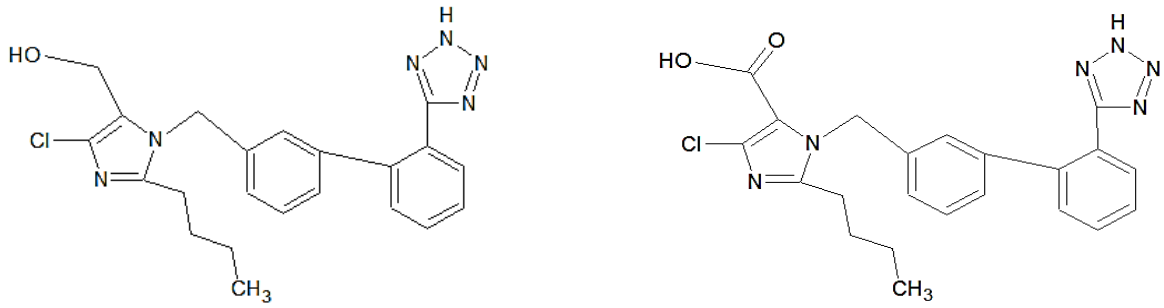

Figure 1. 2D Chemical structure of a losartan and its b active metabolite EXP3174. After oral administration, losartan is absorbed and undergoes first-pass metabolism, forming an active carboxylic acid metabolite and other inactive metabolites.

Widely used in the treatment of hypertension and congestive heart failure, losartan [4,5] selectively binds to the AT1 receptor, blocking the physiological actions of this hormone, without agonistic effects [21].

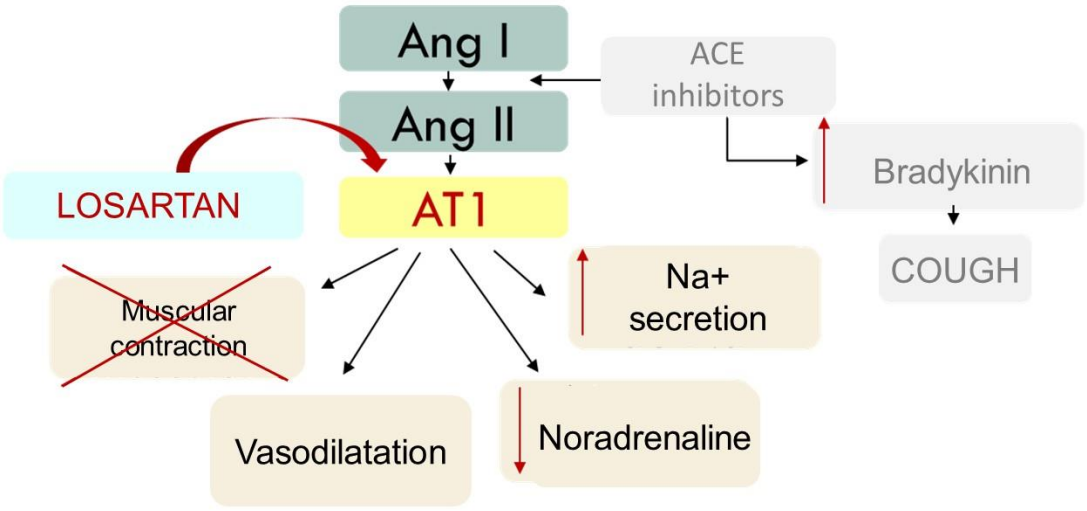

Figure 2. Representation of the mechanism of action of losartan in human body compared to angiotensin conversion enzyme (ACE) inhibitors.

This mechanism inhibits the contraction of vascular smooth muscle caused by angiotensin II. Furthermore, vasodilation and increased sodium secretion and a decrease in noradrenergic activity also occurs (Figure 2). As it does not inhibit angiotensin II type 2 receptors, no increment in renin or angiotensin release is found

[22], With a few adverse effects, ARANG II are well tolerated, with dizziness and orthostatic hypotension being the most common side events [11].

This class of drugs works in a similar way to angiotensin-converting enzyme (ACE) inhibitors, with the advantage of reducingsignificantly the side effects, considering that ARANG II does not affect ACE activity by the degradation of bradykinin, avoiding the side-effects related from this mechanism [23].

Losartan has a usual/initial and maintenance daily dose of 50mg, with the maximum anti-hypertensive effect reached in average after 3 to 6 weeks. The dose can be increased up to 100mg if necessary or reduced to 25mg in cases of intravascular volume depletion, without the need for initial dose adjustment in elderly patients or patients with renal impairement [21]. It is partly bioconverted in EXP3174 (Figure 1b) by the liver (around 14%), making it more effective. While losartan has a half-life ($t_{1/2}$) of 2.5 hours, EXP3174 presents a 6 at 9 hours half-life, reaching the maximum concentration (T_{max}) between 1 to 3 hours [6].

The maximum plasma concentration is approximately 300 µg/L and the EC_{50} (plasma concentration that produces 50% of the maximum antihypertensive response) is 32 µg/L [24]. The high plasma concentration as well as the slow elimination of its metabolite results in anextended antihypertensive activity, also considering the fact that both losartan and EXP3174 strongly binds (99 and 97%, respectively) to plasma proteins [25].

Different patient-specific factors can modify their pharmacokinetics, such as age, body composition and fitness level, among others.For instance, their body composition (mainly lean mass and adipose tissue) has a direct impact on the pharmacokinetics of drugs, as their distribution and uptake can occur to a greater or lesser degree in all body tissues with gradual changes in plasma concentrations after administration, altering the absorption and elimination of these drugs and consequently the plasmatic levels [26, 27].

When evaluating the plasma levels of losartan and EXP3174, the interindividual variation marks significant differences for the peak of the maximum plasma concentration and the area under the plasma concentration curve as a function of time [22], impacting as a consequence the antihypertensive effect.

In a recent study, ca. 80% of nephropathic hypertensive patients had inadequate plasma levels of losartan (subtherapeutic levels). These values may justify high BP levels in clients who adequately and regularly perform the treatment,

considering that losartan wasadministerd under supervision and yet did not reach the therapeutic window due to interindividual variability [28].

Ambulatory blood pressure monitoring (ABPM)

Ambulatory blood pressure monitoring (ABPM), an indirect method of measuring and diagnosing BP, widely used to date, can be performed in two ways: i) the casual method "with an acoustic technique, recorded on the arm, using aneroid or mercury column sphygmomanometer and stethoscope, which is the method used in daily clinical practice" and ii) the intermittent method "with auscultator or oscillometric technique, recorded on the arm, consisting of the ambulatory monitoring of blood pressure for 24 hours" [29].

Both methods commonly applied in clinics to monitor and mainly diagnose cases of hypertension are used after clinical findings or characteristics presented by the patient that predict the possibility of hypertension. When used preventively it can prevent cardiovascular diseases.

Nevertheless, some conditions related to ABPM can impair the diagnosis and make it more difficult to accurately measure blood pressure.When diagnosing in an outpatient, in daily clinical practice, one of the phenomena that makes the process more complex is the condition known as "white coat hypertension" in which the clinical blood pressure is ≥ 140/90 mm Hg in clinical settings on three separate occasions, less than 140/90 mm Hg in non-clinical settings on at least two sets of measurements, and no target organ damage. In these cases, the client benefits little from antihypertensive treatment and may develop sustained hypertension with an increased risk of stroke after six years [30,31].

In contrast, there is also the so-called "masked" hypertension, in which, opposite to white-coat hypertension, clinical BP is normal, while ambulatory BP is high, which may underestimate the risk for cardiovascular events. In a five-year follow-up, it was identified that about a third of patients diagnosed with masked hypertension had a relative risk of cardiovascular events of 2.28 compared to patients whose BP was adequately controlled [32]. In these cases, monitoring through ABPM can be useful for identification.

Nonetheless, ambulatory blood pressure monitoring is not widely used as a criterion or a final point for evaluating the response to antihypertensive treatment,

mainly due to the inconvenience of different ambulatory BP records. The advantages are related to the possibility of evaluating the duration of action of a drug and analyzing its effects on nocturnal BP, but without specifying the reason for the ineffectiveness of the antihypertensive [33].

Although useful for assessing blood pressure, ABPM, when compared to outpatient monitoring, needs some criteria for successful results. It is considerably susceptible to good interpretation when eighty valid readings can be observed (readings recorded by the device) during the twenty-four hours with a deletion rate of less than 20% of the readings. When at least two hours have transcurred without valid readings, the exam will need to be repeated to ensure good results [29].

Other issues related to the use of ambulatory BP monitoring that can limit and hinder the screening and monitoring of SAH are [34]: the very high systolic pressure values making the reading unreliable; those related to physical condition such as unadjusted cuff due to arm circumference or movement disorders such as Parkinson's disease and others related to other conditions such as cardiac arrhythmias, when the pulse is irregular (atrial fibrillation and atrial flutter) and the presence of auscultatory gaps during manual blood pressure measurement.

Analytical methods for TDM of losartan and EXP3174

Among the evaluation methods in the context of hypertension that do not present limitations related to the environment and duration are laboratory tests. Biological samples are collected and used for this purpose, proving to be more practical and useful than conventional outpatient monitoring tests.

As an example, fifty-three 53 adults divided into two groups of hypertensive patients (use of antihypertensive drugs vs. non-use of antihypertensive drugs) showed similar qualitative changes in urinary protein from laboratory tests. Therefore, these should be included among the screening tests in the hypertension monitoring protocol [29] mainly to identify and correlate the inadequacy of a treatment to the clinical conditions of the patient.

Analytical methods enable greater quality control by the determination and quantification of compounds using validated methods and play a key role in pharmacokinetics. The optimization of these techniques and their use in angiotensin II receptor antagonist antihypertensive compounds could help the clinical studies in this field.

Several methods have been proposed for the analysis of drugs and their metabolites, such as the SWV method (square wave voltammetry), liquid chromatography coupled with ultraviolet, fluorimetry and mass spectrometry, among others. A compilation of the analytical methods found in the literature using different matrices, such as synthetic fluids and human plasma for determination and quantification of losartan and/or EXP3174 are listed in Table 2.

Table 2. Analytical methods used forquantification of losartan and EXP3174.

Method used	Molecules	Material analyzed	Detection range	Population	Limitations / objectives	Authors
LC–MS/MS (electrospray ionization mode)	LOSARTAN/ EXP3174	Human plasma	LOS: 0.331 µg/mL EXP 3174: 0.512 µg/mL	n=24	Validation for quantification of losartan and its carboxylic acid metabolite in human plasma using irbesartan as internal standard (IS).	PRASAJA, B. *et al* [35]
LC-MS (electrospray ionization mode)	LOSARTAN	Caco-2 cells	0.008 µg/mL	-	Development of a method for simultaneous determination of antihypertensives: hydrochlorothiazide, losartan, telmisartan, valsartan and the metabolites losartan-5-carboxylic acid, among others, using LC-MS and validation in samples resulting from permeation studies in Caco-2 cells.	DRIGO, L. C. [36]
HPLC-MS/MS	LOSARTAN	Enriched human plasma	0.004 -0,800 µg/mL	-	Use of an appropriate and validated bioanalytical method, of simultaneous determination of two drugs (hydrochlorothiazide and losartan), to assess the relative bioavailability, using the bioequivalence criteria.	MOREIRA, R. F. [37]
HPLC-MS/MS	LOSARTAN/ EXP3174	Human plasma	LOS: 0.027 µg/ml EXP 3174: 0.236 (µg/ml)	n=5	Validated method for simultaneous quantification of Losartan and its active metabolite for plasma monitoring in renal patients, an innovation among the population and field of study.	JUNIOR, A. G.T *et al* [28]
LC-MS/MS (positive electrospray ionization mode)	LOSARTAN/ EXP3174	Human plasma	LOS: 0.191 µg/mL EXP 3174: 0.347 µg/mL	n=36	A pre-dose metabolic phenotype was investigated to interpret inter-individual variations in the characteristics of losartan metabolism.	HE, C. *et al* [38]

LC-ESI-MS	LOSARTAN	Human plasma	0,592 µg/mL	n=24	This analytical method was applied to determine the plasma level of losartan after a single dose in humans healthy volunteers. May be limited to a single dose in a healthy body.	CHOI, Y. *et al* [39]
LC-MS/MS (turboionspray source on positive mode)	LOSARTAN/ EXP3174	Human plasma	LOS: 0,044 µg/mL EXP3174: 0,048 µg/mL	n=6	Simultaneously quantifying seven ARA-II compounds in human plasma and validating this method, including optimizing sample cleanliness and investigating possible suppression effects, to ensure its application in routine analysis.	FERREIRÓS, N. *et al* [40]
HPLC-ESI-MS/MS (operated in the negative ion mode and multiple reactions monitoring mode)	LOSARTAN	Human plasma	0,523 µg/mL	n=28	Simultaneous quantification of these drugs in hundreds of plasma samples obtained from a bioequivalence study of a test LOS-HCTZ formulation (100 mg losartan associated with 25 mg hydrochlorothiazide)	SALVADORI, M. C. *et al* [41]

ESI- electrospray ionization. LOS = losartan

Drugs´pharmacokinetic can be altered by several factors, both biologically and dysfunctional, such as impaired renal function. Therefore, TDM for clinical and toxicological purposes is a key approach to determine the biological level of therapeutic agents in general, such as anticonvulsants, antihypertensives, among others with major impact therapeutic interest [42,43,44,45].

The efficacy and safety of a specific drug can be evaluated regardless of the clinical arrangement, with the main objective of individualizing the therapeutic regimen according to the needs and singularities of each patient [46]. Several methods can be applied to quantify the substances in different biological fluids. According to the pharmacokinetic results, the adequacy of the study to its objective can be evaluated.

Square wave voltammetry was used to quantify losartan in pharmaceutical products and synthetic biological samples, with a sensor made of black carbon, chitosan and epichlorohydrin [47].

A method for quantifying losartan and EXP 3174 was validated and used for therapeutic monitoring in human plasma of CKD patients. HPLC coupled with sequential mass spectroscopy was used and resulted in a feasible method, that reached the minimum parameters of precision and accuracy, with CV values ranging between 0.52 and 9.20% and accuracy values ranging from 85.07 – 113.51%, demonstrating the agreement between spiked plasma concentrations and the expected values [28].

Hence, the determination of losartan in those studies explored different aspects and is a way to explore the circumstances related to the interindividual pharmacodynamic variability, enabling clarification and in-depth studies with the TDM basis.

Special populations

Adverse events, such as decreased efficacy or increased toxicity, may be related to several factors and not just the inappropriate use of drugs or an inefficient diet. Factors related to metabolic alterations in special populations, as well as in patients at greater risk for alterations, must be identified and measures taken.

The interindividual variability of drug metabolism strongly influences the efficacy and the undesirable sideeffects. This aspect can impact the expected therapeutic outcome and may be associated with characteristics such as age, gender, comorbidities and environmental factors. The genetic variation is also an important factor in thepharmacologicalactivity [48]. The clinical response, when found to be related to individual

variations, whether good or inadequate for a specific treatment, reinforces the need of personalized medicine approach, based for example on the genotype of each patient [49]. Interpopulation variability, as well as drug efficacy and adverse reactions are influenced by the difference in pharmacogenomic variants. For instance, the ABCB1 gene, is one of those responsible for drug transporting enzymes of antidepressant, antipsychotic, antihypertensive and analgesic drugs [50].

The miscegenation, from the Paleolithic period to the mixtures of African, European and Native American ancestors resulted in different degrees of genetic and consequently metabolic diversity, generating differences according to the predominant genetic ethnicity as well as gender, age and healthy behavior [51]. Clinical trials for various medical conditions have shown robustly that individuals from different ethnic groups experience diverse responses to therapeutic agents [52].

Another associative characteristic is the drug-nutrient interaction. This type of interaction can be considered clinically significant when an unexpected therapeutic response occurs. [53]. Patients with chronic diseases, polimedicated or long-term treatments are more susceptible to this type of interaction.

The extremes of age are also subject to interactions that influence drug metabolism and the impaired function of noble organs. Poor nutrition and excess of weight also increase the susceptibility to interactions between nutrients and drugs [8].

When body weight is altered, for more or less, it can also be a source of pharmacological changes. In obesity, for example, when the total body clearance approaches or exceeds those in non-obese individuals, it is recommended to use the total body weight for the purposes of dose calculation and maintenance. In addition to provoque changes in progression of chronic diseases, obesity can also influence the drugs´ distribution and metabolism [54].

On the other extreme, subnutrition may be related to ineffective drug activityas it is linked to drug metabolism, genetic, diet, among other predisposing factors. As these factors are identified, the prediction is a tool to reach the proposed therapeutic outcome.

Pharmacometabolomics

The study of metabolome modifications (changes in metabolites over a period of time), in a non-invasive manner, both in physiological and pathological conditions and/or after individual exposure to factors such as environmental, diet and drugs is an investigative technology named metabolomics [55].

