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Application of *Therapeutic Drug Monitoring* to measure drug concentrations in biological fluids in the context of resistant hypertension: clinical validation of an innovative "adherence-test".

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LIST OF ABBREVIATIONS

ABPM: ambulatory blood pressure monitoring
ACE: angiotensin-converting enzyme
ACN: acetonitrile
AD: fully adherent patient
AML: amlodipine
ARB: angiotensin receptor blocker
ATE: atenolol
BMI: body mass index
BP: blood pressure
CAN: canrenone
CCB: calcium channel blocker
CHL: clorthalidone
CLN: clonidine
C_{max}: maximum concentration reached by a drug
CRV: cerebrovascular
C_{trough}: concentration at the end of dosing interval
CV: cardiovascular
DBP: diastolic blood pressure
DMSO: dimethyl sulfoxide
DOT: directly observed therapy
DOX: doxazosin
EE: extraction efficiency
ESI: electrospray ionization
HBPM: home blood pressure monitoring
HCTZ: hydrochlorothiazide
H_2O: water
HPLC: high performance liquid chromatography
HR: heart rate
HT: hypertension
IDP: indapamide
IQR: interquartile range
IS: internal standard
LLOQ: lower limit of quantification
LOD: limit of detection
LOQ: limit of quantification
ME: matrix effect
MeOH: methanol
MRM: multiple reaction monitoring
MS: mass spectrometry
m/z: mass/charge ratio
NAD: totally non-adherent patient
NBV: nebivolol
NFD: nifedipine
OLM: olmesartan
PAD: partially adherent patient
PD: pharmacodynamics
PK: pharmacokinetics
PRHT: pseudo-resistant hypertension
RAM: ramipril
RAM-M: ramiprilat
RAS: renin–angiotensin system
REC: recovery
RHT: resistant hypertension
RSD: relative standard deviation
RT: retention time
QC: quality control
QX: 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline
SBP: systolic blood pressure
SCB: sacubitril
SCB-M: sacubitrilat
SPC: single-pill combination

SPI: spironolactone

STD: standard

TDM: therapeutic drug monitoring

TEL: telmisartan

TQD: triple quadrupole detector

UHPLC: ultra-high performance liquid chromatography

ULOQ: upper limit of quantification

UV: ultraviolet

VAL: valsartan

v:v: volume:volume

WCHR: white-coat heart rate

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ABSTRACT

Background and aims. Arterial hypertension is still among the most frequent causes of cardiovascular and cerebrovascular morbidity and mortality. A significative number of patients show resistance to the antihypertensive treatment and poor adherence to medication regimens is an important cause of treatment failure. Different methods to assess therapeutic adherence are currently available: Therapeutic Drug Monitoring (TDM) of antihypertensive drugs in biological fluids has previously revealed its efficacy and reliability. This work aimed to assess the therapeutic compliance of patients with a diagnosis of resistant hypertension through the TDM across different matrices, other than to identify some key predictors of treatment nonadherence.

Methods. Fourteen antihypertensive drugs and two metabolites were simultaneously tested in plasma, urine and saliva. Analyzed molecules included: atenolol, nebivolol, clonidine, ramipril, olmesartan, telmisartan, valsartan, amlodipine, nifedipine, doxazosin, chlorthalidone, hydrochlorothiazide, indapamide, sacubitril, ramiprilat and sacubitrilat. TDM was performed using ultra-high performance liquid chromatography, coupled to tandem mass spectrometry (UHPLC-MS/MS). The combined protocol has been preliminary applied on a cohort of hypertensive patients.

Results. TDM of plasma samples, which is generally considered the gold-standard, revealed that only 58% of patients were fully adherent; a multivariate logistic regression revealed that office diastolic blood pressure and heart rate could potentially identify real uncompliant patients. Moreover, ROC curve analyses indicated that patients with diastolic blood pressure values higher than 124.5 mm/Hg have higher probability to be uncompliant. Finally, an analytical method for salivary detection of antihypertensive drugs has been developed and validated following European guidelines: when compared to plasma, it demonstrated sensibility and specificity of 98% and 98.1% respectively, with a good feasibility in real life clinical practice.

Conclusions. In our subpopulation, through the TDM analysis and the early detection of non-adherent patients, some invasive practices could probably have been avoided. Considering this, we believe that this tailored therapy may be useful other than cost-effective. Saliva may represent a feasible biological sample for TDM, by non-invasive collection, prompt availability and potential accessibility also in out-of-clinic settings.

In the near future, this study should be confirmed on larger cohorts of patients in order to unveil and better describe drugs pharmacokinetics across different matrices and potentially suggest some therapeutic ranges for antihypertensive drugs.

1. INTRODUCTION

1.1 Hypertension and Resistant Hypertension

Hypertension (HT) is defined as office systolic blood pressure (SBP) values at least 140mmHg and/or diastolic BP (DBP) values at least 90 mmHg and it is considered as the level of BP at which the benefits of treatment (either pharmacological or simply lifestyle changes) unequivocally take priority over the risks of treatment, as documented by clinical trials [1] (Table 1).

Category	Systolic (mmHg)		Diastolic (mmHg)
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension[b]	≥140	and	<90

BP, blood pressure.
[a]BP category is defined according to seated clinic BP and by the highest level of BP, whether systolic or diastolic.
[b]Isolated systolic hypertension is graded 1, 2, or 3 according to systolic BP values in the ranges indicated. The same classification is used for all ages from 16 years.

Table 1: Classification of office blood pressure[a] and definitions of hypertension grade[b] [1].

It is still currently a significant health problem, and its overall prevalence in adults is around 30– 45% [1] with an average prevalence of 70-75% in the population over sixty years of age [2]. In detail, by considering office blood pressure (BP), the global prevalence of hypertension was estimated to be 1.13 billion in 2015, with a prevalence of over 150 million in central and Eastern Europe [1]. With a progressive ageing of adult population, an increasingly more sedentary lifestyle, and a very high percentage of overweight subjects, the prevalence of hypertension worldwide will continue to rise: it is estimated that the number of people with hypertension will increase by 15 – 20% by 2025, reaching close to 1.5 billion [1].

HT is comprised among the most frequent causes of cardiovascular (CV) and cerebrovascular (CRV) events of various severity. Epidemiological associations between BP values and CV risk have been demonstrated starting from non-hazardous levels of BP (i.e. SBP >115 mmHg) [1].

Current antihypertensive treatment is often based on a combination therapy, by the administration of multiple classes of drugs with different mechanisms of action: diuretics, α/β blockers, calcium antagonists, ACE-inhibitors and sartans [3,4]. In particular, a drug treatment algorithm has been developed to provide a simple and pragmatic treatment recommendation for the treatment of hypertension, based on the following key recommendations [1] (Figure 1):

- The initiation of treatment in most patients with a single-pill combination (SPC) comprising two drugs, to improve the speed, efficiency, and predictability of BP control;
- Preferred two-drug combinations are a renin–angiotensin system (RAS) blocker with a calcium channel blocker (CCB) or a diuretic. A β-blocker in combination with a diuretic or any drug from the other major classes is an alternative when there is a specific indication for a β -blocker, for example angina, postmyocardial infarction, heart failure, or heart rate control;
- Use monotherapy for low-risk patients with stage 1 HT whose SBP is < 150 mmHg, very high-risk patients with high–normal BP, or frail older patients;
- The use of a three-drug SPC comprising a RAS blocker, a CCB, and a diuretic if BP is not controlled by a two-drug SPC;
- The addition of spironolactone for the treatment of resistant hypertension, unless contraindicated;
- The use of other classes of antihypertensive drugs in the rare circumstances in which BP is not controlled by the above treatments;

- Information on availability and recommended doses of individual drugs, as well as SPCs and free combinations, can be found in national formularies.

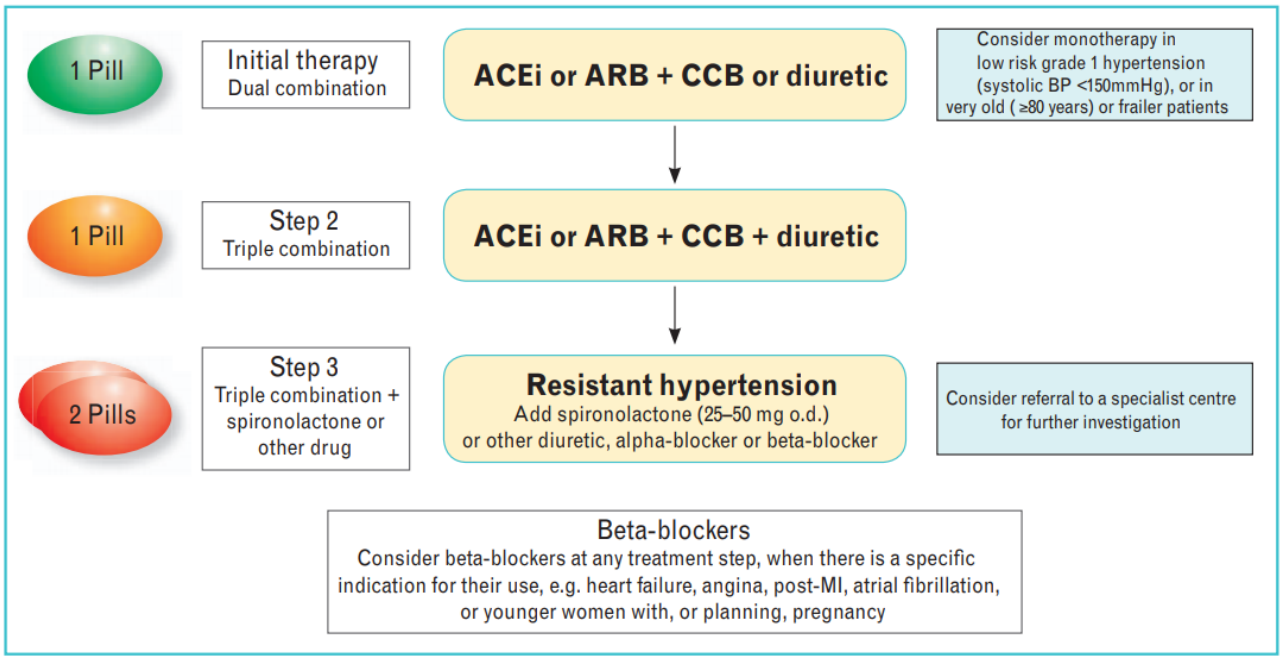

Figure 1: Core drug treatment strategy for uncomplicated hypertension. The core algorithm is also appropriate for most patients with HT-mediated organ damage, cerebrovascular disease, diabetes, or peripheral artery disease. ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; MI, myocardial infarction; o.d., once daily.

While in the vast majority of patients the administration of one to three classes of these drugs is effective enough to control the BP, in some cases the addition of more drugs is necessary (about 15-30% of treated patients) [5]: this condition is called resistant hypertension (RHT) [4,6].

Hypertension is defined as resistant to treatment when "the recommended treatment strategy fails to lower office SBP and DBP values to less than 140 mmHg and/or less than 90 mmHg, respectively, and the inadequate control of BP is confirmed by ambulatory blood pressure monitoring (ABPM) or home blood pressure monitoring (HBPM) in patients whose adherence to therapy has been confirmed" [1]. The recommended treatment strategy should include appropriate lifestyle measures and treatment with optimal or best tolerated doses of three or more drugs, which should include a diuretic, typically an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker (ARB), and a CCB. Pseudo-

resistant hypertension (PRHT) and secondary causes of hypertension should also have been excluded [1].

Very few studies reported a prevalence for RHT, mainly due to variation in the definition used, and the suggested rates ranged from 5 to 30% in patients with treated hypertension. Nevertheless, by applying a strict definition and having excluded causes of PRHT, the true prevalence of RHT is likely to be less than 10% of treated patients [1].

The clinical relevance of this phenomenon is supported by the worse prognosis of these patients and the high costs of more intensive pharmacological treatment and/or invasive surgery, which are often essential in the management of RHT [7-9].

The main difficulty in this case is the discrimination of real cases of resistance from the ones of pseudo-resistance [10,11]. It is therefore of interest to discriminate between RHT and PRHT. The latter may depend on clinician-related factors, such as non-optimal therapeutic regimens or therapeutic inertia [4,12]. More frequently, patient-related factors underlie PRHT including poor therapeutic adherence in a significant proportion of these patients [13-15].

