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Macrolides for KCNJ5–mutated aldosterone-producing adenoma (MAPA): design of a study for personalized diagnosis of primary aldosteronism

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ABSTRACT

Purpose: Aldosterone-producing adenoma (APA) is the main curable cause of endocrine hypertension cause of primary aldosteronism (PA) and it is in up to 66% of all cases investigated with adrenal vein sampling (AVS). Mutations in the KCNJ5 potassium channel involve up to 70% of APA and cause the most florid PA phenotypes. The recent finding that macrolide antibiotics specifically inhibit *in vitro* the altered function of mutated KCNJ5 channels has opened new horizons for the diagnosis and treatment of APA with KCNJ5 mutations in that it can allow identification and target treatment of PA patients harbouring a mutated APA. Thus, we aimed at investigating if clarithromycin and roxithromycin, two macrolides that potently blunt mutated Kir3.4 channel function *in vitro*, affect plasma aldosterone concentration in adrenal vein blood during AVS and in peripheral blood, respectively, in PA patients with a mutated APA.

Methods and design: We designed two proof of concept studies. In study A: consecutive patients with an unambiguous biochemical evidence of PA will be exposed to a single dose of 250 mg clarithromycin during AVS, to assess its effect on the relative aldosterone secretion index in adrenal vein blood from the gland with and without APA. In study B: consecutive hypertensive patients submitted to the work-up for hypertension will receive a single oral dose of 150 mg roxithromycin. The experimental endpoints will be the change induced by roxithromycin of plasma aldosterone concentration and other steroids, direct active renin concentration, serum K⁺, systolic and diastolic blood pressure.

Discussion: We expect to prove that: (i) clarithromycin allows identification of mutated APA before adrenalectomy and sequencing of tumour DNA; (ii) the acute changes of plasma aldosterone concentration, direct active renin concentration, and blood pressure in peripheral venous blood after roxithromycin can be a proxy for the presence of an APA with somatic mutations.

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Introduction and rationale

Primary hyperaldosteronism (PA) affects about 6% of the patients attending the GPs' offices [1], more than 11% of those referred to specialised hypertension centres [2], and over 20% of those with resistant hypertension [3]. Therefore, PA is the main curable cause of human endocrine hypertension. Since 1954, when Jerome Conn first described it, the term “primary” is used to underline the ignorance of its causes and underlying mechanisms, which was scratched only recently when we discovered agonistic autoantibodies against the type 1 angiotensin receptor [4], a down regulation of Twik-Acid Sensitive K channels type 2 (TASK-2) [5], and when Choi et al. identified mutations in the potassium channel Kir3.4

(KCNJ5) [6]. These mutations were thereafter found to involve a significant proportion of aldosterone-producing adenoma (APA), one of the major subtypes of PA. Moreover, according to a recent large meta-analysis, albeit with prominent geographic variations they were found to occur in a substantial proportion (between 18% and 70%) of APA, thus entailing the most prevalent mutations in these tumours [7]. Of note, compared to wild-type APA, patients with KCNJ5 mutated APA are more often women, carry larger tumours and have higher plasma aldosterone concentrations causing a more florid PA phenotype. These features may explain not only why they develop more marked lateralization of aldosterone production at adrenal vein sampling

(AVS) [8], and also a more pronounced cardiac damage [9].

KCNJ5 mutations cause a loss of the K^+ selectivity of the Kir3.4 channel rendering the channel permeable to sodium with increased sodium influx into the APA cells leading to their depolarization, and thereby to opening of the T-type calcium channels and activation in the reverse mode, e.g. Na^+ out and Ca^{2+} in, of the Na^+ - Ca^{2+} exchanger [10]. The ensuing increase of cytosolic Ca^{2+} , and thereby of mitochondria Ca^{2+} , activates the step-limiting enzyme for aldosterone synthesis, e.g. the mitochondrial Ca^{2+} -sensitive aldosterone synthase (CYP11B2), with the final result of a marked activation of aldosterone synthesis.

In June 2017, after a high throughput screening of over 73,000 molecules, the group that discovered KCNJ5 mutations reported that macrolide antibiotics, and some synthesized derivatives without antibiotic activity, specifically inhibit *in vitro* the altered function of G151R and L168R mutated KCNJ5 channels in cells engineered to overexpress these channels [11]. Accordingly, they could show that these molecules blunted the abnormal Na^+ influx in cells with the mutated channel, and thus corrected cell depolarization and the ensuing aldosterone over production in those cells overexpressing the mutated channel. Of utmost importance, such effects were found to be selective in that wild-type channel transfected cells were unaffected.