Metabolomics emerged from studies of the profile of metabolites in different fields of science. The study of metabolites is of great importance when analyzing the compounds that “signal” the biological and chemical variations occurring in the organism [56]. In the late 1940s, mass spectrometry was used efficiently to trace the metabolomic profile of body fluids of schizophrenic clients, just as the use of coupling between chromatographic techniques and mass spectrometry has emerged [57].

Pharmacometabolomics is a recent innovative strategy based on the analysis of pre-dose profiles of metabolic biofluids, which allows reflection on the complex interactions between pathophysiological conditions, gene expression and pharmacological response [58,59,60]to predict possible metabolic changes that are influencing the efficacy of the pharmacotherapy. The application of metabolomics in medicine is growing in the last years, as it is a non-invasive technique, in which biological fluids are used. It can be used as a predictive diagnostic tool and/or to assess the efficacy and toxicity related to the pharmacotherapy, individually evaluating the therapeutic approaches [55].

The compounds of interest are analyzed through identification, confirmation and quantification based on nuclear magnetic resonance and gas or liquid chromatographycoupled with mass spectrometry. Based on that, the results are related to the biological process and the relationship of processes and effectiveness [61].

Metabolic analysis based on ^{1}H NMR can be combined with other analyzes as in a study in which it was combined with pharmacokinetics and multivariate analysis to investigate the relationship between pre-dose metabolic phenotype and characteristics of losartan metabolism, in addition of potential biomarkers and metabolic pathways associated with losartan metabolism [38]. Demonstrating how pharmacometabolomics can be applied in a useful and versatile way, enabling more complete results.

Among the most used biofluids, blood has been widely used for metabolomic analysis, and it is possible to identify and observe from it the dynamics of metabolites in metabolism before elimination [62]. The urinary samples have also been used considering that its composition indicates a wide range of biochemical processes that occur in the kidneys as a final step of the body metabolism [63]. Drug toxicity can also be analyzed from metabolites generated and eliminated by the urinary system.

As an example of this approach, the use of the NMR-based metabolomic suggested six urinary metabolites composed of taurodeoxycholic acid (TDCA), 3-methylhistidine, tyrosine, phenylalanine, argininesuccinic acid and epi-coprostanol for the analysis of toxicity and prediction of nephropathy [64].

In recent studies, it has also been used to identify predictive biomarkers related to interindividual differences regarding the drugs´ pharmacokinetics, such as losartan, what can be used toadjust and personalize the doses according to the metabolomic phenotype [38]

Conclusions

Interindividual variability can be analyzed in different contexts and can directly influence the pharmacokinetics of antihypertensive drugs, their clinical responses and safety.

Losartan has been used as a first-choice anti-hypertensive over decades and is very unlike to cause side-effects. Despite that, no quantification methods are used routinerly for its determination in biological samples. In the last decade, some studies have attempted to propose methodologies in this field. Among those methodologies, mass spectrometry has been the most proeminent, allowing detecting extremely low concentrations of both losartan and its active metabolite EXP3174. Ambulatory blood pressure monitoring and therapeutical drug monitoring are methods used to access the clinical response, based on the clinical outcomes, patients´ adherence and pharmacokinetic aspects.

Recently, pharmacometabolomics emerged as an innovative method based on the analysis of pre-dose profiles of metabolic biofluids and is directly related to biological processes, suggesting drug interactions and the relationship with the environment of each individual. This aspect could be used in the near future, together with the quantification methods of losartan and EXP3174 to predict the pharmacological behavior and support the therapeutic individualization of antihypertensive treatments.

Funding

This study was financed in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and by the Fundação de Amparo a Pesquisa do Estado do Amapá/Conselho Nacional de Desenvolvimento Científico e Tecnológico (Edital PPSUS).

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2 OBJETIVOS

2.1 OBJETIVO GERAL

Revisar a revisar os aspectos mais relevantes da losartana utilizada na terapia anti-hipertensiva, determinar os aspectos socioeconômicos e a percepção associada ao tratamento em pacientes hipertensos e correlacionar o perfil metabolômico e os níveis plasmáticos de losartana e EXP3174 ao controle pressórico em pacientes hipertensos e renais crônicos.

2.2 OBJETIVOS ESPECÍFICOS

- Revisar os aspectos mais relevantes do tratamento com losartana para o controle da hipertensão arterial;

- Determinar o perfil sociodemográfico, clínico dos pacientes hipertensos e renais entrevistados, assim como a percepção associada ao tratamento anti-hiprtensivo;

- Analisar o perfil metabolômico plasmático dos pacientes hipertensos e pacientes hipertensos e renais crônico sem uso de losartana através de espectroscopia de ressonância magnética nuclear;

- Determinar os níveis plasmáticos de losartana e EXP3174 nos pacientes hipertensos e pacientes hipertensos nefropatas através de cromatografia em fase líquida acoplada a espectrometria de massas-massas;

- Correlacionar o perfil metabolômico aos níveis plasmáticos de losartana e EXP3174 e aos níveis pressóricos alcançados pelos pacientes.

Artigo submetido a Revista Atención Primaria

Aspectos socioeconómicos y la percepción asociada al tratamiento antihipertensivo por pacientes de un ambulatorio de atención primaria de Macapa, Brasil

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Sr. Editor,

La hipertensión arterial sistémica (HAS) tiene alta prevalencia, se caracteriza por niveles elevados y sostenidos de presión arterial (PA) igual o superior a 130/80 mmHg[1], é un factor de riesgo y una de las causas de óbito directa o indirecta en el 50% de los pacientes debido a enfermedad cardiovascular[2]. Se estima que cerca del 30% de los brasileños son hipertensos, un promedio de 63,9 millones de personas con esta condición.[3] De esta forma los diferentes condicionantes que conllevan a su control, tales como factores socioeconómicos o bien la percepción de los pacientes, deberían ser investigados de modo a alcanzar una mejor respuesta clínica.

Sendo un de los medicamentos antihipertensivos más utilizados en Brasil, la dosis de losartán es de 50 mg/día y los efectos adversos relacionados con su clase son poco frecuentes y, por esta razón, la mayoría de los usuarios los tolera bien[4].

Este estudio tiene el objetivo de identificar y relacionar el perfil socioeconómico con a HAS y la percepción de pacientes respecto a la farmacoterpia antihipertensiva losartán. Es un estudio transversal, descriptivo, realizado con 40 pacientes hipertensos usuarios de losartana como farmacoterapia principal, atendidos en un ambulatorio de atención primaria de la ciudad de Macapa, ubicada a la orilla del Rio Amazonas, mediante encuesta domiciliar directa bajo consentimiento esclarecido, entre agosto de 2019 y febrero de 2020.

Las mujeres fueran la mayoría de los usuarios entrevistados (65%), en gran parte con más de 60 años (55%). El nivel de instrucción más pronunciado fue la educación

primaria incompleta (45%), seguido del no escolarizado (22,5%). Únicamente el 10% de los entrevistados afirmaron tener un trabajo formal, mientras la mitad son los únicos proveedores familiares, con una renta promedia de 1-3 sueldos mínimos mensuales y cohabitando con 4 o más personas (Tabla 1). De esta manera, se evidencia tanto una instrucción deficitaria, como un bajo poder adquisitivo vivenciado por estos pacientes.

Un panorama semejante ya ha sido evidenciado en otro estudio que han descrito la influencia del nivel socioeconómico en disfunciones fisiológicas como la HAS, una vez que estos aspectos impactan negativamente en la calidad de vida los pacientes[5].

Cuando cuestionados respecto a la actuación de losartán contra la hipertensión, primeramente, se pudo notar dificultad en la contestación, una vez que muchos no supieron relacionar el funcionamiento a la enfermedad (por no experimentaren síntomas específicos), dando poca importancia al tratamiento, y no interactuando activamente en su autocuidado, sobretodo condicionado por su bajo nivel de instrucción.

En este estudio, seleccionamos un único fármaco, dispensado gratuitamente en la atención primaria, de modo a obtener una experiencia terapéutica más, no condicionada al acceso, formas farmacéuticas o incluso posologías diferentes. Por otro lado, debido a la presencia de supuestos síntomas relacionados a la elevación de la presión arterial, el 32,5% de los entrevistados (Tabla 1) ha afirmado que el antihipertensivo no funcionaría de modo regular e incluso no les valdría para controlar la PA.

El descontrol de la hipertensión estaría relacionado a la edad y al nivel de instrucción[6], dos variables identificadas en el presente estudio. La realidad vivenciada por los entrevistados: situación laboral precaria, alta responsabilidad familiar y bajos ingresos puede impactar negativamente en descontrol de la PA.

En paralelo a la edad avanzada y al bajo nivel de educacional, el hecho de no recibir instrucción adecuada, de no tener la PA medida regularmente y no aclarar sus dudas en las consultas, a menudo imposibilitadas por su dificultad de desplazamiento, podría comprometer muchos de estos tratamientos antihipertensivos, los cuales podrían estar siendo incumplidos o bien cumplidos con poco conocimiento y convicción, impactando negativamente en la percepción asociada al tratamiento.

Estrategias de información adecuada y atendimiento individualizado a estos pacientes serian formas de mejorar la percepción y autocuidado frente al tratamiento antihipertensivo.

Registro ante el Comité Local de Investigaciones en Salud nº 18337719.8.0000.0003.

Financiación

Este estúdio fue financiado en parte por la Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) –FinanceCode 001 y por la Fundação de Amparo a Pesquisa do Estado do Amapá/Conselho Nacional de Desenvolvimento Científico e Tecnológico (Edital PPSUS).

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Tabla 1 – Características socioeconómicas y clínicas de los pacientes hipertensos usuarios de losartana

	N	%
SEXO		
Femenino	26	65
Masculino	14	35
RANGO DE EDAD		
< 45 años	8	20
45-59 años	10	25
≥60 años	22	55
ESCOLARIDAD		
No escolarizado	9	22,5
E. primaria incompleta	18	45
E. primaria completa	5	12,5
E. secundaria incompleta	2	5
E. secundaria completa	2	5
E. universitaria incompleta	1	2,5
E. universitaria completa	3	7,5
OCUPACCIÓN		
Jubilado/Pensionista	15	37,5
Trabajo formal	4	10
Trabajo informal	14	35
No informado	7	17,5
RESPONSABILIDAD FAMILIAR		
Unifamiliar	20	50
Matrimonió/pareja	8	20
Matrimonió y otros familiares	12	30
RENTA FAMILIAR (en sueldos mínimos)		
< 1	10	25
1-3	20	50
3-4	2	5
4-10	7	17,5
> 10	1	2,5
NÚMERO DE CO-HABITANTES		
1	4	10
2-3	15	37,5
4-5	11	27,5
≥6	10	25
PERCEPCIÓN RESPECTO A LA LOSARTANA		
Positiva	27	67,5
Neutral	7	17,5
Negativa	6	15

A ser submetido ao Journal of Molecular Medicine

Metabolomic profile and its correlation with the plasmatic levels of losartan, EXP3174 and blood pressure control in hypertensive and chronic kidney disease patients

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Abstract

Systemic arterial hypertension (SAH) is one of the most prevalent chronic diseases worldwide, it presents itself silently and can cause serious complications. Losartan (LOS) blocks all the relevant physiologic aspects of hypertension, acting mainly on the reduction of peripheral vascular resistance. The complications of hypertension include nephropathy, in which the diagnosis is based on the observation of functional or structural renal impairment. Therefore, blood pressure control is essential to attenuate the progression of chronic kidney disease (CKD). In this paper, hypertensive and CKD patients´ ^{1}H NMR metabolomic profiles have been identified. The plasmatic levels of LOS and EXP3174, obtained by HPLC coupled with mass-mass spectroscopy were correlated to the blood pressure control, biochemical markers and to the metabolomic fingerprint of the groups. Several biomarkers could be identified from the plasma samples of hypertensive and CKD-hypertensive patients and used for phenotyping. Also, some of those were correlated to key aspects of hypertension and CKD progression. A trigoneline, urea and fumaric acid, found high, were considered characteristic markers of renal failure. In the hypertensive group, the urea levels found could indicate the onset of kidney injury, when associated with the lack of blood pressure found. Accordingly, the results found in this study point for a new approach to early identify CKD and also to monitor the responses to hypertensive treatments with LOS. These findings can contribute to improve the pharmacotherapy and reduce the morbimortality associated with hypertension and CKD.

Keywords: Hypertension, chronic kidney disease, metabolomics, losartan, EXP3174, plasmatic.

1. Introduction

Systemic arterial hypertension (SAH) is one of the most prevalent chronic diseases worldwide, representing about 30% of the population. During the early stages it presents itself silently, while the progression may cause serious complications. Elevated and sustained levels of blood pressure (BP) are the signs for diagnosis and continuous treatment is used to control and reduce complications [1,2].

The antihypertensive drug losartan (LOS), an angiotensin 2 receptor antagonist, blocks all relevant physiological mechanisms of hypertension, acting mainly on the reduction of peripheral vascular resistance [3]. Its half-life is increased when biotransformed into the active metabolite EXP3174 from 6 to 9 hours, which potentiates the blockade of angiotensin II in vascular smooth muscle and its peak concentration between 1 and 3 hours, thus allowing studies to be carried out in this period [4].

LOS can be administered in daily doses of 25-100mg with good tolerance and few side effects, confirmed by different studies since the1980s [5].

Nephropathy is a cause and a major complication of uncontrolled arterial hypertension. The diagnosis is based on the observation of functional or structural renal impairment [6]. BPcontrol is essential to attenuate the progression of chronic kidney disease (CKD), as angiotensin plays an important role as a mediator of kidney impairment, emphasizing its actions beyond BP control [7].

Some risk factors are related to CKD, such as age over 50 years, genetic predisposition, male gender, obesity, diabetes and its duration/stage and control of SAH. Some tests are recommended for monitoring kidney damage and determine the stage of CKD, such as conventional biochemical analysis, urinalysis and renal/urinary tract ultrasound [8]. Kidney dysfunction often causes systemic damages to the organism. For instance, the drugs´ pharmacokinetics may be altered by the renal disfunction characteristic of the disease, which may be further aggravated by the use of nephrotoxic drugs [9].

Metabolomics come as a novel approach to investigate and propose diseases mechanisms and progression, based on the metabolites´ pathways. This approach allows analyzing the drug *vs.* physiological relationship. More recently, the organism's responses to external stimuli, obtained from plasma samples and biomarkers have been used to

evaluate the effectiveness of pharmacotherapy in some individuals in a non-invasive way [10].

The characterization of pharmacokinetics, as well as nephrotoxicity through metabolomics have been investigated recently [11, 12], but there are still few studies bringing this approach to humans.

Accordingly, the present study aimed to identify the metabolomic phenotypes of hypertensive and CKD patients and correlate that to the plasmatic levels of LOS and EXP3174 and to the clinical outcomes.

2. Material andmethods

2.1 Study design

An observational, case-control study, with a quantitative approach and spontaneous sampling of sixty-seven adult patients was carried out. After the informed consent was signed, blood samples were obtained from hypertensive (n=22)/CKD-hypertensive (n=19) patients. Healthy volunteers (n=26) were also enrolled as a control group (CTRL) for comparison. BP measurements were done immediately before the blood collections. A semi-structured questionnaire was used to inquiry the volunteers about their social, clinical profile (age, sex, other diseases and BP).

The HAS group was evaluated before (fasted) and 1.5 h after the administration of LOS and the CKD group was evaluated before the administration of LOS and before dialysis for those who were undergoing this treatment. This group was not evaluated after administration, due to the expected plasmatic drug loosening due to the dialysis and the difference between the administration regimens according to medical prescription. The CTRL group also only had a single collection (fasted). The BP levels were measured just before the blood collections. The hypertensive participants underwent supervised oral administration of a dose of LOS of 50 or 100 mg (according to their individual medical prescription) with 100 ml of water. The CTRL group only received the corresponding volume of water. Fresh blood samples were collected in a 5 mL heparinized tube. After 30 min at room temperature, the samples were centrifuged at 3,000 rpm for 10 min and the plasma was aliquoted into plastic cryogenic microtubes and stored at -40 °C for further biochemical analysis (urea, creatinine and C-reactive protein) and plasma quantification of LOS and EXP3174. The study was approved by the Research Ethics Committee of Federal University of Amapa (CEP/UNIFAP) under CAAE nº 18337719.8.0000.0003.

2.2 Sample preparation for UHPLC-ToF-MS plasmatic quantification

A previous method validated by our group was used to determine the plasmatic levels of LOS and EXP3174 [13], according to ICH Q2 (R1) guidelines [14], including analytical and bioanalytical steps. The parameters linearity, precision (repeatability), accuracy, limit of quantification and detection, robustness, selectivity and recovery were determined for the analytical stage [13], while linearity, precision (repeatability), accuracy, limits of quantification/detection and ice-thaw cycles in enriched human plasma were used to validate the proposed method for therapeutic monitoring of LOS and EXP3174.

Analytical standards of LOS, losartan acid (EXP3174) and the internal standard (IS, irbesartan) (Synfine®, Canada) were used.

The samples were analyzed in an Ultra High Performance Liquid Chromatograph (Agilent), coupled to aTOF mass spectrometer (Bruker), with chemical ionization at atmospheric pressure (APCI)and atime-of-flight analyzer (TOF) in positive mode.