There are several approaches to assess therapeutic adherence which must be reliable and exempt from potential censorship or manipulation of data by patients [16]. Currently, available methods are classified as indirect or direct [17]: indirect methods include questionnaires, patient interviews, diaries, pill counting and electronic monitoring of pill boxes (MEMS), but the requirement of patient collaboration limits the efficacy of such methods. In contrast, direct methods, that are more intrusive but also more reliable, include Directly Observed Therapy (DOT) or Therapeutic Drug Monitoring (TDM) [15].

1.2 Therapeutic Drug Monitoring

TDM consists in the measurement of drugs concentrations in biological matrices (usually plasma or blood), in order to compare them with known therapeutic ranges, deriving from

pharmacokinetic/pharmacodynamic (PK/PD) studies: this allows to optimize the posology, avoiding concentration-dependent toxic effects and/or therapeutic failures due to underexposure to prescribed drugs [18]. TDM is routine practice for several therapies, such as immunosuppressive, antifungal, antibiotic, antiviral, anticonvulsants, antiarrhythmics and antipsychotic drugs [18-25].

However, the indications for TDM have widened to include efficacy, compliance, drug-drug interactions, toxicity avoidance, and therapy cessation monitoring. Poor compliance can be identified through TDM as a very low/null drug concentration which cannot reflect the prescribed dose, as compared with literature reported data in similar individuals [26]. This evidence becomes stronger when this result is obtained for several different drugs prescribed to the same patient.

Aiming to this, lots of analytical methods have already been developed concerning antihypertensive drugs, but a large part of them is dedicated to a single molecule or to a couple of them [27-34]. However, this solution results poorly applicable to the clinical routine, where there is the need of multiplexed and high-throughput analyses. For this purpose, two analytical multi-drug methods were developed by our group to evaluate patients' compliance to antihypertensive therapy [35,36].

1.3 Instrumental setting

1.3.1 Liquid Chromatography

Liquid chromatography is a well-established technique used in clinical chemistry and pharmacology for the separation of substances contained in a mixture.

The separation principle of High Performance Liquid Chromatography (HPLC) is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded during the flow across the stationary phase. The specific

intermolecular interactions between the molecules of a sample and the packing material define their retention time (RT). Hence, different molecules contained in a sample will be characterized by different RTs. Thereby, the separation of the sample ingredients is achieved. A detection unit (e.g. mass spectrometer, UV detector, …) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data proces-sing unit (Figure 2). The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. The sample is provided to the eluent by the injection valve [37].

Depending on the composition of the mobile phase, two different modes are generally applicable. If the makeup of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system. When the composition of the mobile phase is changed during separation, the HPLC system is defined as a gradient elution system. When the analyte shows a higher affinity to the mobile phase than to the stationary one, it elutes out from the column. The column represents the heart of any HPLC system. It is responsible for the adequate separation of the sample ingredients. The separation efficiency correlates with the column inner diameter, the length of the column and the type and particle size of the column packing material. The HPLC system has been updated and upgraded, leading to a more performant system, named UHPLC, which can work at higher pressures, reducing the duration of analysis and improving the chromatographic separation. The stationary phase of an UHPLC column is made up of particles with a dimeter smaller than 2 μm (while HPLC columns are typically filled with particles that range between 3 and 5 μm).

Finally, the employment of an automatic autosampler allows the analysis of a large number of samples, being analyzed sequentially, with specific calibration curves and internal quality controls (QCs).

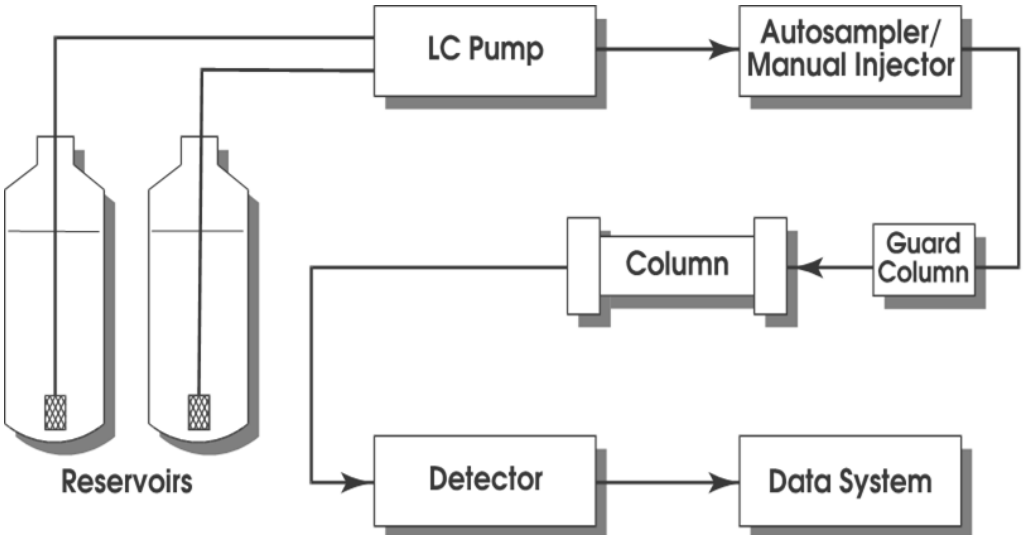

Figure 2: UHPLC instrumental apparatus.

1.3.2 Mass spectrometry

A chromatographic detector has to be capable of establishing both the identity and concentration of eluting components in the mobile phase flow [38]. A broad range of detectors is available to satisfy different requirements: in particular, Triple Quadrupole mass spectrometer (MS) Detector, also known as TQD, has recently become a gold standard for TDM purposes, thanks to its great sensibility and specificity [39]. The first quadrupole (Q1) selects the precursor ion of interest through a magnetic deflection of all other ions, which are so discarded, on the basis of its mass/charge ratio (m/z). The precursor ion moves towards the collision cell (also considered as a second quadrupole, Q2), where usually it is fragmented by energetic collisions with a inhert gas (e.g. argon); then, all the fragmented ions are collimated and enter into the third quadrupole (Q3). As described above, even Q3 performs a mass discrimination of the obtained fragments (daughter scan): in this way a tandem mass spectrum is obtained and it is very analyte-specific. So, the whole liquid

chromatography coupled to tandem mass process results in a sequential increase in specificity: chromatographic separation discriminates temporally (in terms of RT) the target molecules on the basis of their chemical properties; then, at the corresponding RT, the Q1 selects the molecules with the right m/z ratio ("mother" ions) and Q2/Q3 further confirm the identity of target molecules on the basis of their specific molecular structure, which determines the resulting fragments ("daughter" ions). The very low background noise allows the instrument to greatly increase the sensibility of the assay if compared to other detectors (single quadrupole, photodiode array, fluorescence and UV).

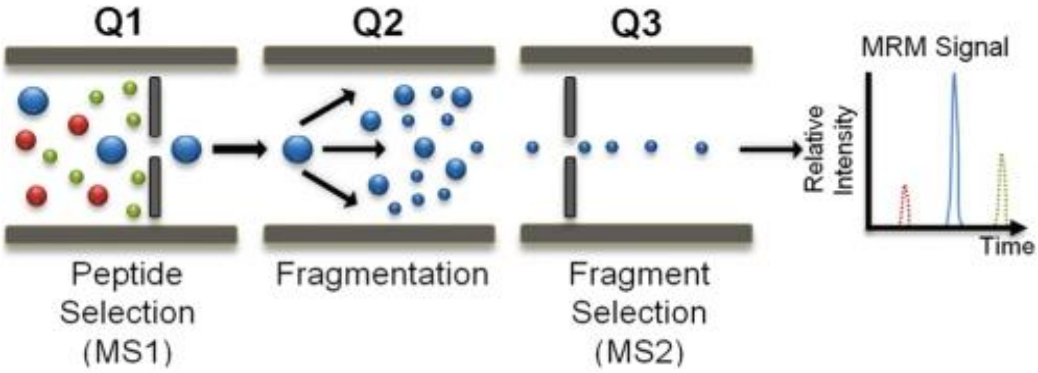

Figure 3: Triple Quadrupole Detector (TQD) overview. After ionization the target molecules, in the first quadrupole (Q1) are collimated through a magnetic field towards the collision cell (Q2), where the collision with inhert gas breaks down the mother ion in fragments; these fragments are selected and collimated through the third quadrupole (Q3) and finally detected.

1.4 Overview of bioanalytical method validation

A full method validation should be performed for any analytical method whether new or based upon literature. Specific guidelines regulate the validation process [40-43]. The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. Generally, a full validation should be performed for each species and matrix concerned.

The main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results are: selectivity, lower limit of quantification, the response function and calibration range (calibration curve

performance), accuracy, precision, matrix effects, stability of the analytes in the biological matrix and stability of the analytes and of the internal standard in the stock and working solutions and in extracts under the entire period of storage and processing conditions.

1.4.1 Reference standards

During method validation and analysis of study samples, a blank biological matrix will be spiked with the analytes of interest by using previously prepared stock solutions to prepare calibration standards and QC samples [40-43]. In addition, suitable internal standards (IS) can be added during sample processing in chromatographic methods in order to correct operator-related errors.

It is important that the quality of the reference standard and IS is ensured, as the purity may affect the outcome of the analysis, and therefore the outcome of the study data. Therefore, the reference standards used during the validation and study sample analysis should be obtained from an authentic and traceable source. A certificate of analysis is required to ensure purity and provide information on storage conditions, expiration date and batch number of the reference standard.

When MS detection is used in the bioanalytical method, a stable isotope-labelled IS is recommended to be used, whenever possible. However, it is essential that the labelled standard is of the highest isotope purity and that no isotope exchange reaction occurs. The presence of any unlabelled analyte should be checked and, if relative amounts of unlabelled analyte are detected, the potential influence has to be evaluated during method validation.

1.4.2 Validation parameters

Method development involves optimizing the procedures and conditions involved with extracting and detecting the analyte; it includes, as requested by guidelines, the optimization of the following bioanalytical parameters to ensure that the method is suitable for validation [40-43]:

- Calibration curve: during method development, the quantitation range of the assay and the concentrations of the calibration standards must be chosen on the basis of the concentration range expected in a particular study. When the method is validated, the calibration curve should be continuous and reproducible. The calibration standards should be prepared in the same biological matrix as the samples in the intended study;
- Quality control samples: they are used to assess the precision and accuracy of an assay and the stability of the samples. QCs should be prepared in the same matrix as the study samples to be assayed with the validated method;
- Selectivity: the analytical method should be able to differentiate the analytes of interest and IS from endogenous components in the matrix or other sample components;
- Specificity: it is the ability of the method to assess, unequivocally, the analyte in the presence of other components that are expected to be present (e.g., impurities, degradation products, matrix components, etc.);
- Sensitivity: it is defined as the lowest analyte concentration in the matrix that can be measured with acceptable accuracy and precision; Limit of quantification (LOQ) is defined as the lowest calibration standard; the lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision, and its signal should be at least 5 times the signal of a blank sample; the Limit Of Detection (LOD) is the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise, and it should be at least 3 times the signal of a blank sample; finally, the upper Limit of Quantification (ULOQ) is the highest standard (STD) of the analytical method;
- Carry-over: it is the appearance of an analyte in a sample from a preceding sample; it should be assessed and minimized during method development procedure;
- Accuracy: it is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. Accuracy is also sometimes termed trueness;

- Precision: it is the closeness of agreement among a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions;
- Recovery: it is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent;
- Matrix effect: it is a direct or indirect alteration or interference in response because of the presence of unintended analytes or other interfering substances in the sample matrix;
- Stability: it is a measure of the intactness an analyte (lack of degradation) in a given matrix under specific storage and use conditions relative to the starting material for given time intervals.