Along this line, exploiting use of an immune separation technique of aldosterone-producing cells isolated *ex vivo* from wild-type and G151R and L168R APA we could show a similar concentration-dependent blunting of CYP11B2 gene expression and aldosterone production with clarithromycin at concentrations between 5 and 50 micromolar [12]. These seminal discoveries might pave the way to new strategies for the diagnosis and treatment of APA with KCNJ5 mutations. However, to date these evidences only pertain to *in vitro* studies and there are no proof-of-concept studies *in vivo*. Therefore, we have conceived two related protocols to investigate the diagnostic and therapeutic effect of clarithromycin and roxithromycin, two of the macrolides found to potentially blunt the dysfunction of mutated Kir3.4 channels.

Methods

This proof-of-principle study will be conducted at the Arterial Hypertension Unit of the University of

Padova, Italy and of the Georges Pompidou European hospital, Paris, France, two reference centers for secondary hypertension labelled by the European Society of Hypertension as Excellence centres. At each of these centers more than 100 new cases of PA are diagnosed on average every year, through systematic screening of referred hypertensive patients.

Study design and setting

Study A: Consecutive hypertensive patients of both sexes with PA, who according to current guidelines [13] must be subtyped by AVS before being referred for adrenalectomy, will be exposed to a single IV dose of 250 mg clarithromycin, the third most potent macrolide found to inhibit *in vitro* the mutated Kir3.4 channel [11]. Clarithromycin will be diluted in 250 ml saline and administered in a peripheral vein as a slow infusion (45 min). This dose of clarithromycin should yield peak plasma concentrations of 2.78 mcg/mL (on average), which are higher than the IC_{50} measured *in vitro* (0.53–1.29 mcg/mL) for blunting aldosterone secretion [11]. The hormonal levels in each adrenal vein and the BP values will be measured twice at baseline (–15 min and 0 min) and again after 15 min at time as usually done when we performed AVS. Sodium and potassium diets will be unrestricted (Figure 1).

Study B: to Investigate the hormonal and hemodynamic effects of roxithromycin, the most potent macrolide found to inhibit *in vitro* the mutated Kir3.4 channel, (see later) 300 consecutive hypertensive patients of both sexes undergoing screening for PA will be exposed to a single oral dose of 150 mg of the antibiotic. To this end, the baseline values of these variables will be recorded and compared to the post-roxithromycin values after 60 minutes. This oral dose of roxithromycin should yield peak plasma concentrations of 5–12 mcg/mL, which are higher than the IC_{50} measured *in vitro* (0.18–0.53 mcg/mL) for inhibition of aldosterone production. Blood samplings will be done with the patient in semi-recumbent position from –30 min to 240 min. Sodium and potassium diets will be unrestricted (Figure 2).

In the hypokalemic patients (about 50% of those with an APA and 20% of those with idiopathic hyperaldosteronism) [2] both Study A and Study B will be undertaken only after normal (between 3.5 and 5.0 mEq/l) serum potassium values have been achieved with oral KCL supplementation to reduce the risk of QT prolongation with the macrolides used.

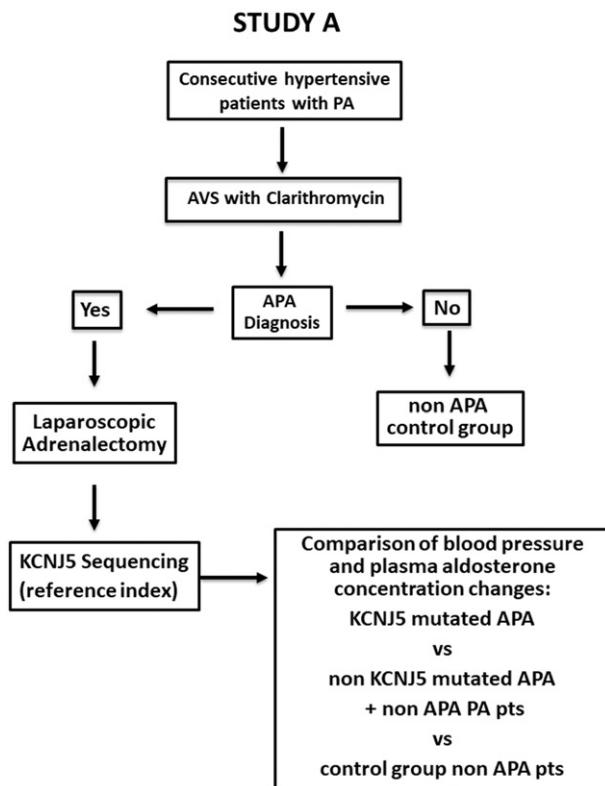


Figure 1. Flowchart of Study A. APA: aldosterone-producing adenoma; AVS: adrenal vein sampling; PA: primary aldosteronism.

Informed consent will be obtained from all individual participants included in the study.