Sample preparation before analysis followed protein precipitation protocols. The plasma obtained from the patients was spiked with the internal standard irbesartan. The analytes were extracted from 300 µL of plasma, which was vigourousmixed with 340 µL of acetonitrile (HPLC grade) and centrifugatedat 3,000 rpm for 10 minutes at 4°C. The supernatant was filtered in a PTFE syringe filter (0.22 µm) and injected into the equipment.

2.3 Sample preparation and acquisition of ^{1}H NMR spectra

The samples for metabolomics analysis were prepared according to the method used by Beckonert et al and Dona et al [15, 16]. An aliquot of 300 µL of plasma and 300 µL of bibasic sodium phosphate heptahydrate buffer ($Na_2HPO_4 \cdot 7H_2O$) were homogenized and centrifuged at 10,000 rpm for 10 min. The supernatant was used to obtain the ^{1}H NMR spectra (Varian Inova 750, Varian ®, USA).

Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence experiments were performed with 154 scans, a total spin-spin relaxation time of 2.5 s, spectrum size of 32 K, acquisition time of 1.95, a spectral width of 8389.26 Hz, 90° for the rotation angle of the radiofrequency pulse and a line amplification factor of 0.3 Hz. All spectra were acquired at 27°C using a sequence solvent pre-saturation pulse, to suppress the water signal, and referenced withtrimethylsilylpropanoic acid -TSP.

The ^{1}H-NMR spectra were aligned, baseline corrected, normalized based on the total spectral area and divided into 81 bands of equal width [(Δδ) 0.04 ppm], between 0.8 and 8.5

ppm using Mestre Nova® software version 12.0. The spectral region comprised between 4.7 and 5.2 ppm was removed due to variability in the suppression of the water signal. A representative spectrum of the classification was loaded in ChenoMX NMR Suite software v8.5 (Chenomx, Inc.) and confirmed by the human metabolome database (HMDB), if found in human plasma, and categorized in the metabolomine, if common or uncommon in the MRI bank plasma metabolism (BMRB) and related literature. Receptor operating characteristic (ROC) analysis was performed to assess the diagnostic accuracy of each candidate biomarker and a panel of predictive biomarkers that showed the highest accuracy was presented based on combinational ROC analysis. A heat map methodology was also used to correlate the metabolites to the analytical and relevant clinical aspects.

2.4 Metabolicpathwayanalysis

To assess the biological roles of the identified metabolites and identify overrepresented pathways in the metabolite list, we used the web-based Metaboanalyst 3.0 (https://www.metaboanalyst.ca/) and the Metabolic Biological Role (MBRole) (https: //csbg.cnb.csic.es/mbrol e2/). These are on-line platforms for metabolite annotation enrichment analysis that calculate the p-values with the cumulative hypergeometric distribution by comparing the number of compounds in the set and in the background with a given annotation [17]. Values of $p < 0.05$ were considered significant. The Human Metabolome Database, Biological Magnetic Resonance and Bruker Biofluid Reference Compound Database Library and the Metaboanalyst 4.0 software were used to confirm the identity, the metabolomic pattern and its correlation with the pharmaco-toxicological parameters of LOS and its active metabolite.

Although the studies of He et al [11] and Uehara at al [12] do not explore a pathway, the remarkable metabolites were also used as a reference for correlating with the pharmacokinetic and clinical aspects: LOS and EXP3174 plasmatic levels, BP values and eventually any sign of renal injury, given the limited number of studies addressing these issues.

2.5 Statisticalanalysis

The data obtained from the questionnaires were tabulated using Microsoft® Excel 2010 for Windows and submitted to descriptive analysis. Plasma levels were assessed in pairs and submitted to analysis of variance (ANOVA) followed by Tukey's post-test. The numerical values obtained from the metabolomic analysis were processed by the orthogonal

signal correction technique (OSC) [18] and also analyzed with the Metaboanalyst 3.0. The O-PLS model was applied to correlate NMR data with biomarkers´ identification.

Two types of multivariant statistical analysis (MSA) methods were used for the NMR bucket data. The first method is the unsupervised principal component analysis (PCA) which was used for the detection of anomalous or outlier samples that have to be discarded because they do not satisfy the quality criteria, possibly due to errors during sample collection, preparation, storage, and/or NMR measurement. The NMR data of the samples not discarded by PCA were subsequently analyzed by the MSA method of Orthogonal-Partial Least Squares- Discriminant Analysis (OPLS-DA). Based on their pattern of intensities, those spectral buckets that best discriminate between groups provided the highest values of VIP scores and loading factors in OPLS-DA. A random test with 2,000 permutations was performed with the derived OPLS-DA model to confirm the robustness of the method. Cross-validation was applied to the OPLS-DA model obtained to determine its accuracy and to derive the confusion matrix of the classification achieved.

Univariant analysis was carried out to study the signal intensity of selected NMR buckets with the highest VIP scores and loading factors found in the MSA calculation by OPLSDA. They are the buckets with the maximum relevance for the group classification achieved by the MSA method. The selected buckets were those with a loading factor comprised in a range from the maximum down to 50% of this value. The distribution of NMR intensities of each one of the selected buckets was represented as Box-plots and the p value of the distribution was calculated with the unpaired T-test. The normality of the data was tested by the Kolmogorov-Smirnov normality test. If data underwent Gaussian distribution performed with Welch's correction, assuming standard deviation was not equal, or otherwise, the Mann-Whitney test was used.

Metabolite assignment was carried out for each NMR bucket that satisfied the same criterion of selection of the univariant analysis, to identify plausible candidates for biomarkers of the disease. The experimental signal profile and intensity at the chemical shift of the bucket were then matched to the ^{1}H patterns of the 750 MHz metabolite ChenoMX® NMR Suite software v8.5database. A metabolite candidate is obtained when the bucket chemical shift, intensity, and signal profile (i.e., pattern of J couplings) can be matched to a given signal of a metabolite in the spectral database, and simultaneously, other additional ^{1}H peaks of the metabolite match or are compatible with the experimental spectrum. Only those metabolites with favorable matching and that could be assigned unambiguously were reported.

3. RESULTS

Participants' characteristics

Sixty-seven adult volunteers were recruited for this study. The case-type patients were divided into subgroups: HAS - 22 hypertensive patients using LOS exclusively, CKD - 19 hypertensive and nephropathic patients using LOS exclusively, being 5 renal outpatients (CKA) and 14 undergoing hemodialysis treatment (CKN) Twenty-six control volunteers were also included in the study.

Blood samples were collected from fasting volunteers before LOS administration and 1.5h after administration in the HAS group. Patients´ sociodemographic and main clinical characteristics are presented in Table 1.

Table 1 Demographic and main clinical characteristics of the study participants (mean ± SD).

Variable	**CTRL (*n* = 26)**	**HAS (*n* = 22)**	**CKD *(n= 19)***
Age (years)	43.9 ± 10.5	54.5 ± 11.6	54.2 ± 11.2
Female gender (%)	61.54	45.45	50.75
Others diseases (%)	0.0	9.09	52.63
Systolic blood pressure (mmHg)[1]	116 ± 9.6	B:143 ± 18.0 A:138 ± 18.7	164 ± 27.0
Diastolic blood pressure (mmHg)[1]	75 ± 8.5	B:91 ± 11.0 A:83 ± 11.7	86 ± 18.8
Patients with plasmatic levels >EC50 (%)[a]		50.0	21.05
Creatinine (mg/dL)[2]	0.8 ±0.2	0.9±0.2	10.2±5.2
Urea (mg/dL) [2]	23.8±5.5	83.5±30.8	109.1±34.2
PCR (mg/dL) [2]	1.8±3.4	2.0±3.1	3.6±3.9

CTRL= controle group, HAS= hypertensive group, CKD= nephropathic group. [a]efficiency concentration (EC_{50}) of losartan is 32 µg/L [19]. *B: before and A: after.* [1]Reference for normal blood pressure values: Systolic pressure ≤120 mm Hg and diastolic pressure ≤80 mm Hg. [2]Complementary tests for biochemical comparison between studied groups. Biochemical markers reference values for adults. Creatinine: 0.40 - 1.30 mg/dL; Urea: 15 - 45 mg/dL; C - reactive protein: ≤6.0 mg/L.

Patients were found to be adults, majorly women. HAS and CKD patients presented high values of BP before the administration of LOS, classified as high and very high, respectively. Moreover, the EC_{50} of losartan was reached by 50% of the HAS patients and limited to 21.05% among the CKD patients.

In a complementary analysis, the BP findings demonstrate values above the normal clinical levels. The HAS group was evaluated before and 1.5 h after the administration of

LOS and the CKD group before the hemodialysis, for those who undewent this treatment and before the administration of LOS. Creatinine and urea were found to be critically above normal values in the CKD group, while HAS group also presented values of urea (83.5 mg/dL) much above the reference range. Control patients presented all the biochemical parameters within the reference range.

Analytical and bioanalytical validation

The method validation for plasma quantification of LOS and EXP3174 was successfully acomplished. The parameters found in the validation of LOS and EXP3174 are displayed in Table 2.

Table 2UHPLC-ToF-MS Losartan and EXP3174 determination in acetonitrile HPLC grade (analytical method) and human plasma matrix (bioanalytical method) validation.

Losartan - Validationparameters µg/mL	**Analytical validation**		**Bioanalyticalvalidation**		
Linearity (n = 3)		R^2: 1.00000		R^2: 0.99689	
Sensitivity		y = 2*107x –9021.5		y = 0.5862x + 2.641	
		LoQ:0.005 LoD: 0.002		LoQ: 0.005 LoD: 0.0005	
		% ± SD		% ± SD	
Accuracy (n = 9)	[1.51] [0.75] [0.025]	92.8 ± 0.05 102.69 ± 0.03 99.4 ± 0.0007	[0.75] [0.15] [0.03]	81.33 ± 0.20 86.67 ± 0.05 133.3 ± 0.06	
		Mean ± %CV		Mean ± %CV	
Precision (n= 9)	[1.51] [0.75] [0.025]	1.39 ± 4.22 0.770 ± 4.89 0.025 ± 3.08	[0.75] [0.15] [0.03]	0.61 ± 0.20 0.13 ± 0.05 0.17 ± 1.33	
		%Change			
Robustness (n = 3)	[0.75][a] [0.75][b]	-5.26 -14.57			
				% after 8h	% after 24h
Ice-thaw cicle			[0.75]	127.16	107.42
			[0.03]	127.90	114.32
EXP3174 - Validationparameters µg/mL	**Analytical validation**		**Bioanalyticalvalidation**		
Linearity (n = 3)		R^2: 0.9966		R^2: 0.99843	
Sensitivity		$y = 1*10^7x – 257159$		y = 0.1825x + 0.09865	

		LoQ: 0.01 LoD: 0.004		LoQ: 0.005 LoD: 0.002	
		% ± SD		% ± SD	
Accuracy (n = 9)	[2.775] [0.925] [0.278]	91.58 ± 0.04 89.42 ± 0.02 88.85 ± 0.001	[0.925] [0.185] [0.037]	82.16 ± 0.23 91.89± 0.08 108.11 ± 0.10	
		Mean ± %CV		Mean ± %CV	
Precision (n= 9)	[2.775] [0.925] [0.278]	2.541 ± 1.59 0.827 ± 3.29 0.247 ± 0.50	[0.925] [0.185] [0.037]	0.76 ± 0.23 0.17 ± 0.08 0.04 ± 0.10	
		%Change			
Robustness (n = 3)	[0.925[a]] [0.925[b]]	-6,80 -10,8			
				% after 8h	% after 24h
Ice-thaw cicle			[0.925] [0.037]	124.21 111.41	89.79 104.25

[a]Acetronitrile HPLC supra gradient grade, [b]non-agitated.

Bioanalytical validation (Table 2), based on ICH Q8 (R2), confirmed the analytical adequacy. The calibration curves obtained from the bioanalytical phase showed a strong linear relationship, with an R^2 values greater than 0.99 for both analytes.

The method also reached satisfactory precision, with CV values between 0.05 and 4.89% for losartan and EXP3174 in analytical and bioanalytical validation. Accuracy values ranged from 81.33 – 133.3 %. The freeze-thaw cycle used to check the stability of the analytes under termic stress conditions resulted in recoveries from127.16% to 127.90% after 8 hours and 107.42% to 114.32% after 24 hoursfor LOS and111.41% to 111.41% after 8 hours and from 89.79% to 104.25% after 24 hours for EXP3174. Very low detection and quantification limits of Losartan (LoD 0.0005µg/mL, LoQ 0.005µg/mL) and EXP3174 (LoD 0.002µg/mL, LoQ 0.005µg/mL) were found (Table 2), confirming the high sensitivity of the method used for human plasma quantification.

According to the patient's plasma analyses, the HAS group did not reach therapeutic levels of LOS and EXP3174 neither before nor 1.5h after the oral administration of LOS tablets (Table 3). This aspect is not very relevant for EXP3174, as its C_{max} is expected to occur up to 3 hours after administration [4].

The plasmatic levels of LOS and EXP3174 found in CKD patients, both outpatients (CKA) and undergoing dialytic treatment (CKN) (Table 3) were also outside the therapeutic range of LOS and EXP3174, except for a single patient (A_4) who presented bioaccumulation

of LOS, probably associated with any type of metabolism disorder; as it was not converted into EXP3174.

Table 3 Plasmatic levels of Losartan and EXP3174 of hypertensive and CKD volunteers.

PATIENT SAMPLE	**LOSARTAN (ng/mL)**		**EXP3174 (ng/mL)**	
HAS	Before administration	Afteradministration	Before administration	Afteradministration
H_1	-	28	4	36
H_2	-	44	4	23
H_3	-	6	15	11
H_4	-	32	24	44
H_5	-	38	7	12
H_6	-	24	-	22
H_7	-	36	-	11
H_8	-	20	-	6
H_9	-	27	4	20
H_{10}	29	-	56	11
H_{11}	-	40	-	7
H_{12}	-	41	-	114
H_{13}	-	-	-	-
H_{14}	-	50	13	59
H_{15}	-	36	-	7
H_{16}	-	76	7	24
H_{17}	-	82	4	6
H_{18}	-	31	1	5
H_{19}	-	-	-	-
H_{20}	5	89	105	222
H_{21}	-	-	-	-
H_{22}	37	35	78	168
CKD	Before administration	Afteradministration	Before administration	Afteradministration
Outpatients (CKA)				
A_1	145	2	234.8[1]	9
A_2	1	2	99	121
A_3	148	3	91	13
A_4	291[1]	13	9	7
A_5	19	-	13	12
Dialytic[2](CKN)				
N_1	-		-	
N_2	-		-	
N_3	-		8	
N_4	-		13	

N_5	126	19
N_6	-	-
N_7	-	-
N_8	-	24
N_9	-	28
N_{10}	-	3
N_{11}	15	75
N_{12}	-	-
N_{13}	-	-
N_{14}	9	96

Results were calculated using the calibration curve and expressed as mean (in ng/mL). Therapeutic range of losartan: 200 – 650ng/mL and EXP3174: 200 – 1200ng/mL; - Not detected; [1]reached the therapeutic window; [2]a single blood collection was performed 24h after drug administration in nephropathic patients undergoing hemodialysis.

NMR metabolomics

NMR metabolomics using both fingerprint and targeting analysis [16, 20] of ^{1}H_T2 was used. Potential biomarkers related to HAS and CKD were searched. The CTRL group was used as reference.

In view of that, the results demonstrating significance after satisfying the spectral quality criterion based on PCA and leading to favorable separation of the groups by MSA and univariate analysis were selected to access first the differences between the three groups and also in pairs. The HAS and CTRL groups were not paired compared, as they did not differ in any buckets, while the group CKD was analysed in the two subgroupds CKA (outpatients) and CKN (undergoing hemodialysis) to acess the differences found in different stages of the renal disease.

NMR of CTRL *vs.* HAS *vs.* CKD groups

The distribution of the PCA score plot for the three groups (Fig. 1) is highly compact and relatively random, with no obvious sample outliers to consider. OPLS-DA analysis found two regions in the spectrum (buckets) with the maximum discrimination power corresponding to urea and trigonelline metabolites (Fig. 1c). The score chart in Fig.1b shows the overlapping between HAS and CTRL groups in parallel with a remarkakbleseparation of CKD group without overlapping and the axes of the 2D scores cover 25.4 and 20.6% of the total variability.