2. AIMS OF THE STUDY

As before mentioned, three analytical methods both for plasma and urine samples had already been developed by our group [36,44,45]; monitored drugs were: atenolol (ATE; β-blocker), clonidine (CLN; α2-agonist), doxazosin (DOX; α1-antagonist), amlodipine and nifedipine (AML, NFD; CCBs), chlorthalidone (CHL; diuretic), hydrochlorothiazide (HCTZ; thiazide diuretic), ramipril (RAM; ACE-inhibitor), olmesartan, and telmisartan (OLM, TEL; sartans/ARBs), spironolactone and canrenone (SPI, CAN; diuretics). Nevertheless, there is the permanent need to keep them updated by adding some more recent molecules, other than introducing less invasive matrices, such as saliva. For these reasons, the primary aim of this project has been the assessment of the clinical usefulness of a routine TDM practice based on the abovementioned methods, in order to discern real RHT patients from cases of poor compliance, and to identify some "predictors" of treatment nonadherence. Contextually, the measurement of drugs concentration in saliva with the possibility to use it as alternative matrix, at least for some "key" drugs, has been performed. The resulting analytical method, although characterized by several limitations in terms of salivary drugs distribution, may be used even outside the clinical routine, for example in the pharmacies or for outpatient visits, resulting in lower white-coat adherence, reduced invasiveness and lower costs. On the other hand, among the secondary aims, there was the improvement of previous LC-MS/MS methods, through the addition of four other antihypertensive drugs (nebivolol, *NBV, β-blocker*; indapamide, *IDP, thiazide diuretic*; valsartan, *VAL, sartan* and sacubitril, *SCB, neprilysin inhibitor* – used in combination with VAL in case of patients with heart failure), two metabolites (sacubitrilat, SBC-M and ramiprilat, RAM-M) and some isotope-labeled internal standards ([$^{2}H_{7}$]-atenolol, [$^{13}C_{8}$]-nifedipine, [^{13}C,$^{2}H_{3}$]-telmisartan and [$^{2}H_{4}$]-amlodipine) to the panel of analytes. Finally, a fast dedicated method was needed for CAN quantification in human plasma: in fact, up to now SPI and its metabolite CAN were quantifiable only in urine, by using an UV detector, due to their tendency to form adducts.

3. MATERIAL AND METHODS

3.1 Chemicals and stock solutions

UHPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR (Radnor, PA, USA). UHPLC grade H_2O was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Blank saliva was obtained from healthy donors. ATE (purity >98%), CLN hydrochloride (overall purity 86.22%), DOX mesylate (overall purity 81.29%), AML (purity 99.52%), NFD (purity 97.64%), CHL (purity 99.9%), HCTZ (purity 99.2%), RAM (purity 99.81%), TEL (purity 99.96%), OLM (purity 99.01%), VAL (purity 99.35%), NBV hydrochloride (overall purity 90.83%), SCB (purity 99.71%), SCB-M (purity 99.85%), IDP (purity 99.61%) and CAN (purity 99.16%) were purchased from MedChem Express (Monmouth Junction, NJ, USA); RAM-M (purity 99.4%) was purchased from Santa Cruz Biotecnology (Dallas, TX, USA). Concerning the internal standards (IS), 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline (QX) (purity 98.5%) and [$^2H_7$]-atenolol (purity 98.7%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA), while [$^2H_4$]-amlodipine maleate (overall purity 78%), [$^{13}C_8$]-nifedipine (purity 95.6%) and [^{13}C,$^2H_3$]-telmisartan (purity 98.4%) were purchased from Alsachim (Illkirch, France). All powders were stored at -20°C or +4°C in the dark, according to the manufacturer's recommendations, in order to prevent any possible degradation.

Stock solutions (1 mg/mL) were prepared singularly as below: DOX, AML, [$^2H_4$]-amlodipine, CHL, HCTZ, NFD, [$^{13}C_8$]-nifedipine, OLM, RAM, RAM-M, SCB-M, IDP, VAL, CAN and QX in a mixture of H2O:MeOH 5:95 (v:v); TEL and [^{13}C,$^2H_3$]-telmisartan in dimethyl sulfoxide (DMSO):MetOH 5:95 (v:v); ATE, [$^2H_7$]-atenolol and NBV in H2O:MetOH 50:50 (v:v), CLN in pure H_2O and SCB in H2O:MetOH:DMSO 1:1:1 (v:v:v). Stock solutions were stored at -20°C until use (less than 6 months).

3.2 Standards and quality controls

3.2.1 Plasma and saliva

Calibration ranges and QC concentrations have been defined according to literature and are summarized in table 2.

Concentration (ng/mL)	CAL 9	QC H	QC M	QC L	LLOQ
CLN	10	8	1	0,1	0,039
NBV	10	8	1	0,1	0,039
DOX	10	8	1	0,1	0,039
AML	20	16	2	0,2	0,078
HCTZ	100	80	7	0,7	0,391
TEL	100	80	10	1	0,391
NFD	100	80	10	1	0,391
RAM	100	80	10	1	0,391
RAM-M	100	80	10	1	0,391
IDP	100	80	10	1	0,391
OLM	250	200	25	2,5	0,977
CHL	1000	700	100	10	3,906
ATE	1000	800	100	10	3,906
SCB	2000	1600	200	20	7,813
VAL	3000	2400	300	30	11,719
SCB-M	8000	6400	800	80	31,250
CAN (only plasma)	200	160	15	1.5	0,391

Table 2: Summary of plasma and saliva concentrations in standards and quality control samples for each drug.

3.2.2 Urine

Calibration ranges and QC concentrations are summarized in table 3.

Concentration (ng/mL)	CAL 9	QC H	QC M	QC L	LLOQ
RAM	200	160	20	2	0.391
CLN	1000	800	100	10	1.953
IDP	2000	1600	200	20	3.906
DOX	2000	1600	200	20	3.906
AML	2000	1600	200	20	3.906
VAL	10000	8000	1000	100	19.531
TEL	10000	8000	1000	100	19.531
SCB	10000	8000	1000	100	19.531
SCB-M	20000	16000	2000	200	39.062
RAM-M	20000	16000	2000	200	39.062
CHL	20000	16000	2000	200	39.062
ATE	20000	16000	2000	200	39.062
OLM	20000	16000	2000	200	39.062
NFD	20000	16000	2000	200	39.062
NBV	20000	16000	2000	200	39.062
HCTZ	40000	32000	4000	400	78.125

Table 3: Summary of urinary concentrations in standards and quality control samples for each drug.

3.3 Sample preparation

3.3.1 Plasma

Concerning the multi-drug method, the analyses were focused on the following drugs: ATE, NBV, CLN, DOX, AML, NFD, CHL, HCTZ, IDP, RAM, OLM, TEL, VAL and SCB, together with two drug metabolites (RAM-M and SCB-M).

For antihypertensive drugs extraction, 200 µL of plasma samples and 40 µL of IS working solution (2 µL of QX, 1 µL of [$^{2}H_{7}$]-atenolol, 0.1 µL of [$^{13}C_{8}$]-nifedipine, [$^{2}H_{4}$]-amlodipine and [^{13}C,$^{2}H_{3}$]-telmisartan stock solution in 4 mL of H_2O:MetOH [50:50]; final concentrations as follow: 500 ng/mL for QX, 250 ng/mL for [$^{2}H_{7}$]-atenolol and 25 ng/mL for [$^{13}C_{8}$]-nifedipine, [$^{2}H_{4}$]-amlodipine and [^{13}C,$^{2}H_{3}$]-telmisartan) are added with 1 mL of ACN in amber PTFE tubes. Samples are then vortex-mixed for 10 seconds and centrifuged at 21000 x *g* at 4°C for

10 minutes. The resulting supernatant is transferred into glass tubes and evaporated at 50°C (for about 1.5 hr) by using a cold-vacuum trap. Dry extracts are then resuspended in 200 µL of H_2O:ACN 90:10 v:v (+ formic acid 0.05%), vortexed for at least 15 seconds and finally transferred in plastic vials: 3 µL of the resulting extracts are finally injected into the UHPLC-MS/MS system. The adoption of plastic material is due to tendency of some molecules (AML, DOX and NBV) to adhere to the glass tube walls, especially in the absence of matrix (e.g. plasma or urine).

The same protocol is followed also for CAN analysis with only two exceptions: the IS in this case contains only QX at the final concentration of 500 ng/mL, and dry extracts are resuspended in 200 µL of H_2O:ACN (+ formic acid 0.05%) 70:30 v:v (instead of 90:10).

3.3.2 Urine

Even in this case, the analyses have been focused on the following drugs: ATE, NBV, CLN, DOX, AML, NFD, CHL, HCTZ, IDP, RAM, OLM, TEL, VAL, SCB, RAM-M and SCB-M.

Due to the high concentration of antihypertensive drugs in urine, sample preparation consists in a simple 1:10 dilution in order to avoid signal saturation and to reduce system contamination and matrix effect: 100 µL of urine spot samples and 40 µL of IS working solution are added with 860 mL of H_2O:ACN 90:10 v:v (+ 0.05% formic acid) in amber PTFE tubes. Samples are then vortex-mixed for 10 seconds and centrifuged at 21000 x *g* at 4°C for 10 minutes in order to eliminate any possible solid residue (eg. uric acid crystals). In some cases the formation of a very small pellet can be observed. The supernatant is finally transferred in bulk vials: 0.3 µL of the resulting extracts are injected into the UHPLC-MS/MS system.

3.3.3 Saliva

3.3.3.1 Preliminary experiments: drug absorption to Salivette® devices

Salivette® (Sarstedt, Nümbrecht, Germany) devices were chosen for saliva collection. Considering the scarcity of data about their employment for antihypertensive drugs TDM purpose, some preliminary experiments have been performed in order to unveil and describe drug retention by Salivette® matrix. In order to describe this phenomenon, separate experiments were performed both in solvent and saliva.

Concerning solvent, three mix containing all sixteen drugs at 3 different concentrations (1000, 100 and 10 ng/mL) were prepared by independently spiking drug stock solutions in a mixture of water and acetonitrile (H_2O:ACN 90:10, v:v) added with 0.05% of formic acid. Two milliliters of each solution were allowed to soak for 1 minute into the Salivette® cotton roll at 37°C (performed in double replicate), followed by centrifugation and collection. A small amount (7 µL) of the resulting volume was directly injected in the UHPLC instrument and analyzed together with the same volume deriving directly from the three mix (1000, 100 and 10 ng/mL), without the passage through the Salivette®.

Concerning saliva, experiments have been focused on the 100 ng/mL concentration: blank sputum from healthy donors was collected in falcon tubes and then spiked with all the 16 drugs at the same concentration. Then, 2 mL were allowed to soak into the Salivette® cotton roll for 1 minute at 37°C (performed in double replicate), and then centrifuged, collected and extracted (according to the protocol below). The remaining volume was directly extracted without the passage through the Salivette®. Resulting peak areas have been compared in order to address the extent of the retention. In order to find possible correlations, a series of molecule-related parameters was collected for each drug: logK (o/w), pKa, molecular weight and retention time.

3.3.3.2 Saliva sample extraction procedure

Saliva samples have been collected by using the Salivette® system, following the manufacturer's instructions: briefly the patient removes the swab from the Salivette® and places it in the mouth for about 60 seconds to stimulate salivation; then, the patient returns the swab into the Salivette® and replaces the stopper; finally, the laboratory staff centrifuge the samples for 2 minutes at 1000 x g in order to obtain a clear saliva sample. Each sample was stored at -20°C until analysis.

Along the extraction procedure, standards/quality controls and unknown samples are treated in two slightly different ways. For the calibration curve and the QCs, 40 µL of IS working solution (final concentrations are as follow: 500 ng/mL for QX, 250 ng/mL for [$^{2}H_{7}$]-atenolol and 25 ng/mL for [$^{13}C_{8}$]-nifedipine, [$^{2}H_{4}$]-amlodipine and [^{13}C,$^{2}H_{3}$]-telmisartan) are added to 40 µL of "calibrating" solution and 200 µL of blank saliva. On the other hand, patients' samples are extracted by adding 40 µL of IS working solution to 40 µL of a blank mixture of H_2O:ACN 90:10 (v:v) and 200 µL of saliva sample. Then, in order to perform the protein precipitation, 1 mL of pure ACN is added to each sample and, in order to equilibrate the salivary pH, 200 µL of ammonium acetate buffer 10 mM (+0.1% acetic acid) are added to each eppendorf tube. Finally, all the samples are vortex-mixed and centrifuged at 21000 *x g* at +4°C for 10 minutes, then transferred into glass tubes and evaporated to dryness at 50°C (for about 1.5 hr). Dry extracts are then resuspended in 200 µL of H_2O:ACN (+ formic acid 0.05%) 90:10 v:v, vortex-mixed and finally transferred in plastic vials: 7 µL of the resulting extracts are injected into the UHPLC MS/MS system.

3.4 UHPLC-MS/MS instruments and chromatographic conditions

A Perkin Elmer LX-50® UHPLC system coupled with Triple Quadrupole QSight 220® (Perkin Elmer, Milan, Italy) is used for the chromatographic analysis.

For the sixteen molecules-analysis, the chromatographic separation is achieved through an Acquity® UPLC HSS T3 1.8 µm 2.1x150 mm (Waters, Milan, Italy), protected by a frit [0.2 µm, 2.1 mm] (Waters, Milan, Italy) precolumn, at 40°C using a column thermostat, with a gradient (Table 4) of two mobile phases: phase A (H_2O + formic acid 0.05%) and phase B (ACN + formic acid 0.05%). The instrument is settled in positive electrospray ionization mode (ESI+) for all drugs, except for HCTZ and CHL, which are detected in negative ionization mode (ESI-).