All procedures that will be performed in the study are in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This protocol was registered to Clinical Trial Registry as 4283/AO/17 and the study will be started after approval of the Ethics Committee of the University of Padua.

Primary endpoints

For Study A the primary endpoint will be the within-patient changes from baseline of the relative aldosterone secretion index (RASI) [14] in adrenal vein blood draining the gland with and without the APA. Secondary endpoints will be the changes of blood pressure values during the procedure and tolerability and safety (QTc interval).

For Study B, the primary endpoint will be the within-patient changes from baseline of plasma aldosterone concentration in peripheral blood. Secondary endpoints will be the changes of direct renin active concentration and serum K^+ , 18-oxocortisol, 18-hydroxycortisol,

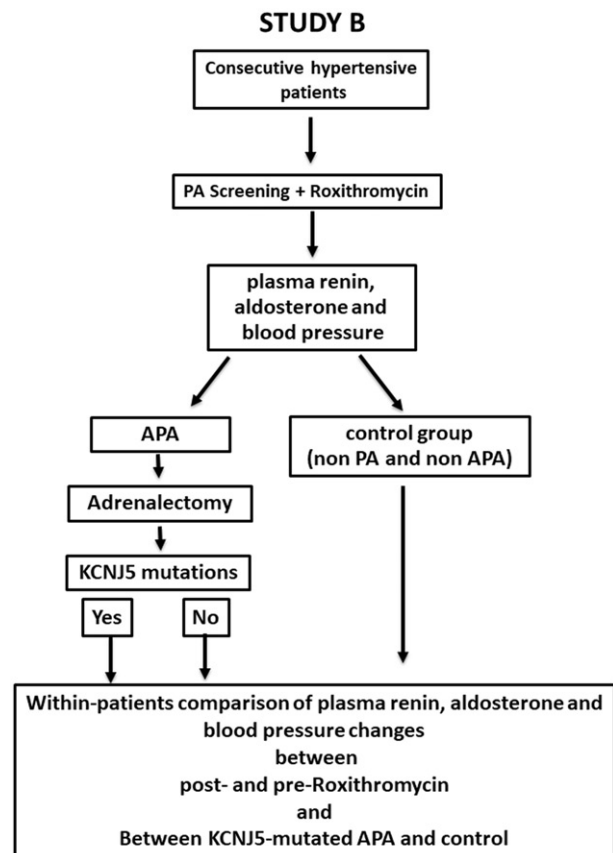


Figure 2. Flowchart of Study B. abbreviations as in Figure 1.

11-deoxycorticosterone, corticosterone, cortisol, blood pressure values and tolerability and safety (QTc interval).

All these changes will be considered as proxy for the effect of the macrolide antibiotics in blunting the altered function of mutated KCNJ5 (Kir3.4) channels in vivo in patients with PA.

Exclusion criteria

The exclusion criteria are listed in Table 1. As regards the concomitant drugs, we will exclude patients who require any drug that increases the QT interval, including (but not exclusively), diuretics, domperidone, sulpiride, serotonin reuptake inhibitors (citalopram, escitalopram), amiodarone and other anti-arrhythmic drugs.

Reference index

For both study A and B, according to the STARD recommendations [15], we will use a reference (gold standard) index. This will be the presence/absence of KCNJ5 mutations, which will be unambiguously

Table 1. General exclusion criteria and exclusion criteria for study A.

General exclusion criteria
History of allergy/intolerance to any macrolides
Refusal of the patient to undergo dynamic testing
Long-QT syndrome (familial and /or drug induced), e.g. a marked baseline prolongation (demonstration of a QTc interval >450 ms)
The use of concomitant medications that prolong the QT/QTc interval
A history of additional risk factors for Torsade de Pointes (TdP) (e.g. heart failure, hypokalemia)
History of syncope and/resuscitated sudden death
Study A exclusion criteria
Patient's refusal to undergo AVS
Contraindications to the general anaesthesia that is required for laparoscopic adrenalectomy
Large tumour suspicious of adrenocortical carcinoma
Cortisol–aldosterone co-secreting adenoma

QT is the QT interval on ECG; QTc: indicates the heart rate – corrected QT interval; AVS: adrenal vein sampling.

determined by sequencing the tumour tissue of the adrenalectomized APA patients.

Sample size

For study A, we calculated that inclusion of 15 patients undergoing AVS, of which about 40% will have KCNJ5 mutations, will provide a >99% power to the study to detect a treatment difference at a two-sided 0.001 significance level, if the true difference between baseline and post-drug for RASI is 4.0, based on the assumption that the standard deviation is 2.0. For Study B, we calculated that with 300 consecutive hypertensive patients, we would detect 42 cases of PA (14% prevalence). Given the rate of surgically curable cases identified at our centres, we expect to have at least about 15 cases of APA, of which about 50% will have KCNJ5 mutations. Under assumptions similar to those made above, we expect to have a power of about 94% to detect a difference in plasma aldosterone concentration of 4.0 ng/dl, based on the assumption that the standard deviation is 2.0 ng/dl.