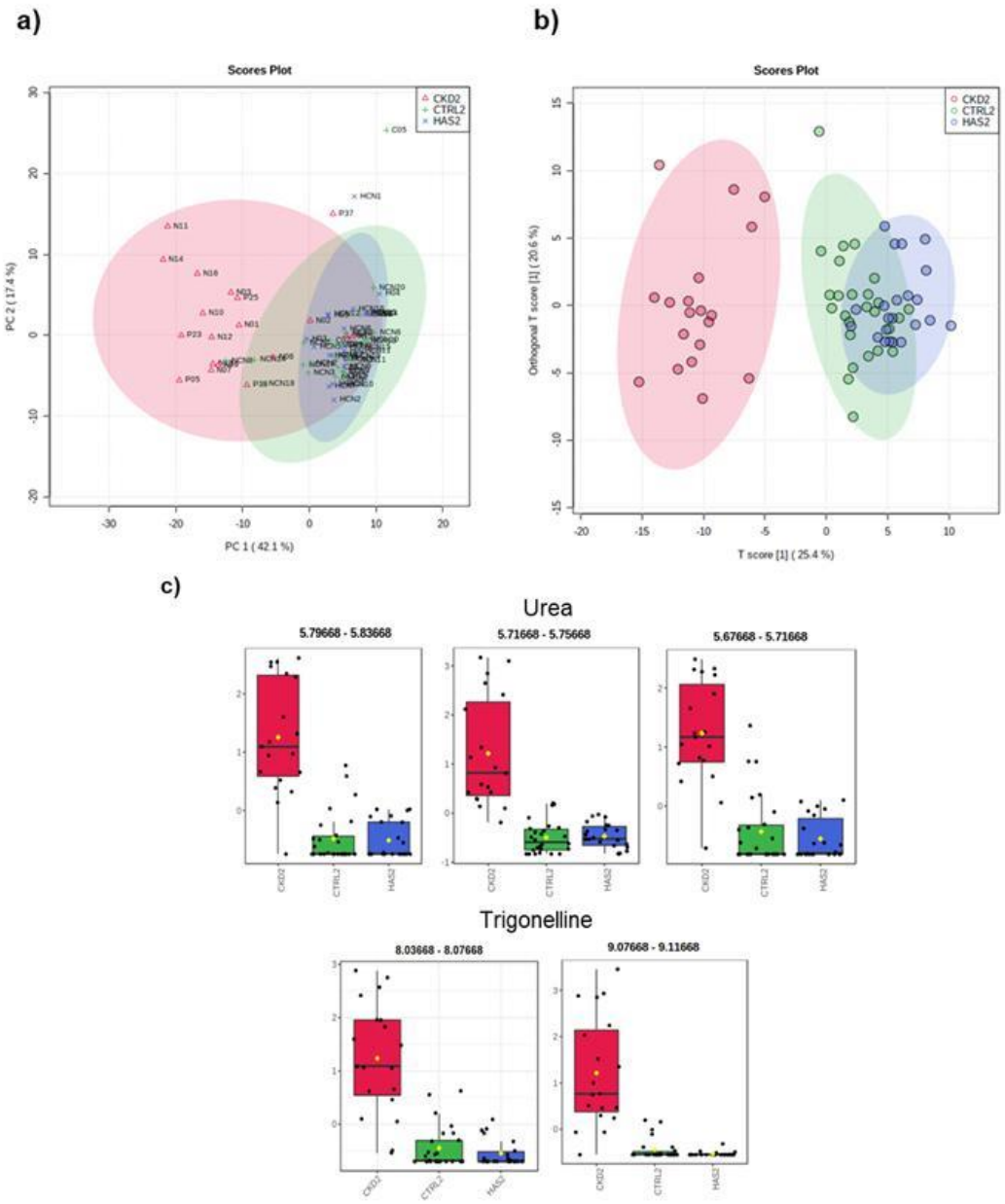

Fig. 1 Statistics of targeted analysis of $^1H\_T_2$ spectra of plasma samples from control group (CTRL, n = 26) *vs*. hypertensive patients (HAS, n=22) and hypertensive patients with renal disease (CKD, n = 19). a PCA score plot. b OPLS-DA score plot. c Bar graphs of the normalized peak intensities of the identified metabolites with differences between the three groups. Their bucket integrals in the spectra were considered relevant for the OPLS-DA classification obtained in b.

In the analysis of the buckets, two metabolites were identified as the most relevant of OPLS-DA: urea and trigonelline. Univariant analysis of the NMR integral in the buckets corresponding to these metabolites in Fig.1c and Table 4 indicate a significant elevation in both metabolites for CKD patients. Trigonelline is an alkaloid product of niacin metabolism, also found in the composition of coffee and teas. Its presence may be related to bioaccumulation after those beverage consumption.

Table 4 Relevant metabolites identified by NMR metabolomics in CTRL vs. HAS vs. CKD on plasma samples.

METABOLITE (Bucket)	HMDB ID	CKD	HAS	CTRL
Urea(5.79668-5.83668)	HMDB0000294	High	Intermediary	Low
Urea(5.71668-5.75668)		High	Low	Intermediary
Urea(5.67668-5.71668)		High	Intermediary	Low
Trigonelline(8.03668-8.07668)	HMDB0000875	High	Low	Intermediary
Trigonelline(9.07668-9.11668)		High	Low	Intermediary

NMR of CRTL vs. CKD groups

The distribution of the PCA score plot for CTRL and CKD groups (Fig. 2) shows considerable differences, emphasizing the peculiarity of the CKD group. The discriminative metabolites found were creatinine, lactate, glycine, trigonelline, choline, urea and fumaric acid. The score chart in Fig.2b shows the evident separation between CTRL and CKD groups, without overlaping, and the 2D score axes cover 24.5% and 23.4% of the total variability.

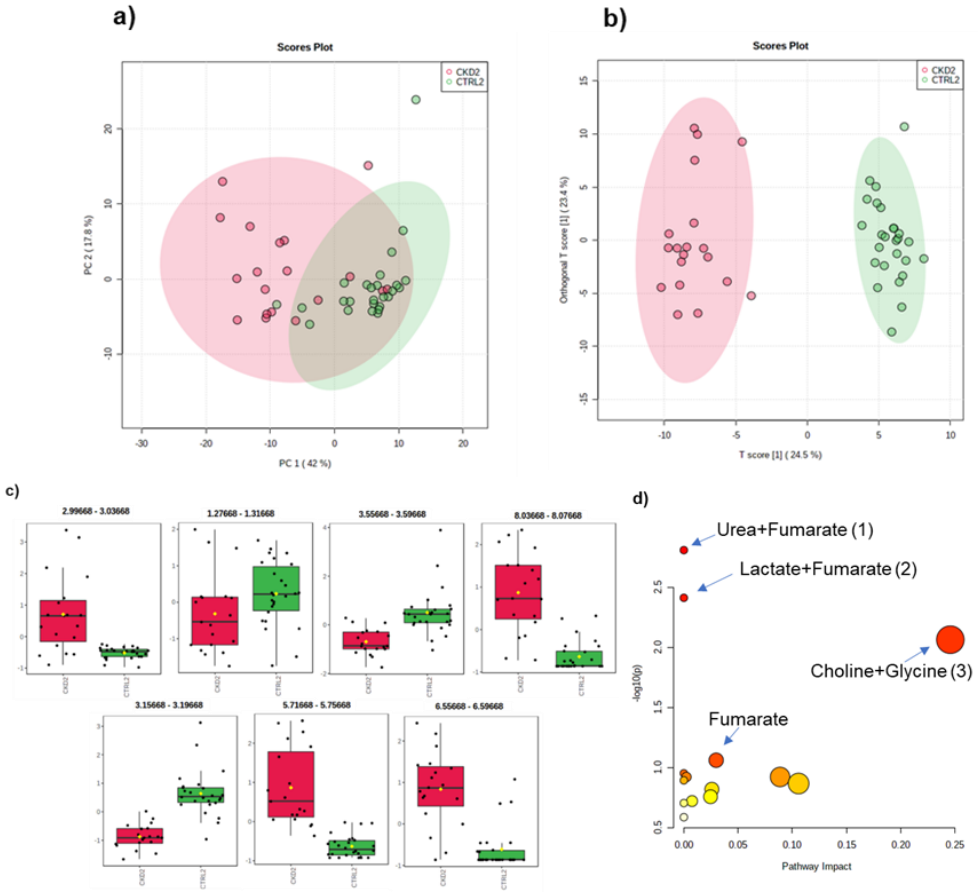

Fig. 2 Statistics from targeted analysis of $^{1}H\_T_2$ spectra of plasma samples from control group (CTRL, n = 26) (in green) vs hypertensive patients with renal disease (CKD, n = 19) (in red). a PCA score plot, b OPLS-DA score plot. c Bar graphs of the distribution of NMR intensities of identified metabolites with differences between the two groups in this order: creatinine, lactate, glycine,

trigonelline, choline, urea and fumaric acid. Their bucket integrals in the spectra were considered relevant for the OPLS-DA classification obtained in b. d Results of the metabolic topology pathway based on potential metabolites in the O-PLS models.

A total of seven metabolites selected as the most relevant were loaded for pathway analysis and hypergeometric testing. According to the pathway analysis, the metabolism of urea+fumarate (1) (p= 0.0015512; FDR= 0.1303) with the metabolic pathway of arginine biosynthesis; the metabolism of lactate+fumarate (2) (p= 0.0038701; FDR= 0.16254) with the metabolic pathway of pyruvate metabolism and the metabolism of choline+glycine (3) (p= 0.0086375; FDR= 0.24185) with the metabolic pathway of Glycine, serine and threonine metabolism were the three most significant pathways found between the CTRL and CKD groups (Fig. 2d).

NMR of HAS vs. CKD groups

The distribution of the PCA score plot for HAS and CKD groups (Fig. 3) indicate are markable separation. OPLS-DA found two regions in the spectrum (bucket) with the maximum discriminating power corresponding to creatinine, lactate, glycine, trigonelline, choline and urea metabolites. The separation evidenced by the score chart (see Fig. 3b) demonstrates the clear distinction between hypertensive patients and nephropathic patients. The 2D score axes cover 30 and 18.8% of the total variability.

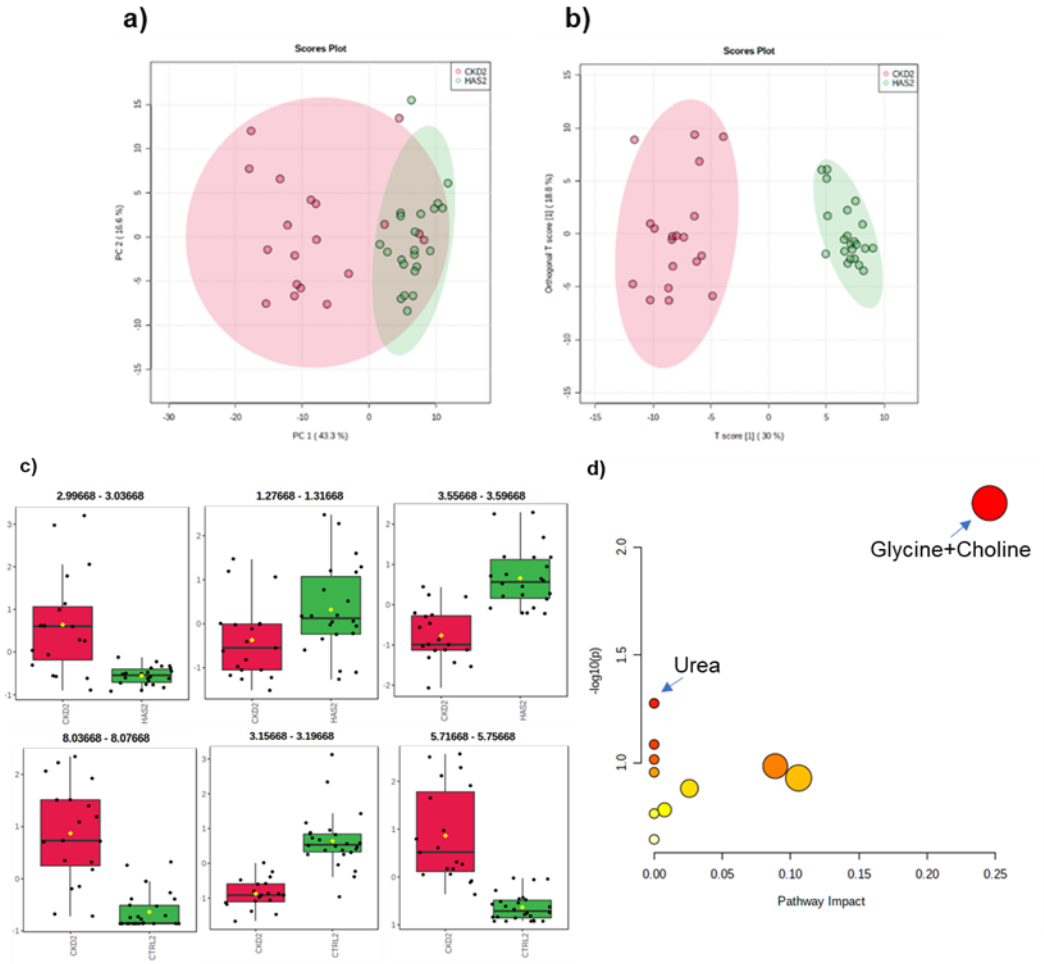

Fig. 3 Statistics from targeted analysis of $^{1}H\_T_2$ spectra of plasma samples from hypertensive patients only (HAS, n=22) (in green) and hypertensive patients with renal disease (CKD, n = 19) (in red). a PCA score plot, b OPLS-DA score plot. c Bar graphs of the distribution of NMR intensities of identified metabolites with differences between the two groups in this order: creatinine, lactate, glycine, trigonelline, choline, and urea. Their bucket integrals in the spectra were considered relevant for the OPLS-DA classification obtained in b. d Results of the metabolic topology pathway analysis based on potential metabolites in the O-PLS models.

A total of 3 metabolites selected as the most relevant were loaded for pathway analysis and hypergeometric testing. According to the pathway analysis, the urea metabolism (p= 0.053068; FDR= 1.0) with the arginine biosynthesis metabolic pathway and the glycine+choline metabolism (p= 0.0062527; FDR= 0.52523) with the Glycine, serine and threonine metabolism were the most significant pathways correlated between HAS and CKD groups (Fig. 3d).

NMR of CKA vs. CKN groups

The best MSA classification of the CKA and CKN groups was obtained with the directed analysis of the $^{1}H\_T_2$ spectra. The distribution of the PCA score plot for the two groups (Fig. 4a) is highly compact and relatively random, with no obvious sample outliers to

consider. The OPLS-DA found seven regions in the spectrum (buckets) with the maximum discrimination power corresponding to creatinine, lactate, glycine, glutamine, anserine, alanine, proline metabolites. The score plot in Fig. 4b shows excellent group separation without overlapping and the 2D score axes cover 15.4 and 32.2% of the total variability.

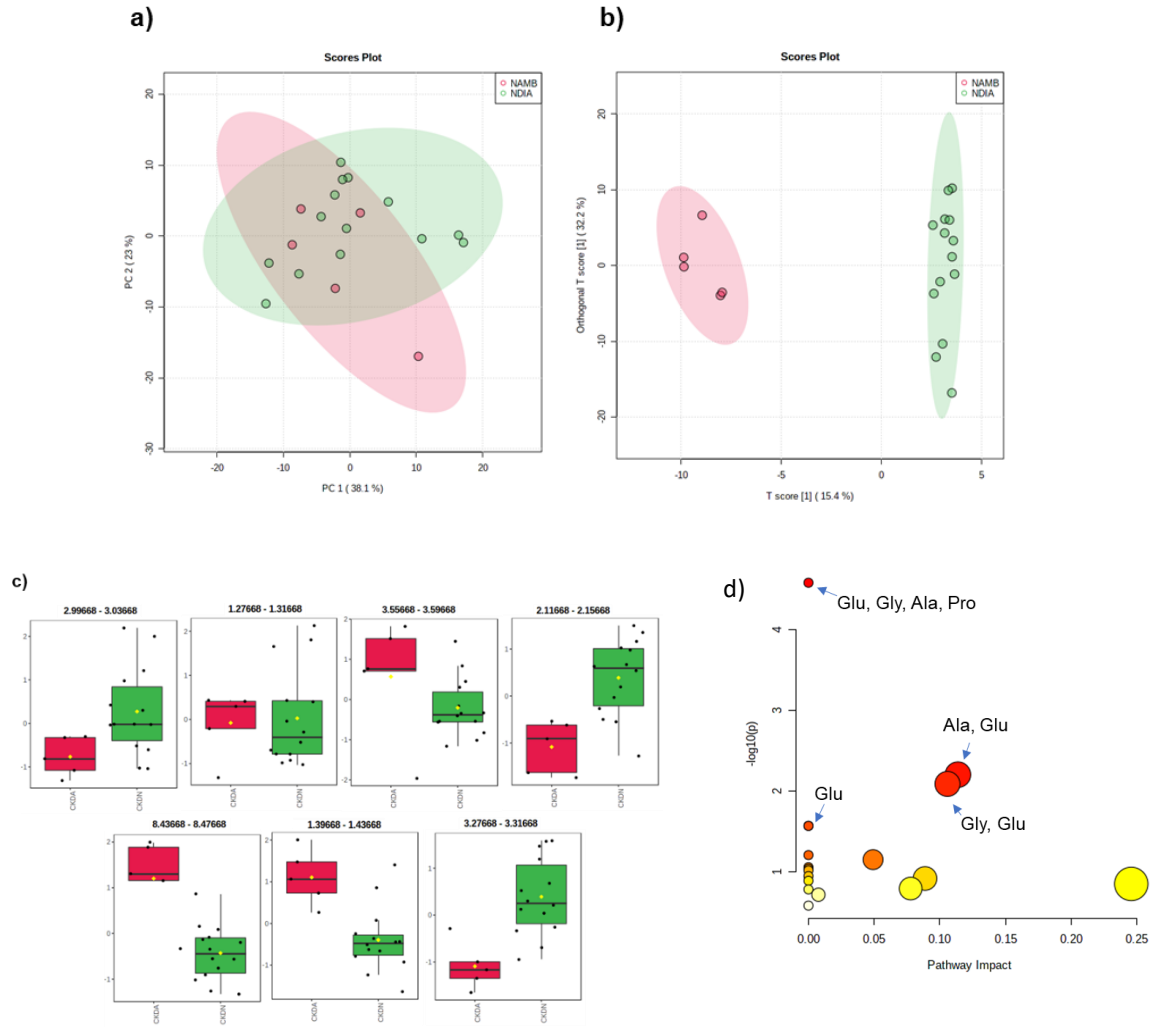

Fig. 4 Statistics from the directed analysis of $^{1}H\_T_2$ spectra. of plasma samples from hypertensive patients with renal disease in outpatient follow-up (CKA, n = 5) (in red) vs. Hypertensive patients with kidney disease undergoing hemodialysis (CKN, n=14) (in green). a PCA score graph, b OPLS-DA score graph. c Bar graph of the distribution of NMR intensities of the identified metabolites of the two groups in this order: creatinine, lactate, glycine, glutamine, anserine, alanine, proline. Their bucket integrals in the spectra were considered relevant for the OPLS-DA classification obtained in b. d Results of the metabolic topology pathway analysis based on potential metabolites between CKD subgroups CKA and CKN in the O-PLS models.