For what concerns CAN quantification, chromatographic separation is obtained on an Acquity® HSS T3 1.8µm 2.1x50mm column (Waters, Milan, Italy), protected by a physical filter ["Frit", 0.2 µm, 2.1 mm] (Waters, Milan, Italy) precolumn, at 40°C using a column thermostat. The mobile phases are the same reported above and the gradient is highlighted in Table 5.

Even in this case, the instrument is settled in positive electrospray ionization mode (ESI+).

General mass settings and multiple reaction monitoring (MRM) traces are resumed in Table 6. Concerning CAN and its IS, the quantification MRM traces (m/z) were: 341.10>187.1 for CAN and 313.20>78.05 for QX.

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.400	90	10
1.10	0.400	90	10
9.00	0.400	25	75
9.60	0.450	5	95
10.60	0.450	5	95
10.65	0.450	90	10
10.90	0.450	90	10
11.00	0.400	90	10
13.00	0.400	90	10

Table 4: Chromatographic gradient of mobile phases A (water + formic acid 0.05%) and B (acetonitrile + formic acid 0.05%) used for the separation of the sixteen drugs in both plasma, urine and saliva samples.

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.400	70	30
2.90	0.400	40	60
3.05	0.400	5	95
4.00	0.400	5	95
4.10	0.400	70	30
5.00	0.400	70	30

Table 5: Chromatographic gradient of mobile phases A (water + formic acid 0.05%) and B (acetonitrile + formic acid 0.05%) used for the separation of CAN and its internal standard in plasma samples.

GENERAL DETECTOR SETTINGS								
Drying Gas Temperature	130°C							
HSID Temperature	270°C							
Nebulizer Gas	350 L/h							
ElectroSpray V1 Positive	5000 V							
Source Temperature	350°C							
Multipole 1 RF	370							
Collision Pressure	410							
ANALYTE SPECIFIC PARAMETERS								
MASS PARAMETERS	**DRUGS**							
	CLN	**DOX**	**AML**	**HCTZ**	**NFD**	**TEL**	**RAM**	**OLM**
Primary Ion Trace (m/z)	230.0>44	452.2>344.2	409.15>238.1	296>205.0	347.15>239.1	515.25>211.1	417.2>117.1	447.2>207.1
Collision Energy	-35	-41	-19	31	-20	-55	-53	-30
Entrance voltage	41	47	10	-40	9	19	21	21
Collision Cell Lens 2	-60	-116	-76	88	-72	-184	-96	-96
Secondary Ion Trace (m/z)	230.0>213	452.2>247.1	409.15>294.1	296>269.05	347.15>195.1	515.25>276.2	417.2>234.2	447.2>235.1
Collision Energy	-35	-54	-17	26	-49	-62	-27	-29
Entrance voltage	39	50	9	-40	9	39	30	20
Collision Cell Lens 2	-92	-160	-84	72	-88	-200	-80	-108
Ionization	ESI+	ESI+	ESI+	ESI-	ESI+	ESI+	ESI+	ESI+
	ATE	**CHL**	**IDP**	**VAL**	**NBV**	**SCB**	**SCB-M**	**RAM-M**
Primary Ion Trace (m/z)	267.15>145.05	337.05>190	366.1>132.1	436.25>235.1	406.2>123.1	412.2>193.1	384.2>193.15	389.2>117.1
Collision Energy	-36	24	-20	-27	-57	-44	-40	-50
Entrance voltage	11	-32	18	18	26	24	6	27
Collision Cell Lens 2	-64	76	-68	-80	-104	-80	-80	-80
Secondary Ion Trace (m/z)	267.15>190.1	337.05>146	366.1>117.1	436.25>207.1	406.2>151.1	412.2>266.2	384.2>266.2	389.2>206.15
Collision Energy	-24	26	-64	-37	-39	-25	-24	-28
Entrance voltage	8	-35	11	18	34	25	16	25
Collision Cell Lens 2	-64	64	-76	-84	-88	-76	-76	-84
Ionization	ESI+	ESI-	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+

Table 6: Summary of instrument settings and MRM transitions.

3.5 Method validation

All developed method should be validated according to FDA and EMA guidelines [40-42,46]. In this project, accuracy, imprecision and limits of quantification have been defined based on six inter-day validation sessions. Moreover, intra-day imprecision was evaluated in 5 intraday replicates. Imprecision was expressed as the relative standard deviation (RSD) at each QC concentration. Integration was performed by considering peak areas for each analyte. Specificity and selectivity were evaluated using six individual sources of the blank matrix (plasma, saliva or urine), individually analyzed, and evaluated for interferences. The ULOQ corresponds to STD 9, the highest calibration standard, for all the analytes; LLOQ were the lowest concentration of analytes in a sample which can be quantified reliably, with a deviation from the nominal concentration (measure of accuracy) and RSD (measure of precision) lower than 20% and with a signal-to-noise ratio higher than 5.

Recovery (REC) was evaluated during six validation sessions at high, medium and low concentrations by comparing peaks areas from extracted QCs (pre-spiked) with those obtained by the direct injection of a chemical mix containing both the drugs and the IS at the same concentrations as the QCs (rec).

Separate matrix samples from six healthy donors were used for the extraction procedure and for the evaluation of matrix effect (ME). The ME was calculated by comparing the signal from the analysis of a post-extraction spiked samples (post-spiked) at high, medium and low QC levels with the ones from the direct injection of the same concentration of analytes without matrix, as described by Taylor [47] and in FDA guidelines (post-extraction addition method).

The extraction efficiency (EE) was measured by comparing the peak areas of pre- and post-spiked samples.

3.6 Design of the study

A complete overview of the study protocol is depicted in Figure 4.

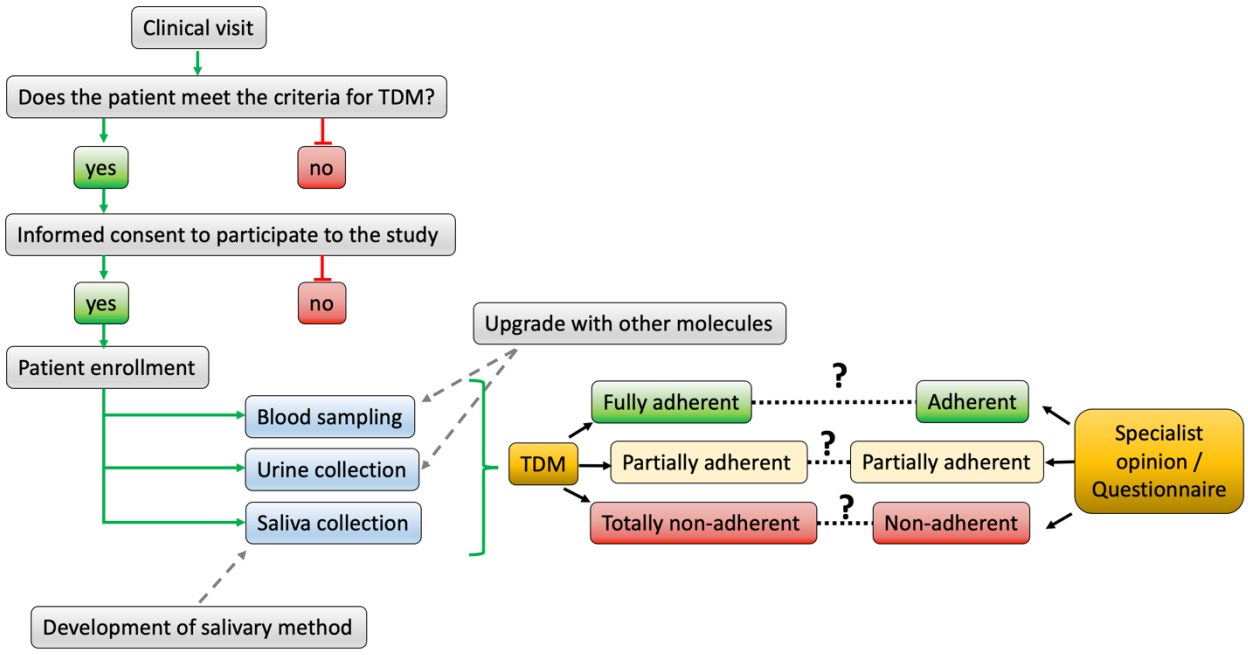

Figure 4: overview of the design of the study.

3.7 Patients' enrollment

Patients with RHT were recruited at the Hypertension Unit of "Città della Salute e della Scienza" in Turin referred to the Hypertension Unit. Blood samples were withdrawn during routine analyses. All patients gave informed consent according to local Ethics Committee indications (TDM-TO study, protocol CS/504 03/09/2015).

RHT was defined as office SBP >140 mm Hg and/or office DBP>90 mmHg, despite regular intake of maximally tolerated doses of ≥3 antihypertensive drugs including a diuretic for at least 6 weeks. In all patients secondary and spurious hypertension causes such as white coat RHT (SBP <130 mmHg and DBP <80 at 24h ABPM), drug related causes or manifest nonadherence, were excluded.

Medical history, anthropometric data and indirect assessment of adherence were collected the same day of blood sampling for TDM. Anthropometric variables such as age, sex, height, weight, Heart Rate (HR), Body Mass Index (BMI), degree and duration of arterial

hypertension, cardiovascular comorbidities, pharmacological therapy were collected for each patient.

Office SBP and DBP measurement were performed the same day of blood sampling for TDM, according to the indications provided by the European guidelines [4].

Moreover, all patients with RHT underwent 24 hours ABPM. In order to limit white-coat adherence, a potential bias of TDM, patients were informed about TDM at short notice, immediately asked for informed consent, and checked at irregular intervals.

In the vast majority of cases, blood sampling was performed at the expected maximum concentration (C_{max}) of antihypertensive medications at 0.5-2 hours after intake, but trough concentrations (C_{trough}, 12 hours after the last drug intake) were also included in this study because the method used for drug measurements could successfully quantify all expected trough concentrations reported in the literature.

Patients were classified into three classes: "fully adherent" patients (AD) had detectable plasma concentrations of all prescribed drugs, "partially adherent" (PAD) patients showed detectable concentrations of only a part of all prescribed drugs, "totally non-adherent" (NAD) patients had undetectable concentrations of all the prescribed drugs.

3.8 Blood pressure and heart rate measurements

Office BP and HR were measured manually with the UA 101 (AND medical) hybrid sphygmomanometer using the appropriate cuff size for patient arm. At least three seated BP measurements taken at least 3 min apart were obtained and the mean of the three measurements were considered.

24-hour ABPM was performed with Spacelabs 90207 Ambulatory Blood Pressure monitors (Spacelabs Healthcare Inc., Snoqualmie, WA, USA). Ambulatory BP monitors were applied by a trained nurse on a routine working day, between 8.00-9.30 a.m. After initialization of devices with patient data, they were set up to measure every 15 minutes, both during day

and during nighttime. Patients were instructed to conduct their normal activities during ABPM, to refrain from intense physical exercise and to avoid moving the arm or talking during cuff inflation. European Society of Hypertension recommendations have been applied to define hypertension, based on ABPM averages (≥130/80 mmHg for the 24-hour average, ≥135/85 mmHg for daytime and ≥120/70 mmHg for night-time) [4].

Finally, by subtracting day time ABPM HR from the office HR, the White Coat Heart Rate increase (WCHR) was calculated [48].

3.9 Indirect evaluation of adherence

Two indirect markers for adherence evaluation were used: the specialist opinion and a home-made questionnaire for self-reported adherence by willing patients (Table 7). On the basis of patient-feedback reporting problems of comprehension with the previously validated original questionnaire, a revised and simplified new one was developed. Furthermore, considering the enrolled population of RHT patients, particular attention was reserved to the polytherapy and a question about social aids was added (this question did not contribute to the final score but was useful for statistical analyses). Three levels of adherence were identified by the questionnaire as follows: scores 0 to < 6 (low, non-adherence); scores 6 to < 9 (medium, partial adherence); scores 9 to 10 (high, full adherence). Clinicians following each patient were asked to note in medical records their hypotheses concerning the level of adherence of the patient on the basis of their personal experience before knowing TDM results.