Statistical analysis

Results will be expressed as mean ± SD, or median and interquartile range, as appropriate. Parametric (student t-test) and non-parametric (Wilcoxon test) statistics will be used for within-patient comparison of quantitative variables as appropriate in the APA and non-APA sub-cohorts.

A Bland-Altman plot will be used to detect systematic biases between baseline and post-macrolide variable values for study B.

In study B, the diagnostic accuracy of the fall in plasma aldosterone concentration in response to

roxithromycin will be assessed by the area (AUC) under the receiver operating characteristic (ROC) curve using the G151R and L168R mutation status as reference. The plasma aldosterone concentration and its fall in response to roxithromycin will be compared in terms of AUC between mutated and wild-type using the method of Hanley & McNeil [16]. Significance will be set at $p < .05$. MedCalc (MedCalc Software Ostend Belgium, vers. 15.8) and SPSS (vers. 24 for Mac, SPSS Bologna, Italy) will be used for data analysis.

Discussion

AVS represents the key test for allocating patients to adrenalectomy or life-long medical treatment according to all guidelines [1,17]. However, being technically challenging and minimally invasive, this test is not widely available and is not offered to all PA patients who should be submitted to it even major referral centres worldwide do [18]. The discovery of KCNJ5 mutations in APA suggested the possibility that patients carrying mutated tumours could be identified without undertaking invasive investigations as AVS, simply by using circulating cell free DNA. Unfortunately, at variance with what achieved in cancer and notwithstanding intensive efforts this hypothesis could not be proven thus far, likely because of the minimal dislodging of cells from APA, 99% of which are benign and show a very slow grow rate.

The recent discovery that G151R and L168R, the most common KCNJ5 mutations render the Kir3.4 channel exquisitely sensitive to inhibition by some macrolide antibiotics and some of their non-antibiotic derivatives. These agents blunted concentration-dependently aldosterone production *in vitro* in HAC-15 cells transfected with heterodimers carrying these two mutations provided ground-breaking evidence that the altered physiology of this channelopathy could be corrected by a specific pharmacologic intervention with commonly used macrolides [11]. These antibiotics feature a many-membered lactone ring with one or more deoxy sugars attached [19] and exerts their antibacterial activity by inhibiting protein synthesis by binding of the desosamine sugar and the lactone ring to bacterial 23S ribosomal RNA [20]. Binding to other targets produces anti-inflammatory activity [21], which can have clinical utility, and stimulation of gastrointestinal motility that can cause side effects [22]. A systematic analysis of 14 different macrolides showed that clarithromycin and roxithromycin were among the most potent macrolide

antibiotics to inhibit the Kir3.4 channel with G151R and L168R mutations [11].

The demonstration that the over secretion of aldosterone can be specifically blunted with macrolide antibiotics in APA cells immuno-magnetically separated ex vivo from human APA with G151R and L168R mutations, represented a first proof of the concept that specific inhibition with macrolides can be clinically useful [12].

Proving the same effects in patients with PA represents, however, a further leap forward to be made. Therefore, in this study we have conceived two protocols to test the effect on aldosterone and other steroids secretion and blood pressure in patients with PA of clarithromycin and roxithromycin, the two macrolides that are most potent in blunting mutated Kir3.4 function. This proof-of-principle study is not only necessary, but also urgent, given the multitude of patients who are currently exposed to the complications of hyperaldosteronism, which commonly causes stage II-III and/or drug resistant arterial hypertension.

As macrolides have been reported to prolong the ECG QT interval [23–25] we have taken the precaution to exclude from the study all the patients with the features listed in Table 1, even though such adverse effect has never been reported with a single dose of the drug, but also with repeated administration [26,27]. This is unlikely to introduce a selection bias since the number of PA patients with known long QT (either familial or drug-induced) or a history of syncope is expected to be very small.

Conclusions

In summary, we believe that this study will allow to prove the concept that in APA harbouring somatic KCNJ5 mutations: (1) acute administration of a common widely used macrolide antibiotic, as clarithromycin, by lowering the relative aldosterone secretion index on the side with a mutated APA, could allow identification of these tumours before undertaking adrenalectomy and sequencing of tumour's DNA; (2) a fall of plasma aldosterone in peripheral venous blood after roxithromycin can be a proxy for the presence of such tumours, helping thus to move forward adrenalectomy in these patients. We find this to be a substantial amount of new knowledge that goes in the direction of precision (personalized) treatment of a common cause of arterial hypertension.

Disclosure statement

The authors declare that they have no conflict of interest.

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