A total of 5 metabolites selected as the most relevant were loaded for pathway analysis and hypergeometric testing. According to the pathway analysis, the metabolism of glutamine+glycine+alanine+proline (p= 2.653E-5; FDR= 0.0022285) with the Aminoacyl-tRNA biosynthesis metabolic pathway; the metabolism of glycine+glutamine (p= 0.0081316;

FDR= 0.22768) with the metabolic pathway of metabolism of Glyoxylate and dicarboxylate; glutamine metabolism (p1=0.026836; FDR1=0.45084; p2=0.026836; FDR2=0.45084) with two metabolic pathways of nitrogen metabolism and D-glutamine andD-glutamate metabolism; and arginine+glutamine metabolism (p=0.006251; FDR=0.22768) with the metabolic pathway of alanine, aspartate and glutamate metabolism were the most significant pathways correlated within the CKD subgroups comparison (Fig. 4d).

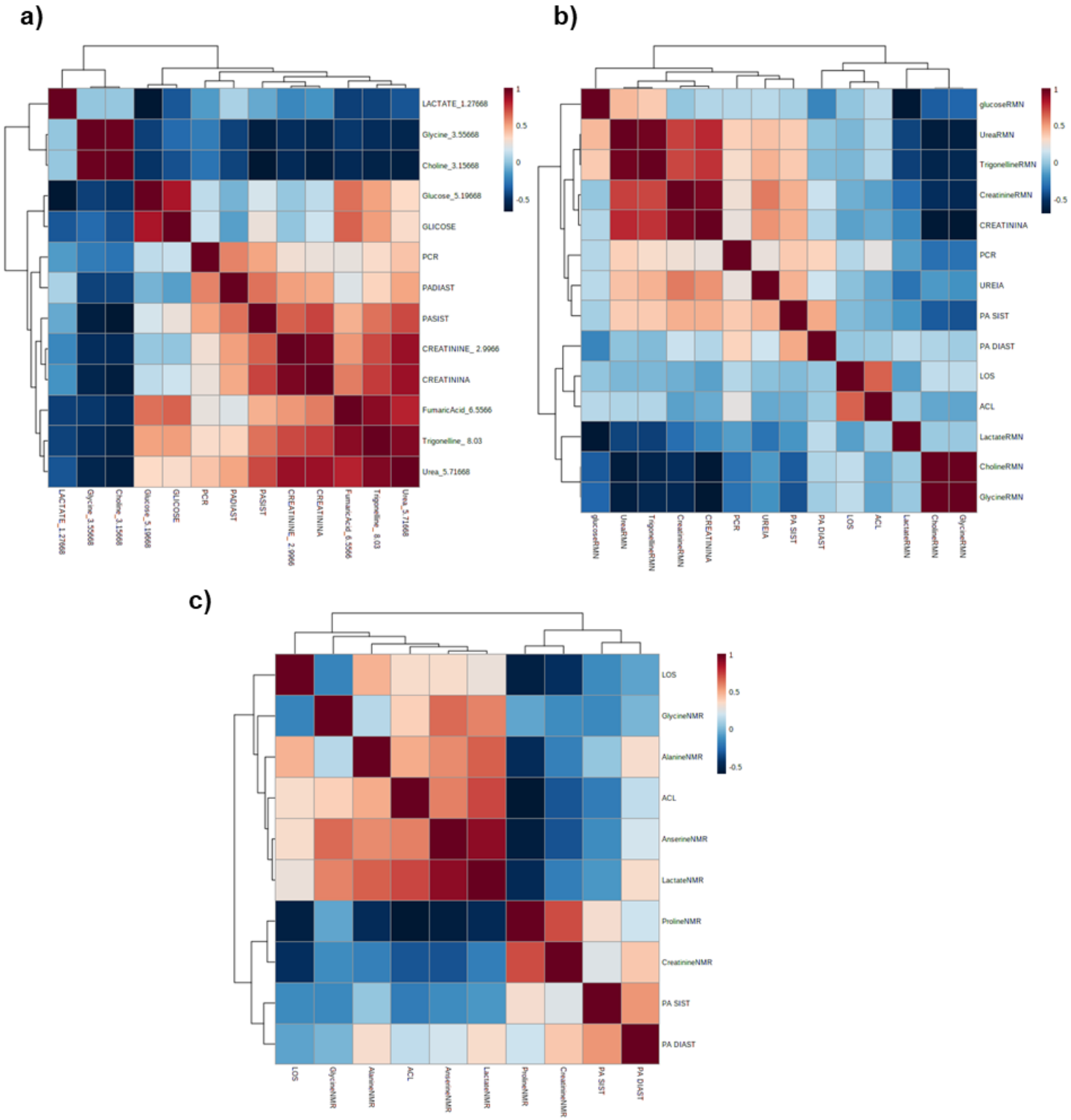

Fig. 5 Comprehensive profile of metabolites identified by CE-TOFMS analysis in plasma was analyzed by hierarchical clustering. Clustering method: UPGMA (unweighted average); similarity measure: Euclidean distance; sorting function: average value. The heat map shows the logarithmic ratio of the base 2 to the means of the corresponding compared groups. a Correlation heat map between the CTRL vs. CKD groups. b correlation between HAS vs. CKD groups. c correlation between CKA vs. CKN groups.

A heat map representation of the correlations made is shown in Fig. 5. The hierarchical grouping of metabolites biomarkers quantified in samples forthe groups in pairs correlated with clinical variables, such as BP, plasmatic levels of LOS and EXP3174 (ACL)

and relevant biochemical parameters.Some metabolites, such as creatinine and urea were found to have high levels in both biochemical analisys and metabolomics, validating these results. Nonetheless, others presented limited levels, such as glycine. A more detailed description of changes observed in the metabolome profile vs clinical aspects examined will be discussed below.

4. DISCUSSION

The determination of biomarkers was performed by ^{1}H NMR plasmatic metabolomics. The NMR data were correlated with biochemical parameters, BP controland plasmatic levels of LOS and EXP3174.

The target BP values varies according to the type of population, being different for hypertensive and non-hypertensive population. The control group presented BP levels of SBP <120 and DBP <80 mm Hg, while the BP of the other two groups (HAS and CKD) showed important changes. The therapeutic target of a hypertensive patient is BP ≤130/80 mm Hg, except for patients at high cardiovascular risk whose target is reduced to≤120/80 mm Hg [21].

After EC_{50} is reached, the BP lowering effects are visible. Nonetheless, only 50% of the patients in the the HAS group reached a satisfactory value for EC_{50} (Table 1), reflecting directly as in the elevation of BP found. This fact may be a risk factor for the onset of renal alterations not yet evident/diagnosedamong these patients. It is suggested the need for dose adjustment and monitoring for reanalysis of the plasmatic levels of losartan. In view of the tenuous relationship between hypertension and kidney disease, monitoring should always be considered an important preventive measure. Early identification of the onset of renal impairement is imperative, to treat in a conservative way. Confusing or silent clinical signs are commonat the early stages, what leads to a later diagnostic, where the CKDis already installed in a more advanced course [22]. Therefore, clinical laboratory tests are recommended for diagnosis and monitoring of both SAH and CKD diseases [23].

Regarding theCKD hypertensive patients, the scenario of goals and treatments are modified. The objective is to reduce BP in order to achieve a PASD <90 mm Hg. Some studies were unable to confirm that lower targets would reduce the risk associated [24].

CKD group had a mean BP of 164/86 mm Hg (Table 1). In addition, only 21.05% reached the EC_{50} of LOS (Table 1), which directly reflects in theuntargeting of PAS. Uncontrolled BP increases the coronary risk [25]. Therefore, more attention should be given to this group in view of the results found.

In association with these findings, the subtherapeutic levels of LOS and EXP3174 in this group (Table 2) may be associated with the elapsed interval (24h) between the administration of LOS and the blood collection, which would be within the normal range if the BP values were adequate. Therefore, dosage adjustment or association with another drug is suggested to adjust BP levels and the need to monitor this drug in renal patients is emphasized.

Regarding the biochemical parameters (Table 1), the CTRL group was found to be within the reference values. As for the HAS group, all parameters are within the normal range, except for the urea values (Table 1). One of the hypotheses for that is the early renal impairement, as hypertension is one of the most frequent causes of CKD, accounting for 34% of cases in 2017 [26]. Elevated BP, associated with arterial stiffness, leads to an increase in pressure in the renal irrigation arterioles, interfering with the renal autoregulation process, causing glomerular hyperfiltration and hyperperfusion, slowly deteriorating its functions [27]. Moreover, all the biochemical parameters evaluated were found to be altered in the CKD group (Tab. 1). Remarkably, the high plasmatic levels of creatinine and ureafound mark the chronic renal impairement [29] and evidence that despite the dialysis treatment, no significant reduction can be reached in those parameters.

In addition to these findings, the PCR results indicate that none of the groups studied was undergoing an acute infectious phase or inflammatory process, whose could interfere with the other variables studied.

Based in the comparisons between groups, some metabolites showed good correlation in the OPLS-DA analysis. When analyzing the three groups together, two metabolites showed greater relevance, urea and trigonelline, notably increased in the CKD group. Urea is directly related to CKD characteristics. Trigonelline presents itself as an innovative finding, associated with the kidney injury, given the high level found within the CKD group. Being a by-product of niacin (vitamin B3) metabolism, trigonelline is, after caffeine, the second largest alkaloid compound in raw coffee beans, being excreted unaltered in the urine [30, 31]. Human exposure to trigonelline occurs through diet, consumption of foods such as oats, potatoes and especially coffee, so it is also used as a biomarker of coffee consumption [30, 32]

Few reports associate this biomarker with injury and/or kidney disease. A study reported trigonelline as a urinary biomarker for renal ischemia and reperfusion in a swine model [33] and another as a marker of kidney damage caused by cisplatin in rats [34].

In our investigation, the increase in trigonelline levels in patients with CKD may be associated with its bioaccumulation from the consumption of foods rich in B3, such as coffee,

reaffirming previous studies of its association with kidney damage, and therefore, avaluable biomarker that could be used in early stages to determine renal impairement.

Urea is synthesized in the liver from ammonia, that comes from amino acids such as arginine. It is the main by-product of protein catabolization and is mostly excreted through the urine, being present in half of all solid products produced by urine [35]. In view of that, urea is another kidney misfunction marker.

In our study, when evaluating the three groups, urea was also present in higher concentrations in the CKD group. Despite not being as specific as creatinine, it is a crucial marker of the initial changes that cause pathologies, because it is more sensitive to primary changes in kidney conditions [36], confirming also its role as a biomarker of kidney injury.

The pathway of arginine and proline metabolism is altered in CKD, as the kidney is directly related to arginine biosynthesis [37]. In our investigation, proline was found at high levels in patients with CKD undergoing hemodialysis when compared to the renal outpatients, which may be a result of the accumulation of orythine (an intermediate of the urea cycle) in advanced CKD [38]. Proline also acts as a (weak) antagonist of glycine receptor and NMDA (N-Methyl D-Aspartate) and non-NMDA [39] ionotropic glutamate receptors.

Glutamine together with glutamic acid plays important roles in ammonia, nitrogen and protein metabolism, being a substrate for gluconeogenesis in the kidneys [40, 41]. In this study, glutamine was found to be increased in CKD dialytic patients. Being converted into glutamate in the kidney and via gluconeogenesis into pyruvate. The elevation found in this group may suggest bioaccumulation associatedwith the LOS treatment, which may be further aggravating indirectly theCKD due to the chain imbalance it may cause.

Pyruvate generated by glutamine chain reaction is further converted to alanine and is a direct participant in muscle energy production [39]. In this study, alanine was found reduced in the CKD dialytic group. This can be related to the bioaccumulation of glutamine, impairing the protein biosynthesis in this group. Anserine, also found at low levels in plasma, when broken down is also converted into alanine and similarly may correlate with low alanine levels [39].

The antihypertensive effect of losartan depends not only on its concentration, but also on the metabolites found in the plasma. In this study, the volunteer with the highest plasma levels of LOS was not the one with the highest levels of EXP3174, demonstrating how complex the human body is and emphasizing the difficulty in quantifying individual differences based on genetic variations solely.

Regarding the metabolomic analysis, no significant differencesin the biomarkers among the CTRL and HAS groups been found. In contrast,remarkable differences between the CKD group when correlated with the two other groups. Lactate was reduced in patients with CKD compared to CTRL and hypertensive patients. In the study of He et al [11], lactate demonstrated a positive correlation with the Cmax ratio achieved after LOS administration in non-hypertensive patients [11]. Higher levels of plasma lactate as well as a hyperglycemic response were also found in rats in which losartan was administered intravenously, suggesting that the angiotensin AT1 receptor may alter the energetic balance [42].

Creatinine was elevated in renal patients compared to the other groups. Also, in the study of He et al [11], this metabolite was found to have a negative correlation with AUC. It is assumed that the pharmacokinetic response of LOS, notoriously impaired in our findings, could be in part related or linked to plasmaticl evels of creatinine, which bioaccumulates due to the inherent characteristic of renal disease. Moreover, high serum creatinine levels would be related to poor survival and lower response to specific treatments in kidney disease [43, 44]. It has already been characterized as a marker of target organ damage and a predictor of cardiovascular risk in hypertensive patients [45, 46]. The higher is its value, the lower the conversion of LOS into EXP3174 [47]. Therefore, creatinine and lactate showed strong correlations with characteristics of LOS metabolism and kidney disease, corroborating the findings of He et al [11].

Comparing the levels of these two metabolites in the subgroups of patients with CKD (CKA - outpatients andCKN - undergoing dialysis) was also relevant. It is known that kidney damage caused by acute kidney injury can result in CKD. Elevated levels of creatinine, urea, sodium, and lactate at emergency admission are independent risk factors for death in patients with acute kidney injury [48], corroborating our findings, as a direct relationship was found between the degree of renal injury and the plasma levels of these metabolites.

Also in the study of He et al [11], the metabolism of glycine, serine and threonine were significantly associated with the AUC/Cmax ratio, with glycine and choline being the main negatively involved metabolites. In another study, an animal model of hypertension showed that regulation of glycine, serine and threonine metabolism occurs from the antihypertensive effect of the drug [49]. When evaluating cardiovascular risk, choline was found to be related to the increased risk of cardiovascular diseases [50], whereas glycine, which is a constituent of collagen and its antioxidant effects, was, in one study, inversely associated with hypertension [51].

When comparing these findings to our data, higher levels of choline and glycine were found in the plasma of hypertensive patients compared to CKD group. Therefore, it could

increase the cardiovascular risk of this group and might be affecting directly the metabolism of LOS. However, when comparing renal outpatients (CKA) with those on dialysis (CKN), glycine levels were higher in outpatients, which may suggest that the permanence of this variable may lead to the progression of the disease as it affects the metabolism of LOS.

Another relevant metabolite found was fumaric acid, which showed higher levels in patients with CKD compared to the CTRL group. This metabolite requires conjugation with glycine as a pathway for its excretion [39]. As glycine was found to be reduced in these patients, it may have contributed for the increased plasmatic level of this metabolite. Therefore, fumaric acid could also be an indirect biomarker of kidney disease.