3.10 Statistical analysis

Statistical analysis was performed to identify associations between clinical/anthropometric/demographic parameters and adherence profile. Associations between categorical variables were tested by a chi-square test. Due to the non-normal distribution of data, differences in continuous variables between groups were tested by non-parametric Kruskal-Wallis (for more than 2 groups) or Mann-Whitney (for 2 groups) tests. The predictive value of clinical parameters for the adherence profile was tested through univariate and multivariate logistic regression analyses. Putative cut-off values for the prediction of adherence profiles were identified by ROC curve analyses. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS software, version 24.0.

COMPLIANCE QUESTIONNAIRE	**YES = 1**	**NO = 0**
Do you ever forget to take even only one medication per day?		
During the last week, how many days did you take the therapy uncorrectly? • 0 – 1 • 2 – 4 • 5 – 7	• 0 – 1 → 2 • 2 – 4 → 1 • 5 – 7 → 0	
Are you always fully honest with your clinician?		
Did you take all your medication yesterday?		
Do you think it is difficult to take all the prescribed drugs every day?		
Do you always remember to bring all your medication when you go away from home?		
Do you feel guilty when you forget to take your therapy?		
From 1 to 5, how much is important for you to treat your hypertension? • 1 – absolutely not importan • 2 – not much important • 3 – quite important • 4 – very important • 5 – essentially important	• 1 → 0 • 2 → 1 • 3 → 2 • 4 → 3 • 5 → 4	
Would you discuss with your clinician before stopping your therapy due to severe side effects?		
Do you receive any social aids for hypertension? [NOT USED FOR SCORE EVALUATION]		

Table 7: overview of the questionnaire used for the evaluation of patients' adherence.

4. RESULTS

4.1 Clinical application of TDM

4.1.1 Patients' features and adherence profiles

Among 1250 patients referred to the Hypertension Unit of Turin, 145 fulfilled criteria for RHT in the study period (Figure 5). Of these, 36 had white coat RHT, 22 had secondary causes of hypertension and 37 had their BP controlled by optimization of antihypertensive therapy. Fifty patients were considered as apparent RHT and included in the study: 29 male and 21 females, with a median age of 56 years old (total range 41 – 79) (Table 8). 39 patients answered the questionnaire, 67% of these resulted AD, 21% PAD and 13% NAD.

TDM on plasma samples revealed that only 58% of patients (n=29; 20 men and 9 women) were AD, 24% (n=12; 5 men and 7 women) were PAD and 18% (n=9; 4 men and 5 women) were NAD, with undetectable concentrations of all prescribed drugs. There were no significant differences between men and women (Table 8).

The agreement between specialist opinion, the questionnaire and the result of plasma TDM was evaluated. A statistically significant concordance (p-value = 0.002) was observed between specialist opinion and TDM results, while no significant association was found between questionnaire and TDM results. Strikingly, all the TDM-defined NAD patients self-defined as AD in the questionnaire.

Baseline patient characteristics, divided by adherence category, are shown in Table 8. Differences in Office DBP and HR among adherence-based categories are shown in Figure 6. Compared with other patients, NAD patients were slightly younger (not significant), comprised significantly more smokers (p-value = 0.027), and had significantly higher office and 24h SBP (p-value = 0.021 and p-value = 0.048 respectively), office and 24h DBP (p-value = 0.010 and p-value = 0.006 respectively), office HR and WCHR (p-value = 0.007 and p-value = 0.001 respectively) and reported a high prevalence of social aids or a prepaid pension (p-

value = 0.045). Among NAD patients, coronary artery disease and previous invasive treatments for hypertension were significantly more frequent (p-value = 0.043 and p-value = 0.001 respectively). Furthermore, a higher fraction (more than 20%) of NAD and PAD patients experienced a previous stroke, compared to ADs (p-value = 0.003).

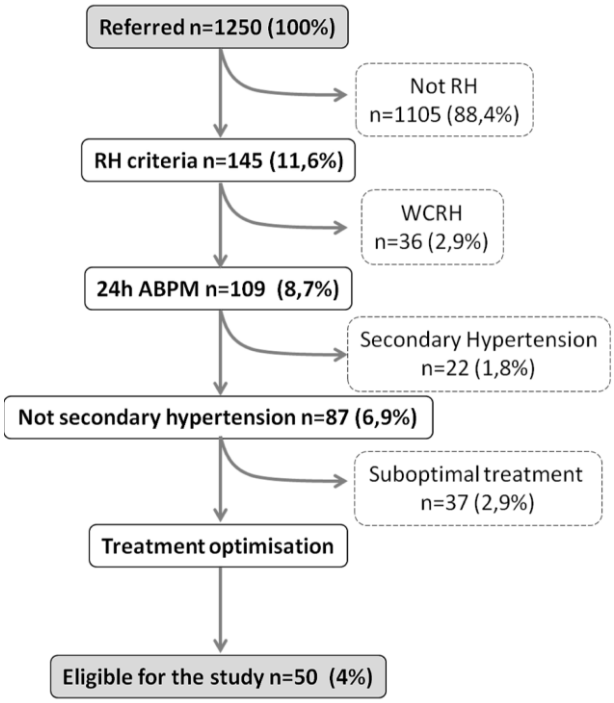

Figure 5: Flowchart showing patient enrolment and inclusion criteria. WCRH = White Coat Resistant Hypertension.

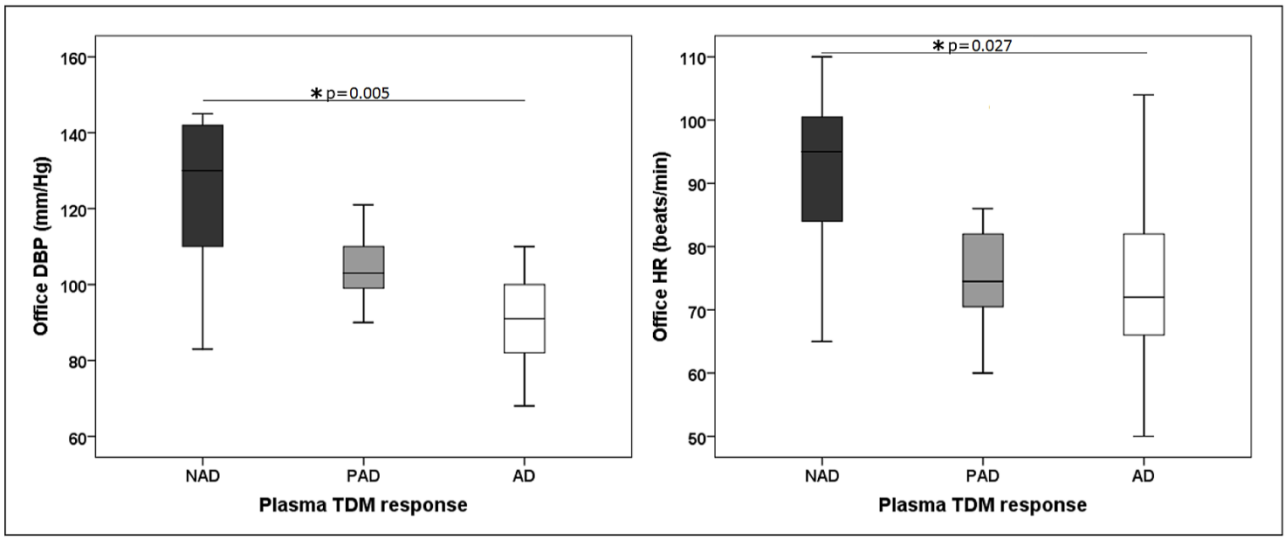

Figure 6: Differences in Office DBP and Office HR values among NAD, PAD and AD patients, according to Kruskal Wallis test.

Total number of patients (50)	Number (%) / Median (IQR)	AD (n=29, 58%) Mean ± SD	PAD (n=12, 24%) Mean ± SD	NAD (n=9, 18%) Mean ± SD	Between 3 groups differences (p-value)
Sex (Male/Female)	29/21 (48% / 42%)	20/9	5/7	4/5	0.180 †
Age (years; median)	56 (IQR 52 – 63)	60 ± 9	54 ± 9	53 ± 5	0.053 ‡
BMI (Kg/m²; median)	30.4 (IQR 26.5 – 33.5)	31 ± 5	31 ± 7	30 ± 4	0.878 ‡
Serum creatinine (mg/dL; median)	0.91 (IQR 0.78 – 1.10)	1.09 ± 0.53	0.79 ± 0.22	1 ± 0.22	0.139 ‡
Smoke (yes/no)	14/36	6/23	3/9	5/4	0.080 †
Duration of hypertension (months)	126 (IQR 60 – 192)	144 ± 104	110 ± 62	144 ± 70	0.569 ‡
Social aids (yes/no)	19/31	7/22	6/6	6/3	**0.046** †*
Number of prescribed drugs	5 (IQR 3.25 – 5)	4 ± 1	5 ± 1	5 ± 1	**0.008** ‡*
Coronary Artery Disease	3 (6%)	1 (3%)	0	2 (22%)	0.121 †
Previous stroke	5 (10%)	0	3 (25%)	2 (22%)	**0.008** †*
Type 2 diabetes mellitus	12 (24%)	8 (28%)	1 (8%)	3 (33%)	0.554 †
Previous invasive treatments (yes/no)	12/38	2/27	4/8	6/3	**0.001** †*
Office SBP (mm/Hg)	155 (IQR 143 – 182)	155 ± 20	165 ± 25	204 ± 49	0.052 ‡
Office DBP (mm/Hg)	95 (IQR 86 – 110)	93 ± 13	104 ± 9	123 ± 25	**0.005** ‡*
Office HR (beats/min)	76 (IQR 67 – 83)	73 ± 13	75 ± 8	91 ± 15	**0.027** ‡*
24h ABPM SBP (mm/Hg)	140 (IQR 128 – 156.5)	138 ± 17	142 ± 20	155 ± 18	0.105 ‡
24h ABPM DBP (mm/Hg)	83 (IQR 73.5 – 90.5)	77 ± 10	88 ± 10	97 ± 13	**0.003** ‡*
24h ABPM HR (beats/min)	72 (IQR 66 – 79)	70 ± 10	77 ± 10	78 ± 5	**0.034** ‡*
WCHR (beats/min)	-1 (IQR -13 – +14)	-3 ± 15	-9 ± 9	22 ± 15	**0.005** ‡*

Table 8: Demographic, anthropometric and clinical features of fully adherent (AD), partially adherent (PAD) and totally non adherent (NAD) patients. IQR= Inter Quartile Range. SD= standard deviation. *= Statistically significant. †= Chi- square test. ‡= Kruskal Wallis test. ABPM = Ambulatory Blood Pressure Monitoring; WCHR = White Coat Heart Rate increase (= "office HR" – "day time ABPM HR").

4.1.2 Predictive parameters of nonadherence

Univariate logistic regression analysis identified the following parameters as putative predictors of NAD (Figure 7): smoking habit (p-value = 0.039), social aids (p-value = 0.056), coronary artery disease (p-value = 0.083), previous invasive treatment for hypertension (p-value = 0.004), office SBP, DBP and HR (p-value = 0.007, p-value = 0.004 and p-value = 0.008, respectively), 24h ABPM SBP and 24h ABPM DBP (p-value = 0.058 and p-value = 0.018, respectively) and WCHR (p-value = 0.012).

A multivariate logistic regression was performed to identify parameters that could potentially identify real NAD patients, considering at most three variables combined. The resulting model included office DBP (p-value = 0.045) and office HR (not significant p-value). To define cut-off values, only considering office DBP, ROC curve analyses indicated that patients with DBP values higher than 124.5 mm/Hg (AUROC = 0.805; p-value = 0.012; sensitivity 71.4%; specificity 97.1%) have higher probability to be NAD (Figure 8).