In view of the results presented, it can be noted that NMR metabolomics allowed finding relevant biomarkers for significant differentiation among HAS and CKD groups, despite the reduced number of volunteers in the study. The analysis of these biomarkers can clarify different mechanisms and from another perspective regarding the groups mentioned, being useful to observe the correlation of biomarkers with the prediction, alterations and stage of hypertension and chronic kidney diseases. Though, it is important to emphasize that the proposal is made with groups in which some variables are not under control (real patients) and that the pathophysiology can vary, for instance the group with chronic kidney disease undergoing dialysis treatment. Population-based studies are suggested to confirm those findings and validating the aspects presented herein.

5. CONCLUSION

The NMR metabolomics study of plasma samples from hypertensive and hypertensive chronic kidney disease volunteers identified several biomarkers that distinguish the groups of patients. Remarkably, higher levels of trigonelline, urea and fumaric acid compared to the other groups were considered characteristic markers of renal impairement. For patients with systemic arterial hypertension, urea levels were alsolikely to be marking the onset of kidney injury, when associated with the high values of blood pressure found.

Regarding blood pressure, patients with SAH and CKD had high BP values before LOS administration, classified as high and very high, respectively. Additionally, the EC50 of losartan was achieved by 50% of patients with SAH and limited to 21.05% among patients with CKD.

In the biochemical tests, we also noticed that creatinine and urea were critically above normal values in the CKD group, while the SAH group also presented urea values well above the reference range.

The differences found by NMR metabolomic analysis among the groups were in agreement with the blood pressure values and provided relevant and consistent data on the pharmacometabolomic profile and the distinction between the hypertensive and chronic kidney disease groups. Clinical protocols for the therapeutic monitoring of losartan can be created from the proposed methodology, efficiently assisting in the pharmacological treatment, especially for renal patients, which can improve the treatment outcomes, increase the quality of life and reduce expenses related to this clinical condition.

Personalized medicine to improve the therapeutic strategies and individual follow-up is a necessary approach. In view of the various possibilities that this type of study presents and the results found, there is a need for more studies covering this topic and complementing the findings to understand the mechanisms involved in hypertension, kidney disease and their relationship and also validate these results using a new cohort. This improvement would make this methodology useful for clinical practice to assess the response based on on molecular biomarkers.

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4CONSIDERAÇÕES FINAIS

O presente estudo investigou as variáveis envolvidas na farmacometabolômica de losartana e a sua correção com a hipertensão e com a doença renal crônica, obtendo resultados relevantes a cerca das particularidades não apenas do fármaco, mas também das interações que coexistem neste processo.

A losartana apesar da eficiência e de ser de bem tolerada pelos clientes durante seu uso pode quando correlacionada com condições externas não ter o resultado esperado no controle pressórico dos pacientes. Condições como situação laboral precária e alta responsabilidade familiar, assim como váriáveis internas relacionas a interindividualidade de cada organismo causam respostas que impactam no metabolismo do fármaco e na progressão da doença, tais como os biomarcadores encontrados, que estavam diretamente relacionados ao controle pressórico e também a injúria renal.

A possibilidade de utilização destes achados para otimização e melhoria do tratamento farmacológico realizado nestes grupos de pacientes poderia trazer mais eficiência e aumentar a sua qualidade de vida, baseado na efetividade e segurança, tendo em vista ainda a possibilidade de prevenir/retardar a evolução da doença renal a partir do controle dos níveis pressóricos em pacientes hipertensos.

Anexo 1 – Parecer do Comitê de Ética

UNIVERSIDADE FEDERAL DO AMAPÁ - UNIFAP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: AVALIAÇÃO DO PERFIL METABOLÔMICO E DOS NÍVEIS PLASMÁTICOS DE LOSARTANA E A SUA CORRELAÇÃO COM O CONTROLE PRESSÓRICO EM PACIENTES HIPERTENSOS

Pesquisador: INGRID SOUZA REIS SANTOS

Área Temática:

Versão: 2

CAAE: 18337719.8.0000.0003

Instituição Proponente: Pró-Reitoria de Pesquisa e Pós-Graduação

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.799.717

Apresentação do Projeto:

Emenda aprovada pelo CEP, Justificativa aceita.

Objetivo da Pesquisa:

Emenda aprovada pelo CEP, Justificativa aceita.

Avaliação dos Riscos e Benefícios:

Emenda aprovada pelo CEP, Justificativa aceita.

Comentários e Considerações sobre a Pesquisa:

Emenda aprovada pelo CEP, Justificativa aceita.

Considerações sobre os Termos de apresentação obrigatória:

Emenda aprovada pelo CEP, Justificativa aceita.

Recomendações:

Emenda aprovada pelo CEP, Justificativa aceita.

Conclusões ou Pendências e Lista de Inadequações:

Emenda aprovada pelo CEP, Justificativa analisada e aprovada.

Considerações Finais a critério do CEP:

UNIVERSIDADE FEDERAL DO AMAPÁ - UNIFAP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1479287_E1.pdf	26/11/2019 10:40:22		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	26/11/2019 10:33:40	INGRID SOUZA REIS SANTOS	Aceito
Folha de Rosto	folhaderostodpq.pdf	29/07/2019 22:53:47	INGRID SOUZA REIS SANTOS	Aceito
Projeto Detalhado / Brochura Investigador	BROCHURA.pdf	25/07/2019 15:27:24	INGRID SOUZA REIS SANTOS	Aceito
Orçamento	ORCAMENTO.pdf	25/07/2019 15:23:58	INGRID SOUZA REIS SANTOS	Aceito
Cronograma	CRONOGRAMA.pdf	25/07/2019 15:22:06	INGRID SOUZA REIS SANTOS	Aceito
Declaração de Instituição e Infraestrutura	autorizacao_institucional.pdf	25/07/2019 15:01:42	INGRID SOUZA REIS SANTOS	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

MACAPA, 14 de Janeiro de 2020

Assinado por:
RAPHAELLE SOUSA BORGES
(Coordenador(a))

Anexo 2 – Normas de publicação dos respectivos periódicos

Clinical Therapeutics

Manuscript File

The manuscript file should contain the following items: title, abstract, clinical trial registration (if applicable), keywords, main text, acknowledgments, figure/table legends, references. These items are described in detail below. All author names and identifying information must be removed from the manuscript file to facilitate double-blind peer review. The manuscript file should be structured according to article type, be double-spaced, include page numbers, and follow AMA style except as indicated below (See Style).

Title
The title should be concise, informative, and focused on the study objective. Statements about the conclusion(s) of the work should be avoided. Randomized controlled trials, meta-analyses, and systematic reviews should be identified as such in the title. Subtitles can be used to provide supplementary information (eg, study design); however, titles should be able to stand alone. Use nonproprietary drug names (See Names of Drugs and Devices); avoid abbreviations and formulae where possible.

Abstract
Abstracts should be structured or unstructured according to article type and word limits as detailed below. Abstracts are often presented separately from the article; therefore, must be able to stand alone. References should be avoided (if essential, the complete reference per AMA style must be given within the lines of text). Non-standard or uncommon abbreviations should be avoided (when necessary, they must be defined at their first use in the abstract).

Structured abstracts are required for Original Research (≤400 words), Reviews (≤400 words), Brief Reports (≤250 words), and Pilot Studies (≤250 words). Structured abstracts should contain sufficient detail as directed by the extension to the CONSORT statement for abstracts, and should be formatted as follows:

Purpose: Briefly provides the frame of reference for the reader and identifies the knowledge gap that the article seeks to address; clearly states the purpose of the research; and identifies the scientific hypotheses and questions being asked.

Methods: Succinctly describes study methodology, including study design, study dates, setting/data sources, inclusion and exclusion criteria, interventions, outcomes, statistical approaches, and adverse event assessment methodology.

Findings: Provides demographics of the study population, including sex, age range, and numbers of participants in each group; reports principle data and outcomes in a quantitative fashion, including effect sizes and confidence intervals or P values; includes adverse events.

Implications: Covers any limitations or problems in interpretation or generalization from the study findings as well as implication and future directions; must be strictly limited to what can be supported directly by the Findings, and what was identified in the Purpose section.

For manuscripts that require clinical trial registration (See Reporting and Registration of Clinical Trials), the name of the trial registry, trial registration number, and URL of the registry should be included immediately following the Implications section of the abstract.

Unstructured abstracts are required for Commentaries (?300 words). Unstructured abstracts should briefly describe the importance and clinical relevance of the topic, the objective, approach, and a summary of key points. Abstracts are not required for Research Letters, Case Reports, and Letters to the Editor.

Keywords
Immediately after the abstract, provide 4-6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Body of Manuscript
The main text should adhere to word limits and structure according to article type as detailed above. All submissions should adhere to journal policies (See EDITORIAL POLICIES) and EQUATOR Reporting

Guidelines for the applicable study design (eg, CONSORT for randomized trials, STROBE for observational studies, CHEERS for economic evaluations, PRISMA for systematic reviews and meta-analyses).

Introduction: State the objectives/hypotheses of the work and provide an adequate background; avoid a detailed literature survey or a summary of the results. Required for all article types except Letters to the Editor.

Methods: Provide sufficient detail to allow the work to be replicated by others. Methods already published should be indicated by a reference: only relevant modifications should be described. For work involving human subjects, describe the (1) study design and randomization procedures; (2) study dates and setting; (3) institutional review board (RB) approval or waiver, including name of the IRB/ethics committee; (4) details of patient consent or assent of youth and children; (5) participants and conditions/factors studied, including full inclusion and exclusion criteria; (6) interventions, if any, with full description of placebo, sham, or control conditions [See Placebos in Clinical Trials]; (7) primary and secondary outcome measures, including whether secondary analyses were pre-specified; (8) detailed statistical analyses, with a priori significance thresholds, methods for handling missing data or outliers if applicable, and any relevant citations. Rationale for inclusion of only one sex or age group should be provided and scientifically justified [See Inclusion of Sex and Gender]. Systematic reviews and meta-analyses should follow PRISMA guidelines. Subheadings (up to two additional levels) are encouraged. A Participants and Methods section is required for Original Research, Brief Reports, Pilot Studies, and Research Letters. A Methods section is required for Reviews.

Results: The Results should be clear, concise, and relevant to the stated objectives/hypotheses. Describe the study population first, including sex, age, and other relevant demographic characteristics of subjects. All numeric data should be reported with descriptive and/or inferential statistical test results (including exact p-values, if available). For each outcome, report results for each group, the effect size, and its precision (ie, 95% confidence interval). Do not discuss implications or limitations in this section. Tables, figures, and subheadings (up to two additional levels) are encouraged. Results are required for Original Research (including meta-analyses), systematic Reviews, Brief Reports, and Pilot Studies; optional for narrative Reviews.

Discussion: The Discussion should explore the importance and relevance of the results of the work, not repeat them. Authors should critically examine the work, describe unexpected and/or contradictory findings, and address any limitations of the study design or statistically indeterminate results here. All inferences must be supported by evidence presented in the Results section. Authors should also discuss generalizability and clinical implications of findings, as well as any future studies needed. Avoid extensive citations and discussion of published literature. Subheadings (up to one additional level) are permitted. A Discussion is required for all article types except Letters to the Editor; a combined Results and Discussion section is often appropriate for narrative reviews.

Conclusions: The main conclusions of the study should be present

Acknowledgments
Collate acknowledgments in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided materials or assistance during the research (eg, language help, writing assistance, or proofreading the article, etc). Do NOT list the names of authors or funder(s) in this paragraph.

Disclosure of Funding Support
All financial support for the submitted work must be reported in a separate section below the Acknowledgements. Authors should declare the role of study sponsors, if any, in the study design; in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Figure Captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used. If figures are not original, it is the author's responsibility to obtain permission from the original publisher; the source and a statement that permission has been obtained must be included in the caption.

References

Permissible Sources
References should include only published works or articles in press. Citation of a reference as 'in press' implies that the item has been accepted for publication and a DOI number must be included. Abstracts, unpublished data, and personal communications are not permitted in the reference list. Unpublished results and data published in abstract form may be mentioned in the text only if the methodological details and data are made available in supplemental digital content. Research datasets that have been assigned a global persistent identifier number are permitted.

Citation in Text
In-text citations should be indicated sequentially by superscript number(s) or in brackets. The authors can be referred to, but the reference number(s) must always be given. Please ensure that every reference cited in the text is also present in the reference list (and vice versa).

Reference List Format
There are no strict requirements for formatting the reference list at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. Journal names should be abbreviated according to the list of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Data Reference
[dataset] 1. Oguro, M, Imahiro, S, Saito, S, Nakashizuka, T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. http://dx.doi.org/10.17632/xwj98nb39r.1.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Tables

Number tables consecutively in accordance with their appearance in the text. Each table should have a concise and descriptive caption. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article. Submit each table as a separate file in its original file format.

Figures

Figures should be numbered consecutively in accordance with their appearance in the text and uploaded as individual high-resolution files. Brief Figure Captions are required and should be supplied separately above the References section in the manuscript file (See Figure Captions). Authors are urged to consult the detailed guide on electronic artwork and adhere to the following:

Electronic Artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
• Ensure that color images are accessible to all, including those with impaired color vision.

Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color Artwork
Please make sure that artwork files are in an acceptable format (TIFF [or JPEG], EPS [or PDF]), or MS Office files) and with the correct resolution. For further information on the preparation of electronic artwork, please see https://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Supplemental Material

Supplementary material such as applications, images, and sound clips, can be made available online via links published with your article. Supplementary material relevant to the work, but not critical to support the primary findings, is allowed for Original Research, Reviews, Brief Reports, and Pilot Studies. All supplemental materials, including tables and figures, must be mentioned in the main text and must be numbered consecutively in accordance with their appearance in the text (eg, Table S1, S2, Figure S1). Submitted supplementary items are published online exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. Authors are encouraged to submit a single, consolidated file containing a table of contents followed by all supplemental material in order of appearance in the text. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

STYLE

Manuscripts submitted to Clinical Therapeutics should be prepared in accordance with AMA Style unless otherwise noted. See American Medical Association Manual of Style: A Guide for Authors and Editors, 10th Edition.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Names of Drugs and Devices

Drugs should be referred to by their universally accepted generic names, not by proprietary names, unless the specific trade name is essential to the methods or discussion. In such cases, use the proprietary name once and the generic or descriptive name thereafter. US adopted names (USANs) are acceptable. If unnamed compounds are referred to, as much information as possible (eg, class of compound) should be included and published references to the compound should be provided. If this is not possible because of intellectual property reasons, then this should be stated.

For drug-device combinations, capitalize the first letter of each term. At first mention in the abstract and in the main text, include the active ingredient and dose in parentheses following the product name.

Where proprietary names of drugs and drug-device combinations are used as permitted above, capitalize only the initial letter of the trademarked word (unless the trademarked name is an abbreviation) and do not include trademark symbols.

Units of Measure

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Currency Values

Clinical Therapeutics endeavors to make all manuscripts readily understandable by using universally accepted chemical names, structures, spelling, abbreviations, and formatting. For Pharmacoeconomics submissions, results are sometimes reported in the currency of the author(s)'s country. The relative value of currencies from some countries may not be obvious to some readers. Therefore, we now require that equivalencies in US dollars (USD) and European Union euros (EUR) be added in parentheses after other currencies. For example, 68 Indian rupees or INRs would be followed by ($1.00 USD/0.85 EUR). We recognize that currency values fluctuate. Therefore, equivalencies should reflect values at the time of submission.

Language (usage and editing services)

Please write your text in standard, grammatically correct English. If English is not your first language, authors are encouraged to consult with a colleague or professional whose native language is English to improve grammar and syntax prior to submission. Alternatively, authors may wish to visit Elsevier's language editing and copyediting services which are available both pre- and post-submission at: https://webshop.elsevier.com/language-editing-services/language-editing/ or our customer support site at https://service.elsevier.com for more information. Articles that are not cogent and clearly written will be returned to the author.

Use of Inclusive Language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Usage of Sex vs Gender

The term sex should be used when describing biological factors and the term gender should be used when referring to sociocultural factors.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Atención Primaria

CARTAS AL DIRECTOR

En esta sección se publicarán preferentemente y con la mayor rapidez posible cartas que comenten artículos aparecidos recientemente en la revista. La carta será enviada a los autores del artículo al que se refiere y, si éstos desean contestarla, la carta y su réplica se publicarán simultáneamente.

También se aceptarán cartas al director que presenten experiencias y opiniones de interés para la atención primaria como informes cortos de estudios de investigación y descripciones de series de casos clínicos. En el texto de este tipo de cartas se debe hacer referencia a los objetivos, diseño del estudio, mediciones, resultados y conclusiones principales. texto de este tipo de cartas se debe hacer referencia a los objetivos, diseño del estudio, mediciones, resultados y conclusiones principales.

El comité editorial se reserva la decisión de publicar las Cartas al Director en papel o on-line. En ambos casos la referencia del articulo saldrá publicada en el sumario.

El número máximo de autores será de 4.

La estructura de los trabajos debe ser la siguiente:

- Carta de presentación (véase normas generales).

- Primera página (véase normas generales).

- Texto (máximo: 600 palabras sin contabilizar la bibliografía ni las tablas).

- Tabla y/o figura (máximo: 1) (véase normas generales).