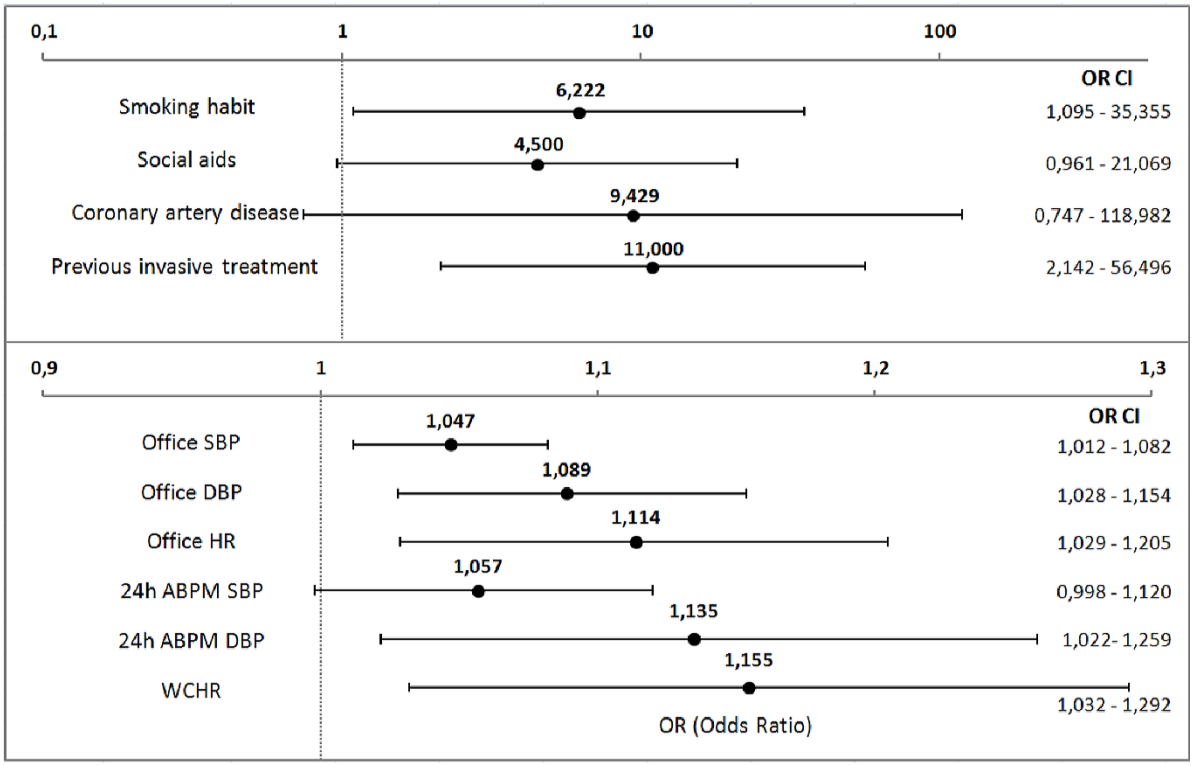

Figure 7: Predictive parameters of nonadherence obtained by univariate logistic regression analysis with odds ratios and 95% confidence intervals (CI).

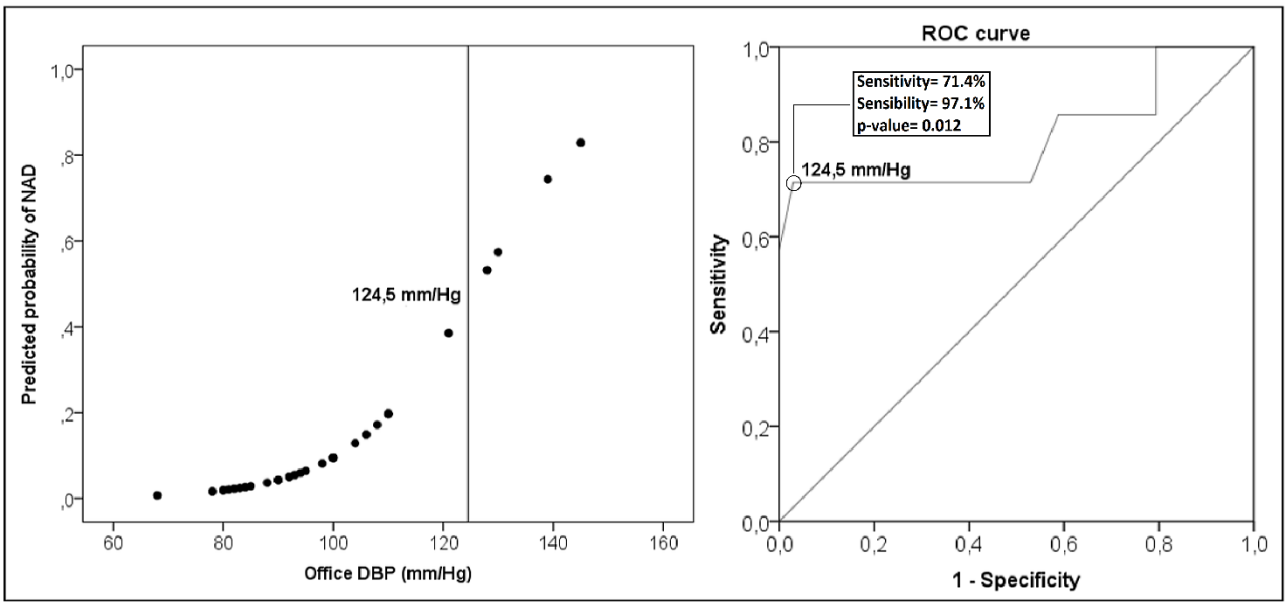

Figure 8: Predicted probability to be NAD according to Office DBP values obtained through ROC curve analysis.

4.2 Methodological upgrade

During this phase, NBV, IDP, VAL, SCB, SCB-M and RAM-M and some isotope-labeled internal standards ([$^{2}H_{7}$]-atenolol, [$^{13}C_{8}$]-nifedipine, [^{13}C,$^{2}H_{3}$]-telmisartan and [$^{2}H_{4}$]-amlodipine) have been added to the panel of monitored drugs.

Nevertheless, some technical issues have been observed for what concerns AML, TEL, DOX and NBV due to the very low plasma concentration of those molecules. In particular, AML, DOX and NBV show the tendency to attach to the glass tube walls, especially in the absence of matrix (e.g. plasma or urine) and in presence of pure solvents (H_2O and ACN). This represents a problem especially during extraction recovery evaluation.

As a consequence, plastic tubes have been tested and the problem seems to be partially solved. For what concerns TEL, a very high matrix effect has been observed in plasma, but it was very stable and reproducible across different matrix lots.

4.2.1 Plasma method re-validation

All method validation parameters are resumed in Table 9; chromatographic peaks and retention times of the 14 drugs (without metabolites) resulting from the injection of a STD 9 are depicted in Figure 9.

Drug	QC	Accuracy (%)	Imprecision		Mean Recovery (RSD%)	Mean Matrix Effect (RSD%)	Mean Extraction Efficiency (RSD%)
			Intra-day (RSD %)	Inter-day (RSD %)			
CLN	H	99.83	1.7	1.82	95.9 (2.5)	100.4 (2.5)	95.5 (0.3)
	M	92.93	2.6	2.59	88.8 (4)	95.8 (3,6)	92.7 (2.2)
	L	95.25	5.6	4.72	92.5 (6)	93.3 (7)	99.4 (6)
DOX	H	98.48	0.4	0.55	137.6 (5.6)	130.6 (4.8)	105.3 (1.4)
	M	88.63	3	2.82	281.7 (4.5)	258.9 (4.8)	108.9 (3.7)
	L	96.25	8.4	6.91	343.4 (7)	281.2 (6.3)	122.2 (4.9)
AML	H	88.15	1.1	1.02	136.4 (8.7)	114.9 (6)	118.7 (4.8)
	M	92.21	6.9	6.53	217.6 (7.1)	178.8 (6.6)	121.8 (6.6)
	L	98	19.7	16.91	250 (18.9)	154.1 (8.6)	124.2 (3.9)
HCTZ	H	102.19	1.3	1.64	107.8 (0.8)	123.2 (0.4)	87.5 (0.5)
	M	93.33	3.5	3.38	99.6 (4.1)	123.1 (2.6)	80.9 (2.2)
	L	93.08	1.5	2.46	100.8 (7.7)	121.6 (14.2)	82.7 (4.9)
NFD	H	110.96	4	3.93	62.6 (2)	100.9 (1.5)	62 (2.8)
	M	110.52	1.2	5.68	50.3 (3.3)	97.3 (4.1)	51.7 (3.7)
	L	109.05	10.1	11.45	66.6 (18.7)	100.6 (13.8)	62.1 (9.5)
TEL	H	95.33	0.4	1.48	179.1 (3.5)	188.7 (1.3)	94.9 (2.9)
	M	97.15	2.7	2.49	160.1 (6.6)	263.9 (5.6)	60.7 (2.8)
	L	95.35	4.4	5.51	188.1 (11.1)	234.7 (8.8)	80 (3.2)
RAM	H	99.7	0.7	0.64	110.6 (2.1)	112.1 (1.8)	98.6 (0.7)
	M	97.34	1.4	1.46	109.5 (1.3)	113.4 (1)	96.6 (1)
	L	87.53	5.5	4.52	110.8 (2)	113 (0.4)	98 (2)
OLM	H	104.23	0.8	1.15	96.7 (1.9)	103.9 (0.9)	93.1 (1.1)
	M	98.39	0.7	0.69	96 (0.4)	107.9 (0.8)	89 (1)
	L	98.05	5.3	4.92	101.4 (2.3)	108.5 (3.9)	93.6 (5)
ATE	H	97.47	0.4	0.59	97.2 (0.9)	98 (0.5)	99.1 (0.7)
	M	90.94	1.1	1.06	89.8 (1.7)	109 (1.5)	82.4 (1.7)
	L	98.04	2.6	2.22	99.9 (0.8)	133.3 (2)	75 (2.2)
CHL	H	97.79	1.9	1.63	88.9 (0.9)	94.9 (0.6)	93.7 (0.4)
	M	86.26	1	0.98	80.8 (0.7)	93.2 (1.2)	86.8 0.8)
	L	80.69	8.2	6.80	83.4 (6.4)	93.6 (1.5)	89 (4.9)
NBV	H	91.30	2.3	2.97	143.7 (11.9)	139 (7.7)	103.1 (4.2)
	M	89.23	10.6	9.17	326.1 (11.4)	311.2 (3.9)	104.6 (7.6)
	L	62.25	5.2	6.99	392.6 (34.7)	302.1 (37.9)	137.9 (10.6)
SCB	H	109.5	3.2	4.65	109.4 (4.3)	106.7 (0.6)	102.5 (3.8)
	M	99.74	1.7	4.12	95.5 (5.5)	107.5 (3.2)	88.8 (2.3)
	L	103.17	2.8	3.22	118.8 (5.2)	109.7 (4.4)	108.3 (1.8)
VAL	H	89.61	2.2	2.57	104.2 (1.3)	115.7 (2.7)	90.1 (1.9)
	M	102.15	1.3	5.38	75.4 (2.2)	103.7 (2)	72.7 (0.6)
	L	93.29	1.5	1.35	78.4 (4.7)	108.2 (4.5)	72.5 (1.8)
IDP	H	95	0.2	0.39	90.6 (2.2)	95.9 (1.9)	94.5 (0.7)
	M	90.92	2.1	2.32	85.3 (2.2)	88.1 (2.7)	96.8 (3)
	L	93.48	3.8	3.46	84.7 (2.1)	87.5 (5.5)	97 (6.1)
SCB-M	H	99.81	0.9	0.8	93.2 (0.7)	102.5 (0.2)	90.9 (0.6)
	M	93.94	0.9	1.14	67.7 (1)	101 (0.6)	67.1 (1.4)
	L	99.05	3.1	3.22	64 (2.4)	114.9 (1.5)	55.7 (2.8)
RAM-M	H	109.24	0.6	1.87	87.4 (2.7)	103 (1.8)	84.8 (1.2)
	M	98.46	2.4	2.65	77.6 (2.9)	102.3 (1.5)	75.9 (2.1)
	L	93.60	4.8	4.11	49.9 (8.1)	102.7 (5.7)	48.6 (2.7)

Table 9: Plasma method upgrade and validation parameters: red characters stand for observed anomalies.