Cada una de las partes anteriores debe iniciarse en una página nueva.

El número máximo de referencias bibliográficas es 6.

En el caso de cartas que se refieran a un artículo publicado, una de las referencias debe corresponder a este artículo.

Estructura del artículo
Apartados
Organice su artículo mediante apartados y subapartados claramente definidos, precedidos por un encabezado conciso (como Introducción, Material y métodos, Resultados y Discusión) en una línea aparte. Los encabezados deben utilizarse para realizar referencias a apartados concretos del texto.

Introducción
Describa los objetivos del trabajo, basados en los fundamentos apropiados. Evite realizar una revisión detallada de la literatura o resumir los resultados.

Material y métodos
Proporcione detalles suficientes para permitir que un investigador independiente pueda reproducir el trabajo. Si los métodos ya están publicados, se incluirá la referencia y un resumen de los mismos. En caso de realizar citas textuales, el texto se colocará entre comillas e incluirá la referencia a la fuente original. Si se han realizado modificaciones sobre un método publicado, deberán describirse claramente.

Resultados

Deben ser claros y concisos

Discusión
Debe analizar el significado de los resultados, pero no repetirlos. A veces es apropiado combinar ambas secciones en una sola. Evite las citas demasiado extensas, así como el comentario de artículos publicados.

Apéndices
Si existe más de un apéndice, deberá numerarse como A, B, etc. Si incluyen fórmulas y ecuaciones, se utilizará una numeración independiente: Eq. (A.1), Eq. (A.2), etc, Eq. (B.1), etc. Del mismo modo para tablas y figuras: Tabla A.1, Fig. A.1, etc.

Primera página
¿ Título. Conciso e informativo. El título se utiliza en los sistemas de recuperación de la información (índices). Evite incluir fórmulas y abreviaturas en el mismo siempre que sea posible. Se incluirá también el título en inglés.
¿ Nombres y filiaciones de los autores. Indique nombre y apellidos de cada uno de los autores y asegúrese de que los proporciona en la forma ortográfica correcta. Detrás de la transliteración inglesa, puede añadir entre paréntesis los nombres en su escritura original. Incluya los datos de filiación de cada uno de los autores (nombre y dirección de la institución en la que se realizó el estudio) debajo de los nombres. Indique todas las filiaciones mediante una letra minúscula en superíndice al final del apellido de cada autor. La misma letra debe preceder los datos de la institución. Indique la dirección postal completa para cada filiación, sin olvidar el país, así como la dirección de correo electrónico de cada autor, si es posible.
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. Consideraciones Éticas, Financiación y Conflicto de Intereses. Ver sección Tipos de artículo.

Resumen estructurado
El resumen estructurado a través de encabezados debe proporcionar el contexto o los antecedentes para la investigación y en él se debe mencionar su propósito, los procedimientos básicos (selección de sujetos para el estudio o animales de laboratorio, métodos observacionales y analíticos), los hallazgos principales (aportando los tamaños de efectos específicos y su importancia estadística, si es posible) y las conclusiones principales. Debe hacerse hincapié en aspectos nuevos e importantes del estudio u observaciones.

La estructura que deberá seguirse es: Objetivo, Emplazamiento, Participantes, Intervenciones, Mediciones principales, Resultados y Conclusiones.

Resumen gráfico
El resumen gráfico es opcional, pero aconsejamos su remisión porque genera más atención sobre el artículo online. El resumen gráfico sintetiza los contenidos del artículo de forma ilustrada y concisa y su función es captar la atención de un amplio conjunto de especialistas. La figura de resumen se remitirá en un archivo individual de, como mínimo, 531 x 1328 pixels (altura x anchura). Estas dimensiones pueden incrementarse de forma proporcional. Esta imagen tiene que ser legible en un tamaño de 5x13 cm y en una pantalla de resolución de 96 dpi. Se recomienda utilizar los siguientes formatos: TIFF, EPS, PDF o documentos de MS Office. Puede ver Ejemplos de resumen gráfico en nuestra web. Los autores pueden utilizar el Servicio de Ilustración y Mejora de Elsevier para presentar las imágenes con el mejor diseño posible y cumpliendo todos los requisitos técnicos: Servicios de ilustración.

Palabras clave
Incluir un máximo de 6 palabras clave después del resumen, utilizando inglés británico, evitando términos generales, plurales y multiplicidad de conceptos (como por ejemplo el uso de 'y' o 'de'). Solamente pueden utilizarse abreviaturas como palabras clave en el caso de que estén firmemente establecidas en la especialidad que corresponda al artículo. Las palabras clave se utilizan en la indexación del artículo.

Abreviaturas

Defina las abreviaturas que no son estándar en su especialidad en una nota a pie de página en la primera página del manuscrito. Asegúrese de que utiliza las abreviaturas de forma consistente a lo largo de todo el artículo.

Agradecimientos

Sitúe los agradecimientos en una sección aparte al final del manuscrito y antes de las Referencias bibliográficas. No los mencione en ninguna otra parte del artículo. Incluya aquellas personas que colaboraron en la realización del artículo (por ejemplo, revisando la redacción o la traducción del mismo).

Formato de las fuentes de financiación

Enuncie las fuentes de financiación utilizando el siguiente formato estándar requerido por las entidades financiadoras:

Financiación: El presente trabajo ha sido financiado por los NationalInstitutesofHealth [beca número xxxx, yyyy]; la Bill & Melinda Gates Foundation, Seattle, WA [beca número zzzz] y los UnitedStatesInstitutesofPeace [beca número aaaa].

No es necesario incluir descripciones detalladas sobre el programa o el tipo de beca o asignación. Cuando la financiación proceda de una beca a nivel regional o nacional, o de recursos de universidades u otras instituciones dedicadas a la investigación; incluya el nombre de la institución u organización que financió el estudio.

Si no se ha recibido financiación alguna, le rogamos que incluya la siguiente frase:

La presente investigación no ha recibido ayudas específicas provenientes de agencias del sector público, sector comercial o entidades sin ánimo de lucro.

Unidades

Utilice las reglas y convenciones aceptadas internacionalmente, como el sistema internacional de unidades (SI). Si menciona otro tipo de unidades, por favor, proporcione su equivalente en el SI.

Imágenes

Manipulación de imágenes

Aunque se admite que a veces los autores tienen que retocar las imágenes para hacerlas más claras y comprensibles, no se acepta la manipulación de las mismas con intención fraudulenta. Esto constituye una infracción de la ética científica y se actuará en consecuencia. La revista aplica la siguiente normativa para las imágenes: no se puede mejorar, oscurecer, desplazar, eliminar ni añadir ningún elemento de las mismas. Se permite realizar ajustes de brillo, contraste o equilibrio de colores siempre y cuando no oscurezcan o eliminen ninguna información visible en la imagen original. Si se realizan ajustes no lineales (como cambios en los parámetros gamma) debe indicarse en el pie de figura.

Formatos electrónicos

Consideraciones generales.

• Asegúrese de que presenta sus ilustraciones originales de forma uniforme en cuanto a tamaño y leyendas.
• Incruste las fuentes en el archivo, si la aplicación que utiliza lo permite.
• Procure utilizar las fuentes: Arial, Courier, Times New Roman, Symbol, u otras que se asemejen en sus ilustraciones.
• Numere las ilustraciones de forma correlativa.
• Elija una nomenclatura lógica para denominar los archivos de imágenes.
• Proporcione los textos para el pie de cada figura en una lista separada.
• Utilice un tamaño similar al que deberían tener las imágenes en la publicación.
• Envíe cada figura en un archivo independiente.
• Compruebe que las imágenes en color son accesibles para todo el mundo, también para las personas con transtornos de la visión en color.

Obtendrá información más detallada sobre cómo preparar las imágenes en la guía sobre ilustraciones electrónicas

Le recomendamos que visite dicha página. A continuación incluimos un pequeño resumen.

Formatos.

Si ha utilizado una aplicación de Microsoft Office (Word, PowerPoint o Excel), por favor remita la imagen en el formato propio del archivo.

Si ha utilizado otras aplicaciones, una vez la figura esté terminada, por favor haga un 'Guardar como' o bien exporte o convierta cada uno de los archivos de imágenes a alguno de los formatos siguientes (tenga en cuenta la resolución requerida para dibujos de líneas, medios tonos o combinaciones de ambos que se detalla más abajo):

EPS (o PDF): imágenes vectoriales. Incruste todas las fuentes que haya utilizado.
TIFF (o JEPG): fotografías en color o escala de grises (halftones), con una resolución de 300 dpi/ppp como mínimo.
TIFF (o JEPG): bitmap, pixeles en blanco y negro puros, con una resolución de 1.000 dpi/ppp como mínimo.
TIFF (o JEPG): combinaciones de líneas bitmap e imágenes halftone (color o escala de grises), con una resolución de 500 dpi/ppp como mínimo.

Le rogamos que no remita
• Archivos que no son óptimos para su utilización en pantalla (GIF, BMP, PICT o WPG, por ejemplo, suelen tener una baja resolución y un número limitado de colores).
• Archivos con baja resolución.
• Gráficos de tamaño desproporcionadamente grande en relación con su contenido.

Imágenes en color
Por favor, compruebe que los archivos de imagen tienen el formato adecuado (TIFF (o JPEG), EPS (o PDF) o archivos de Microsoft Office) y la resolución necesaria. Si ha remitido figuras en color utilizables, Elsevier las publicará en color en la edición electrónica de la revista (por ejemplo, ScienceDirect y otras páginas web) sin cargo adicional. Más información sobre la preparación de ilustraciones digitales.

Servicios de ilustración
El Servicio para autores de Elsevier ofrece servicios de ilustración para aquellos autores que los requieran. Los expertos ilustradores de Elsevier pueden realizar imágenes científicas y técnicas, así como una amplia variedad de tablas, diagramas y gráficos. La web también ofrece servicios de optimización de las imágenes para que alcancen un nivel estándar profesional. Visite la web para obtener más información.

Pies de figura
En un documento aparte, redacte un pie para cada una de las figuras y compruebe que no falta ninguno. El pie debe contener un título corto (que no debe aparecer en la ilustración) y una descripción de la figura. Intente que la presencia de texto en la figura sea mínima, y no olvide incluir en el pie la definición de todos los símbolos y abreviaturas utilizados en la misma.

Tablas
Remita las tablas como texto editable, y no como imágenes. Puede colocarlas dentro del manuscrito, cerca de la parte del texto donde se mencionan, o también en páginas aparte al final del manuscrito. Numere las tablas de forma consecutiva según su aparición en el texto y coloque las notas correspondientes debajo de cada tabla. Limite la utilización de tablas y compruebe que los datos que presenta en las mismas no duplican resultados ya descritos en el texto. No utilice pautas verticales ni celdas sombreadas.

Referencias bibliográficas
Citación en el texto
Compruebe que cada referencia dada en el texto aparece en la lista de referencias (y viceversa). No se recomienda incluir comunicaciones personales o trabajos no publicados en la lista de referencias (y, en caso de hacerse, deben seguir las convenciones estándar sustituyendo la fecha de publicación con la mención 'Resultados no publicados' o bien 'Comunicación personal'), pero pueden mencionarse en el texto. La mención de una referencia como 'En prensa' implica que el manuscrito ha sido aceptado para su publicación.

Enlaces online a las referencias
Los enlaces online a las referencias favorecen la diseminación de la investigación y el nivel de calidad del sistema de revisión por pares. Para poder crear enlaces a servicios de indexación y consulta como Scopus, CrossRef y PubMed es necesario que los datos proporcionados en la lista de referencias bibliográficas sean correctos. Tenga en cuenta que errores en los nombres de autor, el título de la publicación, el año de publicación y las páginas pueden impedir la creación del enlace al manuscrito citado. Cuando copie una referencia tenga en cuenta que puede contener errores. Recomendamos encarecidamente la utilización del DOI.

El DOI nunca cambia, y por ello puede utilizarse como enlace permanente a un artículo electrónico. Ejemplo de citamediante DOI: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. JournalofGeophysicalResearch, https://doi.org/10.1029/2001JB000884. Tenga en cuenta que estas citas deben tener el mismo estilo y formato descrito en las normas para el resto de referencias bibliográficas.

Referencias a páginas web

Como mínimo, debe proporcionarse la URL completa y la fecha en que se accedió por última vez a la referencia. Deberá añadirse también cualquier otra información conocida (DOI, nombres de los autores, referencia a una publicación fuente, etc). Las referencias a páginas web pueden presentarse en una lista aparte, a continuación de la lista de referencias bibliográficas, o bien pueden incluirse en la misma.

Referencias a un conjunto de datos
Le invitamos a referenciar los conjuntos de datos que ha utilizado o son relevantes para su artículo. Para ello, debe incluir la cita en el texto, así como en el listado de Referencias bibliográficas, al final del artículo. Las referencias a conjuntos de datos constan de los elementos siguientes: nombre(s) del (los) autor(es), nombre del conjunto de datos, nombre del repositorio, versión (si procede), año e identificador global permanente. Añada la etiqueta [dataset] justo antes de la referencia, para que podamos identificarla como una referencia a datos. Esta etiqueta no aparecerá en la versión publicada del artículo.

Referencias en un número extraordinario
Compruebe que añade la mención 'este número' a las referencias de la lista (y a su mención en el texto) que citan artículos del mismo número extraordinario.

Software para la gestión de referencias
La mayoría de revistas de Elsevier cuentan con una plantilla bibliográfica disponible en los programas de gestión de bibliografía más habituales. Nos referimos a cualquier programa compatible con los estilos Citation Style Language, como Mendeley, por ejemplo. Mediante las extensiones para procesadores de texto que ofrecen estos productos, los autores simplemente tienen que seleccionar la plantilla correspondiente a la revista para que el procesador de texto aplique automáticamente el estilo bibliográfico de la revista a las citas y a las referencias bibliográficas. Si esta revista todavía no dispone de plantilla, le rogamos que consulte la lista de referencias y citas de muestra que se ofrece en esta guía para utilizar el estilo correspondiente a la revista. Si Vd. utiliza programas de gestión bibliográfica, por favor, asegúrese de que elimina los códigos de campo antes de enviar el manuscrito electrónico. Más información sobre como eliminar los códigos de campo en distintos programas de gestión bibliográfica.

Si usted utiliza la plataforma Mendeley Desktop, puede instalar facilmente el estilo de referencias de esta revista a través del siguiente link:

Cuando esté preparando su manuscrito, puede seleccionar el estilo mediante el plug-in de Mendeley para Microsoftt Word o LibreOffice. Para mas información sobre el estilo de citación, consulte http://citationstyles.org.

Formato de las referencias
Texto: Indique las referencias mediante números en superíndice dentro del texto. El autor puede mencionarse si se desea, pero el número de la referencia es imprescindible.
Lista: Numere las referencias en la lista en el mismo orden en que aparecen en el texto.
Ejemplos:
Referencia a un artículo de revista:
1. Van derGeer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J SciCommun2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.

Referencia a un artículo de revista con número de artí-culo:
2. Van derGeer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon. 2018;19:e00205. doi:10.1016/j.heliyon.2018.e00205.

Referencia a un libro:
3. StrunkJr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Referencia a un capítulo de libro:
4. Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.
Referencia a una página web:
5. CancerResearch UK. Cancerstatisticsreportsforthe UK, http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/; 2003 [consultada el 13 de marzo de 2003].
Referencia a una base de datos:
[dataset] 6. Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. https://doi.org/10.17632/xwj98nb39r.1.
Nótese la forma abreviada para el último número del rango de páginas (por ejemplo, 51–9), así como que, en el caso de más de 6 autores, el séptimo y siguientes se mencionan como “et al”. Encontrará información más

detallada en 'UniformRequirementsforManuscriptssubmittedtoBiomedicalJournals' (J Am MedAssoc1997;277:927–34) (véase también Ejemplos de formato de referencias).

Abreviaturas de las revistas
Los nombres de las revistas deben abreviarse de acuerdo con la Lista de abreviaturas de palabras para títulos.

Vídeos
Elsevier acepta vídeos y secuencias animadas que apoyen o realcen la investigación científica. Los autores que deseen enviar vídeos o animaciones con sus manuscritos deben incluir referencias o enlaces a los vídeos dentro el cuerpo del artículo, en la misma forma que lo harían para una figura o tabla, esto es, refiriéndose contenido del vídeo o la animación e indicando en el cuerpo del texto dónde debería aparecer. Todos los archivos que se entreguen deben estar correctamente etiquetados, de forma que pueda identificarse directamente el contenido del archivo. Para garantizar la visualización directa de los vídeos o animaciones, es necesario proporcionar el archivo en alguno de los formatos recomendados y con un tamaño máximo ideal de 150 MB por archivo, y de 1 GB en total. Los vídeos y animaciones se publicarán online en la versión electrónica del artículo en los productos web de Elsevier, como ScienceDirect. Le recomendamos que remita también imágenes estáticas, que puede elegir entre cualquier imagen del vídeo o bien crear por separado. Estas imágenes se utilizarán en lugar de los iconos estándar para personalizar el enlace a los videos. Encontrará instrucciones más detalladas en nuestra página Instrucciones para videos. Dado que los vídeos y animaciones no pueden reproducirse en la edición impresa de la revista, le rogamos que proporcione textos para las ambas ediciones, la electrónica y la impresa, que puedan ser incluidos en las partes del artículo donde se hace referencia a este contenido.