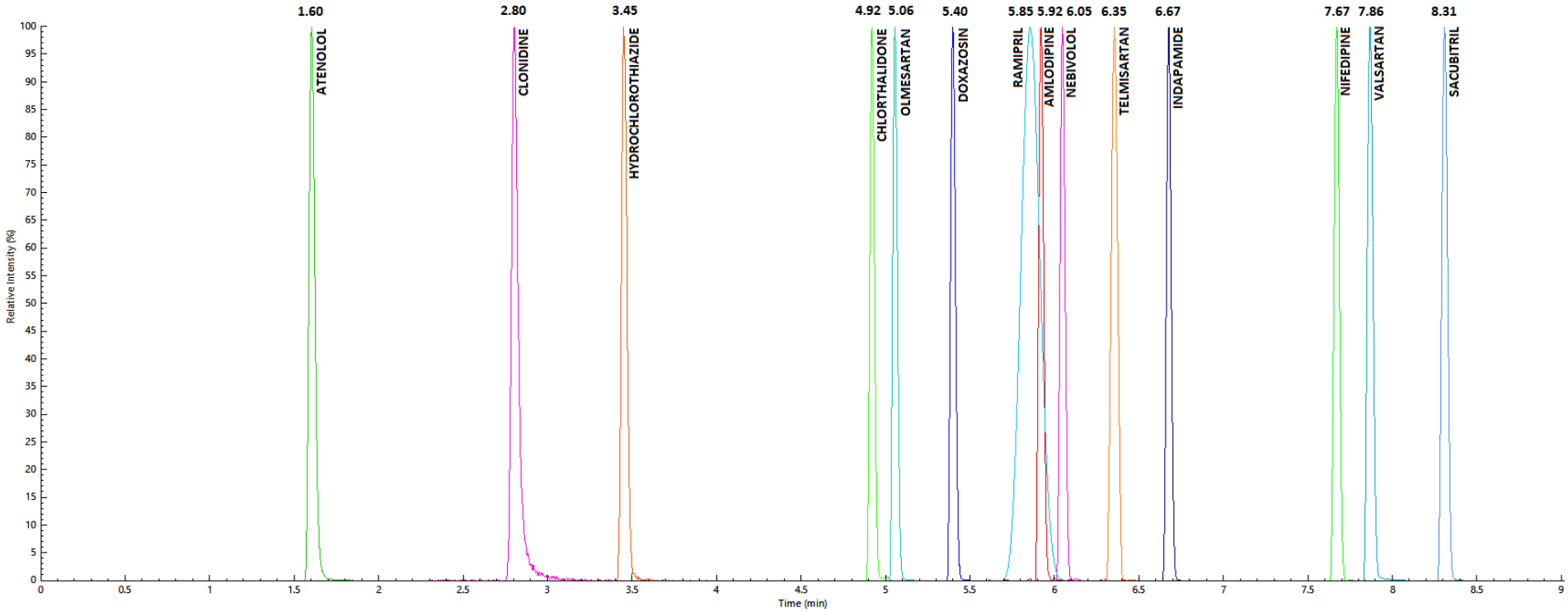

Figure 9: Chromatographic peaks and retention times of the 14 drugs (no metabolites) resulting from the injection of the highest point of the plasma calibration curve.

4.3 Canrenone quantification in human plasma

The validation process of this analytical method is not yet fully complete. Nevertheless, it has been used to confirm drug presence (yes/no) and thus therapeutic adherence in a subset of patients enrolled in the ETRURIA study. Chromatographic peaks and RT of CAN and of its IS (QX), resulting from the injection of a STD 9, are depicted in Figure 10.

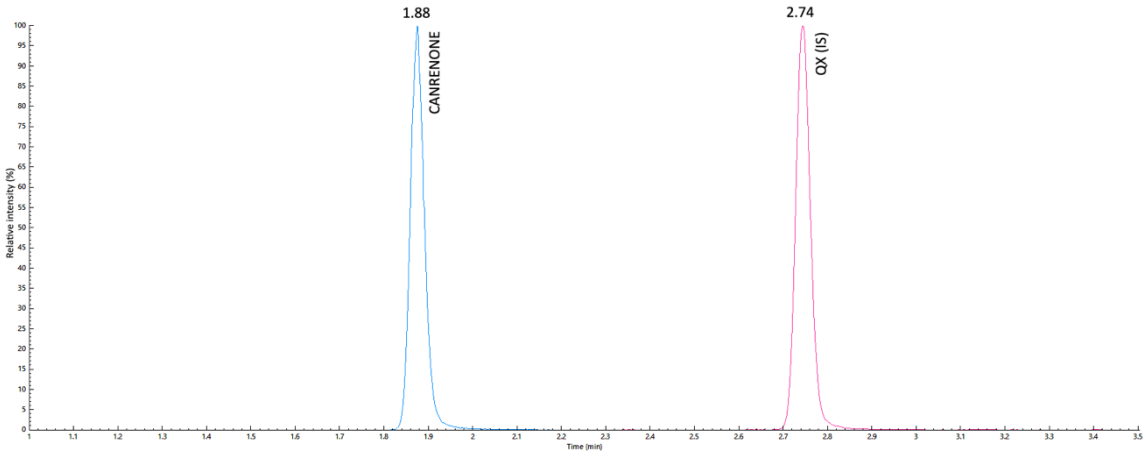

Figure 10: Chromatographic peaks and retention times of of CAN and its IS resulting from the injection of the highest point of the plasma calibration curve.

4.4 Saliva method: development, validation and clinical application

4.4.1 Recovery from the Salivette® devices

Concerning the experiment performed in solvent, AML, TEL, DOX and NBV showed a >90% loss after the passage through Salivette®; SCB, SCB-M, CLN, HCTZ, RAM and ATE showed a reduction comprised between 20 and 50%; finally, OLM, CHL, VAL, NFD and RAM-M showed a <20% variation, consistent with a casual error. Unaccountably, IDP showed a moderate increase in both replicate experiments.

On the other hand, for what concerns the experiment performed with saliva, many results were very similar to the ones described above: AML, TEL, DOX, NBV but also HCTZ showed a >50% loss after the passage through Salivette®; CHL, NFD and ATE showed a reduction comprised between 20 and 50%; finally, OLM, SCB, SCB-M, VAL, RAM and RAM-M showed a <20% variation, consistent with a casual error. IDP confirmed its strange increase, in this case accompanied by CLN.

All the results are detailed in table 10, together with a series of molecule-specific parameters, which presented any correlations with drugs retention.

	Mean solvent loss (%)	Mean saliva loss (%)	logD (o/w)	pKa	MW	RT
AML	-94	-97	3.00	8.60	408.9	5.92
TEL	-100	-62	8.42	3.86	514.6	6.35
DOX	-99	-100	3.30	6.52	451.5	5.40
NBV	-97	-98	4.04	8.13	405.4	6.05
HCTZ	-33.5	-54	-0.07	7.90	297.7	3.45
ATE	-46.5	-21	0.16	9.60	266.34	1.60
SCB	-30	-14	3.94	4.18	411.5	8.31
RAM	-31	2	3.32	3.74	416.5	5.85
OLM	-1.5	2	3.63	4.30	446.5	5.06
CHL	-14	-30	0.85	9.36	338.8	4.92
CLN	-28.5	24	1.59	8.05	230.1	2.80
SCB-M	-22.5	-2	n.a.	n.a.	383.4	7.04
VAL	-13	-10	4.00	4.73	435.5	7.86
NFD	-13	-38	2.20	3.93	346.3	7.67
RAM-M	-12	13	1.69	n.a.	388.5	5.00
IDP	19.5	33	2.06	8.80	365.8	6.67

Table 10: recovery experiments from Salivette devices; logD (o/w): distribution coefficient, that is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in octanol/water; pKa (acid dissociation constant); MW: molecular weight.

4.4.2 Saliva method validation

Mean retention times (± 0.05 min) for the considered analytes were as follows: 1.60 min for ATE, 2.80 min for CLN, 3.45 min for HCTZ, 4.92 min for CHL, 5.00 min for RAM-M, 5.06 min for OLM, 5.40 min for DOX, 5.85 min for RAM, 5.92 min for AML, 6.05 for NBV, 6.35 min for TEL, 6.67 min for IDP, 7.04 min for SCB-M, 7.67 min for NFD, 7.86 min for VAL and 8.31 min for SCB.

Analytes accuracy and imprecision values well fitted guidelines recommendations at different concentrations and are summarized in Table 11. Recovery, extraction efficiency, matrix effect and IS-normalized matrix effect were in accordance with guidelines recommendations for almost all QC levels of all drugs and are resumed in Table 11.

Calibration curves fitted quadratic regression models by passing through the origin of the axes; a weighting factor *1/X* was used, to ensure high accuracy at low concentrations. Regression coefficients (r^2) of calibration curves were all above 0.996.

			IMPRECISION					
		ACCURACY %	INTRA-DAY (RSD%)	INTER-DAY (RSD%)	RECOVERY MEAN (RSD)	EXTRACTION EFFICIENCY MEAN (RSD)	MATRIX EFFECT MEAN (RSD)	IS-n-ME MEAN (RSD)
ATE	H	95.9	0.8	0.7	89.8 (4.3)	94.2 (5.1)	-4.5 (6.3)	-5.8 (3.4)
	M	95.4	1	1.0	90.0 (1.3)	90.5 (6.9)	-0.1 (7.7)	-3.2 (2.8)
	L	85.6	2.5	2.4	95.1 (4.7)	93.3 (8.9)	+2.6 (9.6)	-1.2 (5.0)
CLN	H	94.6	3.4	2.8	84.8 (1.3)	89.7 (4.7)	-5.2 (5.6)	-7.5 (6.5)
	M	91.9	3.5	3.2	85.8 (6.0)	89.0 (7.0)	-3.2 (7.9)	-5.2 (9.8)
	L	58.8	14	13.1	108.3 (18.2)	80.2 (10.2)	+36 (20.6)	+40.8 (13.2)
HCTZ	H	96.4	2.7	3.6	106.4 (2.4)	86.6 (10.9)	+23.8 (8.6)	+22.1 (9.2)
	M	108.2	0.9	1.3	135 (1.7)	92.9 (13.4)	+46.8 (11.4)	+48.5 (10.9)
	L	86.9	25.6	30.3	142.3 (2.2)	84.8 (8.2)	+69.1 (9.7)	+69.3 (9.6)
DOX	H	98.4	2.2	3.2	133.9 (1.2)	98.8 (0.4)	+35.6 (1.6)	+29.6 (0.6)
	M	109.5	4.3	6.8	149.6 (1.8)	103.3 (1.3)	+44.9 (3.2)	+41.9 (3.2)
	L	89.7	3.2	9.4	159.5 (14.0)	118.9 (11.0)	+33.9 (3.0)	+27.9 (3.8)
OLM	H	97.6	2.6	3.6	97.2 (2.1)	97.4 (4.3)	-0.1 (6.6)	-5.5 (16.0)
	M	93.5	2.3	3.4	108.9 (6.8)	97.0 (7.1)	+12.7 (12.7)	+8.0 (6.8)
	L	81.9	3.1	4.7	112.3 (8.8)	95.4 (9.3)	+18 (13.3)	+18.4 (10.0)
CHL	H	96.7	4.3	5.0	73.9 (7.4)	93.7 (12.8)	-20.2 (13.6)	-34.7 (6.8)
	M	97.1	3.8	5.0	79.6 (2.5)	95.0 (6.6)	-15.9 (11.8)	-19.5 (13.9)
	L	92.7	5.9	8.4	84.1 (15.5)	98.0 (8.6)	-13.4(19.6)	-22.3 (16.2)
TEL	H	100.3	2.7	3.1	115.1 (8.4)	113.0 (7.1)	+2.2 (14.6)	-7.2 (6.3)
	M	97.9	3.8	8.0	118.7 (17.6)	120.0 (7.8)	-1.6 (12.7)	-4.9 (8.5)
	L	103.0	22.9	27.4	134.1 (34.3)	149.8 (31.2)	-9.6 (24.9)	-4.6 (22.1)
AML	H	93.7	3.7	7.4	96.3 (3.0)	88.9 (2.0)	+8.3 (1.2)	+4.7 (2.5)
	M	94.6	6.5	7.3	105.0 (6.3)	102.4 (20.0)	+13.8 (13.7)	+11.8 (2.4)
	L	73.2	35.1	32.9	94.0 (10.0)	101.1 (19.3)	-6.2 (9.4)	-16.8 (11.3)
RAM	H	101.9	2.3	2.2	85.1 (6.5)	92.8 (4.9)	-8.4 (5.8)	-13.6 (14.7)
	M	105.1	0.9	0.9	87.0 (6.1)	94.4 (3.0)	-7.9 (6.1)	-9.4 (10.1)
	L	90.0	2.7	4.2	89.5 (3.3)	99.6 (8.6)	-9.7 (8.6)	-6.3 (13.9)
RAM-M	H	101.6	2.3	3.5	94.9 (2.2)	95.2 (4.9)	-0.1 (5.9)	-4.7 (6.7)
	M	101.2	3.6	3.0	96.1 (7.2)	94.8 (8.6)	+1.7 (7.6)	-2.1 (9.3)
	L	78.1	4.7	5.1	108.5 (18.2)	96.8 (12.3)	+11.9 (12.6)	+7.6 (12.1)
NBV	H	89.1	3.2	10.6	102.3 (2.9)	99.8 (5.0)	+2.6 (2.2)	-1.4 (3.3)
	M	62.2	6.3	12.0	112.6 (4.2)	110.5 (8.4)	+2.5 (12.5)	-1.7 (12)
	L	51.7	4.2	10.0	108.6 (0.4)	95.9 (14.3)	+14.4 (14.8)	+13.5 (15.0)
NFD	H	109.9	7.2	8.7	102.6 (1.7)	124.7 (4.4)	-17.7 (2.7)	-20.5 (4.1)
	M	108.2	5.7	5.4	99.4 (4.3)	123.1 (4.7)	-19.1 (8.9)	-19.5 (20.1)
	L	100.3	10.8	10.4	118.5 (11.0)	125.0 (15.6)	-4.8 (4.7)	-11.2 (9.7)
SCB	H	100.4	2	2.2	93.8 (2.9)	100.4 (2.7)	-6.5 (1.2)	-9.3 (1.4)
	M	101.6	0.6	0.5	64.9 (1.9)	98.1 (2.5)	-3.3 (2.4)	-5.7 (3.6)
	L	113.6	1.2	1.4	96.7 (4.3)	97.5 (5.9)	-0.7 (4.6)	-2.8 (2.8)
SCB-M	H	93.4	1.2	1.8	92.5 (1.6)	97.0 (1.0)	-4.6 (1.2)	-7.8 (2.3)
	M	110.1	0.3	0.5	87.8 (2.1)	93.9 (2.0)	-6.5 (3.4)	-9.2 (3.9)
	L	107.2	1.8	1.9	89.3 (7.1)	95.2 (6.0)	-6.3 (8.3)	-9.9 (6.5)
IDP	H	97.8	2.1	1.7	79.5 (5.6)	88.3 (5.9)	-9.9 (6.5)	-14.7 (7.2)
	M	113.4	2.6	2.5	78.6 (7.4)	90.4 (6.6)	-13.0 (8.5)	-17.3 (9.0)
	L	110.4	5.6	5.7	84.9 (10.9)	99.6 (14.8)	-13.6 (13.7)	-16.1 (13.0)
VAL	H	99.0	3.2	2.7	91.7 (1.3)	96.5 (0.7)	-5.0 (0.9)	-14.9 (11.1)
	M	110.0	1.5	1.9	91.0 (2.9)	95.1 (1.2)	-4.2 (3.4)	-8.5 (10.9)
	L	89.6	1.2	2.0	92.4 (4.3)	94.7 (4.2)	-2.4 (3.9)	-1.4 (13.9)

Table 11: Overview of validation parameters for the salivary method: red characters stand for observed anomalies.

4.4.3 Clinical application

A total of 57 saliva samples from patients enrolled in the TDM-TO study have been tested with the described protocol (Table 12). A very high inter-patient variability was observed, but this could depend on water consumption, time-from-dose and salivary pH. All patients, whose adherence had been previously verified, have been randomly tested on purpose, in order to evaluate the overall method performance in a "real-life" clinical context (extemporaneous sampling).

	N of samples	Median (ng/mL)	IQR (interquartile range)
ATE ng/ml	1	38,60	n.a.
AML ng/ml	21	6,13	2,92 - 9,24
OLM ng/ml	7	0,22	0,12 - 0,51
TEL ng/ml	13	2,60	0,49 - 26,84
NFD ng/ml	2	1,77	1,51 - 2,02
HCTZ ng/ml	6	6,23	1,38 - 58,20
CHL ng/ml	4	5,35	3,17 - 17,27
DOX ng/ml	6	3,01	1,26 - 4,44
RAM ng/ml	6	0,13	0,09 - 0,20
RAM-M ng/ml	6	0,11	0,10 - 0,27
VAL ng/ml	2	2,19	1,27 - 3,12
NBV ng/ml	6	0,74	0,18 - 8,77
IDP ng/ml	1	n.d.	n.a.

Table 12: Number of patients tested for each drug and median saliva concentrations from patients enrolled in TDM-TO study.

Finally, on a subset on 27 patients who had a time-matched saliva and plasma sampling, we demonstrated that the large majority of analyses (48 out of a total of 53 matches) resulted fully confirmed.

4.5 Future perspectives: plasma, urine and saliva contextual analyses

A preliminary analysis concerning the simultaneous quantification of the fourteen drugs across the three different matrices (saliva, plasma and urine spot) was conducted on a total of 24 patients.

Of these, 12 had a prescription for AML, 9 for TEL, 5 for RAM, 4 for DOX, 2 for OLM, NFD, HCTZ CHL, VAL and NBV, 1 for ATE and none for IDP, SCB and CLN.

Out of a total of 50 matches, 39 were fully confirmed, and 48 were confirmed on two out of three matrices. Mean measured concentrations are reported in Table 13.

	n=	Median plasma concentration (ng/mL)	Median saliva concentration (ng/mL)	Median urine spot concentration (ng/mL)
ATE ng/ml	1	100,72	38,60	4484,00
AML ng/ml	12	5,57	7,42	177,93
OLM ng/ml	2	363,32	0,51	1039,36
TEL ng/ml	9	45,82	0,79	8,33
NFD ng/ml	2	36,22	1,77	n.d.
HCTZ ng/ml	2	10,82	50,69	4229,50
CHL ng/ml	3	95,60	7,10	1224,00
DOX ng/ml	4	7,12	1,58	17,98
RAM ng/ml	5	1,65	0,14	26,83
RAM-M ng/ml	5	11,12	0,17	721,00
VAL ng/ml	2	749,78	2,19	2094,50
NBV ng/ml	3	21,17	0,74	51,37

Table 13: Number of patients tested for each drug and median plasma, saliva and urine spot concentrations from patients enrolled in TDM-TO study.

5. Discussion

In the course of this project the clinical usefulness of a TDM practice for antihypertensive drugs has been demonstrated in a routine context for the discrimination of real RHT patients from cases of poor compliance.

Contextually, the already existent LC-MS/MS methods have been improved, through the addition of newer drugs, metabolites and isotope-labeled internal standards in order to achieve better performances.

Simultaneously, a simple and fast method, dedicated to CAN, has been developed for human plasma: it revealed very useful to monitor adherence to diuretics, that are often characterized by poor compliance, due to their heavy side effects.

Finally, antihypertensive drugs distribution through saliva has been investigated and a new analytical method has been developed and validated on this matrix.

5.1 Usefulness of TDM for the discrimination of real RHT

RHT has become increasingly investigated to define its real prevalence. The impact of poor therapeutic adherence, which often impairs the results of clinical trials, is relevant but a multitude of discordant data are reported in the literature. RHT patients are at high risk of nonadherence because of the high number of prescribed antihypertensive medications [49]. Moreover, fixed combinations of drugs are often unsuitable for patients or unavailable in some countries and the addition of further drugs, with the worsening of side effects, often results in further nonadherence [49]. Nonetheless RHT patients exert a high economic impact on the Italian health system, because of the number of reimbursed visits, hospitalizations and prescribed pills. Moreover, these patients may potentially undergo invasive treatments and are at a higher CV risk, thus there is a substantial clinical utility to accurately discriminate between RHT and PRHT.

Herein we show that 42% of RHT patients were non-adherent, of whom 24% resulted partially adherent and 18% were totally non-adherent, largely in accordance with other studies with similar inclusion criteria [50-52]. These findings greatly reduce the prevalence of true RHT and confirm that nonadherence is frequent in Italy where the health system provides medical reimbursement and widespread health educational programs. Some studies have proposed even higher percentages of NAD but the inclusion criteria, that are particularly crucial for this kind of evaluation, were different. Florczak *et al.* enrolled only patients with tachycardia [53]: in our work, tachycardia was an important predictor of poor adherence. In fact, all our NAD patients were prescribed either a β-blocker or a centrally acting drug (or both of them), that commonly induce a HR decrease, and their non assumption causes a consequent HR higher than expected [54]. Similarly, Ceral *et al.* used a severe increase in BP values (BP>150/95 mmHg) as an inclusion criterion, thereby introducing a bias for cases of poor adherence, as suggested by our data.

Another source of bias in estimating the real prevalence of RHT comes from "historical" indirect methods often used to measure adherence, such as questionnaires. Such methods require patient collaboration and are not reliable for the detection of intentional poor adherence. In particular, the main factor that limits the accuracy of questionnaires is the inclination to over-report adherence [55]. Therefore new methods have been developed to directly measure therapeutic adherence including TDM [56]. Nevertheless, in some cases, questionnaires can be adopted for their educative value and can be useful to build a constructive dialogue with patients to emphasize the importance of adherence [17].

In this study we compared "traditional" methods for adherence assessment with TDM. We highlight that the clinicians opinion usually agrees with the results of TDM and we confirm the over-estimation of adherence and unreliability of questionnaires. In practice, only 34% of patients declared concerns with adherence in the questionnaire, but these patients mainly comprised those with several comorbidities who may find difficult to cope with a complex

pill burden and/or the related side effects even if they are adherent. In contrast, all NAD patients (confirmed through TDM) declared full adherence in the questionnaire.

We also described clinical/anamnestic, demographical and anthropometrical characteristics of non-adherent patients to identify a common profile. No significant gender differences were observed. On the other hand, significant differences in age, DBP and SBP and HR were observed between different adherence groups. In particular, the worst adherence profiles were associated with globally higher BP and HR values. An association between poor adherence and previous invasive treatments was found, suggesting that the lack of effectiveness may be related to a preexisting problem of poor compliance to therapy [57,58]. Furthermore, total or partial nonadherence was associated with an increased prevalence of previous acute CRV events, confirming other studies [59-61]. As highlighted by Kronish *et al.* suboptimal levels of adherence are associated with a wide visit-to-visit BP variability, with significant fluctuations in reported BP values, a phenomenon that affects CV outcomes [62]. This could be explained by patients who had experienced stroke and invasive treatments underestimating the importance of treatment adherence. Finally, we demonstrated that office DBP and HR were the best predictors of total NAD, and HR may be explained by the total nonadherence to centrally acting drugs or β-blockers [63], instead of a misplaced attitude towards therapy.

5.2 Methodological improvement

The re-validation processes led to a series of analytical upgrades: first of all, the addition of other molecules widened the method exploitability and the number of analyzable patients; then, the new chromatographic gradient, which is three minutes longer than the previous, offers a better peak resolution, with a consequent reduction of possible cross-talks.

The new methods are still robust but even more multiplexed.

Furthermore, the easy and fast method for CAN quantification allows the application of TDM to a large fraction of patients affected by primary aldosteronism, often prescribed with SPI and CAN. In fact, thanks to the rapid conversion of SPI to CAN, the adherence to the therapy can be assessed by monitoring only the metabolite.

5.3 Saliva employment

The use of saliva resulted really welcomed by the patients due to the low invasiveness. Moreover, it is very manageable even from the laboratory staff point of view, since does not require any training course or invasive practices.

The preliminary experiments demonstrated an extensive absorption of many drugs by the Salivette® devices. Even though some authors already suggested that β-blockers retention seems to be correlated to the logarithm of the drug partition coefficient [64], we did not find any possible correlation between drug characteristics and retention extent. We thus hypothesize that the phenomenon could be explained by secondary bounds at the molecular level.

By observing the validation results, some values resulted out-of-range for the lowest level (QC L) of some analytes, nevertheless the first aim of this method is the adherence assessment in the least invasive way. For its own nature, a salivary method will never be as quantitatively precise as a plasmatic one: many factors such as water consumption (dilution), smoke, food, co-medications (diuretics) and oral pH could potentially affect drug concentrations within saliva. By contrast, preliminary experiments demonstrated a good concordance with plasma (above 90%) and this can already justify a method that does not require a blood withdrawal. As a purpose, plasma can always be considered the gold standard to be adopted for doubt cases.

6. Conclusions

This is the first study aimed to simultaneously apply the TDM of antihypertensive drugs to different matrices, in accordance with the clinical routine needs.

The use of TDM as a direct method for evaluating adherence is currently producing promising results and is already became standard practice in the context of resistant hypertension [1].

The phenomenon of PRHT, which is still difficult to identify and manage without a systematic approach, is frequent and often undervalued. As a consequence, the improper use of invasive strategies of treatment in patients with PRH has caused worse pharmaco-economical consequences. In our subpopulation, through the TDM analysis and the early detection of NAD patients, some invasive practices could probably have been avoided. Considering this, we propose that this tailored therapy is useful other than cost-effective.

In the near future, this study should be confirmed on a larger cohort of patients in order to unveil and better describe drugs pharmacokinetics across different matrices and potentially suggest some therapeutic ranges for antihypertensive drugs.

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