Material suplementario
Los autores pueden remitir material suplementario (aplicaciones, imágenes o archivos de audio) para mejorar su artículo. El material suplementario se publicará online tal y como lo ha remitido (los documentos Excel o PowerPoint aparecerán online como tales). Por favor, remita el material junto con el manuscrito y proporcione un breve texto descriptivo para cada uno de los archivos. Si desea hacer cambios en los datos suplementarios en cualquier fase del proceso, deberá remitir un nuevo archivo actualizado con las correcciones, y no simplemente anotaciones en el archivo original. Asegúrese de desactivar la herramienta de `Control de cambios' en los archivos de Microsoft Office, pues de lo contrario éstos se visualizarán cuando estén publicados como material suplementario.

Depósito y enlace de datos
Elsevier anima y ayuda a los autores a compartir los datos en bruto subyacentes al trabajo de investigación que se publica siempre que sea apropiado y, con este fin, permite el enlace online de artículos y datos. Más información sobre como depositar, compartir y utilizar de datos de investigación.

DATOS CIENTÍFICOS
La revista recomienda compartir los datos que los autores han utilizado en su trabajo de investigación, cuando sea procedente, y permite la realización de enlaces con los artículos publicados. Por datos científicos entendemos los resultados de las observaciones o experimentos que validan los hallazgos realizados. Con el fin de facilitar la reproducibilidad de la investigación y la reutilización de datos, la revista recomienda también compartir código, tipo de software, modelos, algoritmos, protocolos, métodos y cualquier otro material útil relacionado con el proyecto.

A continuación encontrará distintas opciones para asociar datos con su artículo o bien para hacer una declaración sobre la disponibilidad de los mismos durante el envío de su manuscrito. Cuando elija alguna opción de compartición de datos, no olvide incorporar la cita correspondiente tanto en el manuscrito como en la bibliografía. En el apartado ¿Bibliografía¿ encontrará información sobre la citación de datos. Puede obtener más información sobre como depositar, compartir y utilizar datos u otros materiales científicos relevantes en la página datos científicos.

Journal of Molecular Medicine

Manuscript lengths and formats
The main text body of an Original Article should not exceed 3500 words and 50 references.

The number of display items may not exceed 8 figures including tables.

Reviews should not exceed 15 printed pages including figures (1 page corresponding to 800 words or 2 figures/tables or 40 references). Color figures in reviews are published free of charge.

Commentaries may refer to previous papers in Journal of Molecular Medicine, or other matters of interest. They do not have an abstract, nor do they have sections such as Introduction, Materials & Methods, Results and Discussion. The title should include the message, i.e. the finding which makes the commentary worth publishing. Commentaries must not exceed 1,000 words and 2 printed pages, including figures, tables (if any) and references. One printed text page corresponds to about 800 words.

Title Page
Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information
The name(s) of the author(s)
The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
A clear indication and an active e-mail address of the corresponding author
If available, the 16-digit ORCID of the author(s)
If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract
Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

Trial registration number and date of registration for prospectively registered trials
Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials
Keywords
Please provide 4 to 6 keywords which can be used for indexing purposes.

Important note on Manuscript Title
The manuscript title should be clear, concise and capture the conceptual significance for a broad audience. It should not exceed 12 words and include sufficient detail for indexing purposes but be general enough for readers outside the field to appreciate what the paper is about. The use of jargon, uncommon abbreviations, acronyms, or punctuation should be avoided. For publication, titles may be revised by the Editorial Board to ensure clarity and accessibility to our wide readership, with the final approval of the authors.

Examples
Inhibition of autophagy enhances anticancer effects of bevacizumab in hepatocarcinoma
Critical role of proteostasis imbalance in pathogenesis of COPD and severe emphysema
Ras regulates interleukin-1 beta-induced HIF-1 alpha transcriptional activity in glioblastoma
Regenerative potential of glycosaminoglycans for skin and bone
Important note on Abstract
The abstract may not exceed 200 words and should provide a clear synopsis of the reported findings that is accessible to a non specialist reader. It should state the rationale, objectives, findings, and conclusions of the manuscript. References and primary data should not be presented in abstracts, and nonstandard abbreviations must be defined. For publication, abstracts may be revised by the Editorial Board to ensure clarity and accessibility to our wide readership, with the final approval of the authors.

Key Messages
Key messages are mandatory for original articles in this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'key messages' in the file name and include 3 to 5 bullet points that describe the essence of the research (maximum 85 characters, including spaces, per bullet point).

Text
Text Formatting
Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.
Use italics for emphasis.
Use the automatic page numbering function to number the pages.
Do not use field functions.
Use tab stops or other commands for indents, not the space bar.
Use the table function, not spreadsheets, to make tables.
Use the equation editor or MathType for equations.
Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).
Headings
Please use no more than three levels of displayed headings.

Abbreviations
Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes
Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments and Funding Information
Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full. In addition, please provide the funding information in a separate step of the submission process in the peer review system. Funder names should preferably be selected from the standardized list you will see during submission. If the funding institution you need is not listed, it can be entered as free text. Funding information will be published as searchable metadata for the accepted article, whereas acknowledgements are published within the paper.

References
Citation
Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list
The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

Journal article
Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

Book
South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter
Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document
Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation
Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

Authors preparing their manuscript in LaTeX can use the bibliography style file sn-basic.bst which is included in the Springer Nature Article Template.

The following statements must be included in your submitted manuscript under the heading 'Statements and Declarations'. This should be placed after the References section. Please note that submissions that do not include required statements will be returned as incomplete.

Funding

Please describe any sources of funding that have supported the work. The statement should include details of any grants received (please give the name of the funding agency and grant number).

Example statements:

"This work was supported by […] (Grant numbers […] and […]). Author A.B. has received research support from Company A."

"The authors declare that no funds, grants, or other support were received during the preparation of this manuscript."

Competing Interests

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work.

Example statements:

"Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M. Dr. C has received speaker honorarium and research funding from Company M and Company N. Author D has received travel support from Company O. Non-financial interests: Author D has served on advisory boards for Company M and Company N."

“The authors have no relevant financial or non-financial interests to disclose.”

Please refer to the “Competing Interests” section below for more information on how to complete these sections.

Author Contributions

Authors are encouraged to include a statement that specifies the contribution of every author to the research and preparation of the manuscript.

Example statement:

“All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.”

Please refer to the “Authorship Principles ” section below for more information on how to complete this section.

Data Availability
All original research must include a Data Availability Statement. Data Availability Statements should provide information on where data supporting the results reported in the article can be found. Statements should include, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. When it is not possible to share research data publicly, for instance when individual privacy could be compromised, data availability should still be stated in the manuscript along with any conditions for access.

Example statements:

“The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT LINK TO DATASETS]”

“The datasets generated during and/or analysed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.].”

Please refer to the “Research Data Policy and Data Availability” section below for more information on how to complete this section.

In addition to the above, manuscripts that report the results of studies involving humans and/or animals should include the following declarations:

Ethics approval

Authors of research involving human or animal subjects should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee and reference number, if available). For research involving animals, their data or biological material, authors should supply detailed information on the ethical treatment of their animals in their submission. If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

“This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No....).”

“This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.”

For detailed information on relevant ethical standards and criteria, please refer to the sections on “Research involving human participants, their data or biological material”, “Research involving animals, their data or biological material”.

Consent to participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript.

Example statement:

“Informed consent was obtained from all individual participants included in the study.”

“Written informed consent was obtained from the parents.”

Please refer to the section on “Informed Consent” for additional help with completing this information.

Consent to publish

Individuals may consent to participate in a study, but object to having their data published in a journal article. If your manuscript contains any individual person’s data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. This is in particular applicable to case studies. A statement confirming that consent to publish has been received from all participants should appear in the manuscript.

Example statement:

“The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.”

Please refer to the section on “Informed Consent” for additional help with completing this information.

Tables
All tables are to be numbered using Arabic numerals.
Tables should always be cited in text in consecutive numerical order.
For each table, please supply a table caption (title) explaining the components of the table.
Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.
Please note:
If a cited article has more than 10 authors, please list only the first ten authors in the reference list followed by “et al”.

Journal article:

Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, Mckenzie B, Kleinscheck MA, Owyang A, Mattson J, Blumenschein W et al (2006) IL-23 is essential for T-cell mediated colitis and promotes inflammation via IL-17 and IL-6. J ClinInvest 116:1310–1316 “

Apêndice A – TCLE

Atendendo a todos os critérios da **Resolução 466/2012**, convidamos você a participar da pesquisa: **AVALIAÇÃO DO PERFIL METABOLÔMICO E DOS NÍVEIS PLASMÁTICOS DE LOSARTANA E A SUA CORRELAÇÃO COM O CONTROLE PRESSÓRICO EM PACIENTES HIPERTENSOS**, sob a responsabilidade do pesquisador Prof. Dr. Francisco Fábio Oliveira de Sousa da Universidade Federal do Amapá e da mestranda Ingrid Souza Reis Santos.

O estudo tem como objetivo geral: avaliar o perfil metabolômico e os níveis plasmáticos de losartana e ácido losartana e a sua correlação com o controle pressórico em pacientes hipertensos. Sua participação é voluntária, previamente agendada de acordo com sua disponibilidade, e se dará por meio da aplicação de questionário, realização de teste de bioimpedância e coleta de material biológico para de coleta de dados. O questionário *BriefMedicationQuestionnaire* (adaptado) tem perguntas relacionadas ao perfil sociodemográfico, clínico e farmacoterapêutico. Este questionário servirá para realizar a triagem e averiguar a adequação ao grupo correspondente, sendo então agendada posteriormente o teste de bioimpedância junto com a coleta de amostra sanguínea e urinária a ser utilizada para as demais análises.

O teste de bioimpedância servirá para avaliação antropométrica, assim como a coleta de material biológico servirá para análise dos metabólitos no sangue relacionado a medicação ingerida, na qual será coletado no tempo de (0 h) e 1,5 e 3,5 horas após a administração do seu medicamento prescrito pelo médico. Estas análises serão agendadas previamente, de acordo com a disponibilidade e será realizado em um laboratório da Universidade Federal do Amapá, dentro das condições e técnicas adequadas para realização. Você tem liberdade de se recusar a participar e ainda se recusar a continuar participando em qualquer fase da pesquisa, sem qualquer prejuízo para você. Sempre que quiser poderá pedir mais informações sobre a pesquisa através do telefone do coordenador e, se necessário através do telefone do Comitê de Ética em Pesquisa.

A realização da pesquisa oferece o risco de desconforto/constrangimento. Para diminuir esse risco a aplicação do questionário ocorrerá em um espaço reservado no qual o voluntário sinta-se seguro e confortável para responder as questões. A coleta da amostra sanguínea ocorrerá em um dos laboratórios da universidade (ambiente privativo, iluminado e climatizado) dentro das condições e técnicas adequadas para realização por profissional capacitado e a coleta da amostra urinária será realizada por você após orientações.Você poderá interromper a aplicação do questionário a qualquer momento.O benefício esperado é que você tenha a possibilidade, a partir do conhecimento dos níveis séricos e urinários do losartana e ácido losartânico, de adequar a farmacoterapia de modo que a efetividade seja garantida, com mínimos riscos associados. E posteriormente os pesquisadores possam retornar os resultados da pesquisa aos profissionais de saúde que atendem vocês hipertensos (sem identificá-los), buscando minimizar as consequências de uma hipertensão descontrolada, esclarecer dúvidas, potencializar o conhecimento já obtido, e/ou minimizar anseios e vulnerabilidades relacionadas ao conhecimento. Servirá, portanto para a formulação de estratégias que ampliem e possibilitem os tratamentos oferecidos para o controle da PA e redução das morbimortalidades dela decorrentes.

Você não terá nenhuma despesa e também não receberá nenhuma remuneração. Os resultados da pesquisa serão analisados e publicados, mas sua identidade não será divulgada, sendo guardada em sigilo.

Os formulários serão guardados pelo coordenador da pesquisa por um período de 5 anos e depois serão destruídos. Para qualquer outra informação, você poderá entrar em contato com:

Pesquisadores: Francisco Fábio Oliveira de Sousa. Universidade Federal do Amapá, Coordenação da Farmácia. Rod. Juscelino Kubitschek, km 2, Jardim Marco Zero Macapá-AP, CEP: 68903-419. E-mail: phabio_oliveira@yahoo.com.br, Fone (96) 99913-3132.

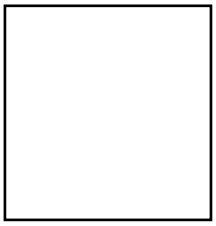
Ingrid Souza Reis Santos. Universidade Federal do Amapá, Coordenação de Pós-graduação em Ciências Farmacêuticas. Rod. Juscelino Kubitschek, km 2, Jardim Marco Zero Macapá-AP, CEP: 68903-419. E-mail: reisingrid@gmail.com, Fone (96)98134-4771.

Comitê de Ética em Pesquisa – CEP/UNIFAP: Rod. Juscelino Kubitschek, Km 2, Jardim Marco Zero Macapá – AP, CEP 68.903-419. Centro Integrado de Pesquisa da Amazônia – Unifap. E-mail: cep@unifap.br, Fone: (96)4009-2804.

CONSENTIMENTO PÓS–INFORMAÇÃO

Eu,_____, fui informada sobre o que a pesquisadora quer fazer e porque precisa da minha colaboração, e entendi a explicação. Por isso, eu concordo em participar da pesquisa, sabendo que não vou ganhar nada e que posso sair quando quiser. Este documento é emitido em duas vias que serão ambas assinadas por mim e pela pesquisadora, ficando uma via com cada um de nós.

Macapá, Data: ___/ ____/ _____.



Impressão do dedo polegar
Caso não saiba assinar

_____
Assinatura do participante

_____
Assinatura do Pesquisador Responsável

_____
Mestranda Responsável

Apêndice B – Questionário

Paciente nº:

Nome:
_____

Sexo: (M) (F) Idade (em anos completos): _____ Peso (em Kg): _____ Altura (em cm): _____

Telefone de contato: _____

Bairro:_____
Cidade:_____

Dados sociodemográficos relacionado ao paciente

- Qual seu grau de escolaridade:

( ) Analfabeto
Ensino Fundamental ( ) Completo ( ) Incompleto
Ensino Médio ( ) Completo ( ) Incompleto
Ensino Superior ( ) Completo ( ) Incompleto

-Você possui alguma ocupação?
( ) Não ( ) Sim Qual?

- Você reside com sua família?
( ) Sim ( ) Não

- Quantas pessoas residem com você?
_____

- Quem é o principal contribuinte financeiro da família? _____
- Qual o valor de sua renda familiar mensal?

( ) Até R$ 788,00 ( ) De R$ 788,01 até R$ 2.354,00 ( ) De R$ 2.354,01 até R$ 3.940,00
( ) De R$ 3.940,01 até R$ 11.820,00 ( ) Mais de R$ 11.820,00 ( ) Não sabe informar

Você possui alguma outra doença?
( ) Não ( ) Sim
- Caso sim, citar as outas doenças.

Doença	Tempo	Controlada?

Dados relacionados aos medicamentos: Instrumento adaptado *BriefMedicationQuestionnaire*

1) Quais medicações que você usou nos últimos 15 DIAS?

Entrevistador: Para cada medicação anote as respostas no quadro abaixo
Se o entrevistado não souber responder ou se recusar a responder coloque NR

NA ÚLTIMA SEMANA					
a) Nome do medicamento e dosagem	b) Quantos dias você tomou esse remédio?	c) Quantas vezes por dia você tomou esse remédio?	d) Quantos comprimidos ou doses você tomou em cada vez?	e) Quantas vezes você esqueceu de tomar alguma dose?	f) Como esse medicamento funciona para você 1 = Funciona Bem 2 = Funciona Regular 3 = Não funciona bem

2) Algum dos seus medicamentos causa problemas para você? (0) Não (1) Sim

a) Se o entrevistado respondeu SIM, por favor, liste os nomes dos medicamentos e quanto elas o incomodam

Quanto esse medicamento lhe incomodou?					
Medicamento	Muito	Um pouco	Muito pouco	Nunca	De que forma você é incomodado por ela?

3) Agora, citarei uma lista de problemas que as pessoas, às vezes, têm com seus medicamentos.

Quanto é difícil para você	Muito difícil	Um pouco difícil	Não muito difícil	Comentário (Qual medicamento)
Abrir ou fechar a embalagem				
Ler o que está escrito na embalagem				
Lembrar de tomar todo remédio				
Conseguir o medicamento				
Tomar tantos comprimidos ao mesmo tempo				

- Além dos medicamentos prescritos, você faz uso de algum outro medicamento fitoterápico ou não, como chás, xaropes e entre outros.

( ) Não ( ) Sim

Quais?_____

- Pra que você o(s) utiliza?