MAcrolides for Precision Medicine Management of Aldosterone-Producing Adenoma (MAPA Study)

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SUPPLEMENTAL MATERIAL

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Patients

The patients were unequivocally diagnosed with PA according to the current guidelines.(1,2) Diagnosis of APA included the 5 corners criteria: (1) lateralization of aldosterone secretion at AVS, (2) surgery, (3) pathology, (4) outcome of adrenalectomy at follow-up, and demonstration of a CYP11B2-positive adenoma at pathology.(3) Biochemical evidence of PA correction at the follow-up included normalization of hypokalemia without K^+ supplementation and the aldosterone-to-renin ratio, along with correction of renin suppression and decrease of plasma aldosterone concentration (PAC) after removal of the responsible adrenal gland, documented in Figure S7.

The baseline and the post-roxithromycin values of PAC, direct active renin concentration (DRC), cortisol concentration (PCC), ACTH, and systolic and diastolic BP were compared within-patient, with the patient in semi-recumbent position after 60 min of quiet resting. To mimic real-life conditions, dietary sodium and potassium intake was unrestricted; normokalaemia was achieved with oral KCl supplementation in hypokalemic patients to avoid the risk of macrolide-induced QT prolongation.(4)

All biochemical measurements were performed in the ISO 9001-certified central laboratory of the University Hospital of Padua. DRC and PAC were measured shortly after blood sampling in the ad hoc collected samples using an automated system (DiaSorin, LIAISON. XL instrument), the LIAISON. Direct Renin kit (DiaSorin, Saluggia, Italy) and the LIAISON. XL Aldosterone kit. Normal ranges and antibody cross-reactivity for the hormonal measurements have already been reported.(5)

Plasma roxithromycin measurement

Measurement of plasma roxithromycin concentration by high performance liquid chromatography (HPLC). In brief, 100 µL of internal standard solution (clarithromycin, 0·1 mg/mL) were added to 1 mL of plasma. The sample was alkalinized with 100 µL of a Na_2CO_3 solution (pH 9·2) and extracted with 6 mL exane/propanol (98:2). After centrifugation, the organic phase was back-extracted in 300 µL of

acetic acid (0·1%) and 10 µL were injected in a Kromasil 100-5 C18 (250x4·6 mm) chromatographic column.(6) The mobile phase consisted of a mixture (v/v) of acetonitrile (37%) and KH_2PO_4 buffer (63%, pH 4·5), flow rate 1 mL/min. The effluent was analysed with a UV detector set at 210 nm. Calibration curves were linear in a range of 0·5-10 µg/mL and the coefficient of determination R^2) was always > 0·99. Recovery reached 76·3% for roxithromycin and 88·9% for clarithromycin and the limits of detection, defined as a signal-to-noise ratio of 3:1, were 0·10 µg/mL and 0·33 µg/mL, respectively.

Tumour analysis

Tumour specimens were collected in the operating room, immediately after surgery, with utmost precaution to ensure the preservation of nucleic acids, tissue structure, and morphology.(7)

After DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Milan, Italy), adrenal specimens were sequenced using Sanger sequencing of KCNJ5 with standard methods following polymerase chain reaction (PCR) amplification, as reported.(7) Briefly, PCR was performed to amplify genomic fragments of the KCNJ5 gene using Sso Fast EVA Green Supermixes (Bio-Rad, Los Angeles, CA, USA) and specific primers (forward, 5'-CGACCAAGAGTGGATTCCTT-3', and reverse, 5'-AGGGTCTCCGCTCTCTTCTT-3'). Purification of PCR products was done using the PCR Purification Kit (Qiagen, Milan, Italy).

Direct sequencing of PCR products spanning amino acids 122 to 199 was performed using the ABI Prism Big Dye Terminator version 3·1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3700 DNA analyzer (Applied Biosystems, Milan, Italy).

Next generation sequencing (NGS) was systematically performed in all excised adrenals to search for KCNJ5-mutated or wild-type APA, after CYP11B2 immune-histochemical identification of the responsible nodule(s).

NGS was performed using a custom panel of biotinylated oligonucleotides (xGenTM Hyb Panel, Integrated DNA Technologies, Coralville, Iowa, USA), as recently reported.(7) The custom panel

consisted of 487 amplicons covering coding regions and flanking exon/intron boundaries of 12 PA putative genes (KCNJ5, ATP1A1, ATP2B3, CTNNB1, CACNA1D, APC, CACNA1H, PRKACA, ARMC5, GNA11, GNAQ, CLCN2), which provided an average gene coverage > 99% at 500X. The libraries for targeted capture were prepared using the Lotus DNA library prep Kit (IDT). Briefly, 100 ng of DNA was quantified by Qubit DNA HS assay (Life Technologies, Milan, Italy). Genomic DNA was enzymatically cut; fragments were then ligated with stubby P5 and P7 adapters, following the manufacturer's instructions.

AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to purify the libraries, which were quantified by LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer, Boston, MA, USA). A multiplexing pool of 500 ng of each library from 12 samples was used for hybrid capture with the biotinylated DNA oligonucleotides custom panel. After hybridization, Streptavidin Dynabeads were used to capture biotinylated DNA. Purified DNA was amplified using the Bio-Rad CX200 thermal cycler (Bio-Rad Laboratories, Hercules, USA). The library pool was further purified using AMPure XP beads (Beckman Coulter, Brea, USA) and quantified by Qubit DNA HS assay. Amplicon length average size was 254. Finally, libraries were sequenced on an Illumina MiSeq at a final concentration of 12 pM using a v3 cartridge (2 × 151 reads) following the manufacturer's instructions. This high-resolution NGS method was found to provide a high Qscore of 30 (Q30) of 99·9%, indicating that the base call accuracy (ie, the probability of a correct base call) is higher compared with other available methods

Sample size and power calculation

Considering that the PA prevalence at our centre was 14% when the study was planned, we calculated that about 300 consecutive hypertensive patients were needed to detect 42 cases of PA. Of them, at least 25 cases were expected to have a surgically curable APA.(7) We also estimated that about 50% of them could harbour the mutations, a prediction confirmed after introduction of NGS, which is more

sensitive for detecting KCNJ5 mutations than Sanger sequencing.(7) Based on these calculations, we estimated that 12 patients per group would furnish to the study a 94% power to detect a mean fall of PAC values of 4·0 ng/dl after roxithromycin (with a SD of 2·0 ng/dl), at α=0·05 using a Wilcoxon test for comparison.

After one year, an ad interim auditing indicated that 150 mg roxithromycin did not produce effective plasma levels of the drug in some patients. Therefore, it was decided to double the dose to 300 mg and to examine results in an 'intention-to-treat' analysis comprising all patients, and in a 'per protocol' analysis confined to those that showed a plasma concentration of roxithromycin above the drug IC50.

Hence, the following analyses were performed:

1. Intention-to-Treat (ITT) analysis: All the patients, regardless of having reached or not effective plasma levels of roxithromycin after dosing;

2. Per-Protocol (PP) analysis: Patients who achieved effective plasma levels of roxithromycin after dosing.

Missing data

Only 8 of 307 BP values were missing in non-PA patients. No replacement was done.

No data lacked in KCNJ5-mutated or wild-type APA patients.

Ex vivo studies on NO-mediated vasodilation

Mice studies design

We investigated the vascular effects of macrolides in mice *ex vivo* in isolated aorta segments and *in vivo*. The protocols were approved by the Ethical Committee of Research of the Universidad

Autónoma de Madrid and by Dirección General de Medio Ambiente, Comunidad de Madrid, Spain (PROEX 183.2/20).

Animals' handling followed the Spanish Policy for Animal Protection RD53/2013 and European Union Directive (2010/63/UE) recommendations. Experiments were conducted in accordance with the National Institutes of Health (NIH) guidance (NIH Publication No. 85-23, revised 1996).

Male, 13- to 15-weeks old, C57BL/6J mice were used to avoid possible interferences by female hormones. Mice were maintained in the animal facility of the Universidad Autónoma de Madrid (UAM) under controlled temperature and humidity conditions, fed with standard diet and water, and submitted to 12-hour light/dark cycles. In *ex-vivo* experiments, animals were randomly assigned to different treatment arms (roxithromycin, clarithromycin, azithromycin). Azithromycin was used for *in-vivo* experiments. Mice were euthanized at 13 to 15 weeks of age by CO_2 inhalation.

Immediately after sacrifice, the heart and aorta were excised en block, fat and connective tissue were removed, and aortas were separated from hearts and placed in cold (4°C) Krebs-Henseleit solution (KHS) (mmol/L: 115 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·2 $MgSO_4.7H_2O$, 2·5 $CaCl_2$, 1·2 KH_2PO_4, 11·1 glucose, and 0·01 Na_2EDTA). About 2 mm-long segments of the aorta were mounted in a wire myograph (Danish MyoTech, Aarhus, Denmark). Arteries were first equilibrated for 30 min in oxygenated KHS continuously bubbled with 95% O_2 and 5% CO_2, and then stretched to their optimal lumen diameter for active tension development. To test the functional integrity of the arterial segments, they were initially exposed to a 120 mmol/L K^+ solution; endothelium integrity was verified by relaxation of phenylephrine (1 nmol/L–0·1 mmol/L)-precontracted vessels with exposure to acetylcholine [10^{-5}M]. After that, endothelium-intact concentration-response curves for roxithromycin, clarithromycin, azithromycin, and the vehicle dimethyl sulfoxide (DMSO) (1 nmol/L–30 µmol/L) of phenylephrine-precontracted arteries were performed (n= 5-6 mice).

To assess the endothelial-dependently macrolide vasodilatation effect, the endothelium of the vessel was mechanically removed. Effectiveness of the removal was proven by lack of acetylcholine-induced relaxation in phenylephrine-precontracted vessels. To test the role of nitric oxide in the relaxation effect of macrolides, in parallel experiments vessels were incubated for 30 minutes with NG-Nitroarginine Methyl Ester (L-NAME) [10^{-4}M] followed by precontraction by phenylephrine and relaxation by macrolides (n=5-12 mice). All macrolides were dissolved in dimethyl Sulfoxide (DMSO). All drugs were purchased from Sigma-Aldrich Roxithromycin 95·0-102·0% (HPLC) R4393-1G, clarithromycin ≥95% (HPLC): C9742-250MG, azithromycin dihydrate ≥98% (HPLC): PZ0007-5MG phenylephrine hydrochloride (Cat-No. P6126), acetylcholine chloride (Cat-No. A9101).

Studies on the antihypertensive effect of macrolides

This animal study complied with the 3Rs (Replacement, Reduction, and Refinement) principle. For this *in-vivo* study, the animals were randomly assigned to 4 different experimental groups (7-10 animals per group, total n=32 mice): 1) control vehicle treated mice; 2) azithromycin treated (azithromycin dihydrate ≥98% mice by intraperitoneal injections for 14 days, 50 mg/kg/day); 3) angiotensin II-infused mice (Ang II; 1·44 mg/Kg/day, in subcutaneously implanted Alzet osmotic minipumps (Durect Corp, Cupertino, CA), 14 days); 4) angiotensin II-infused and azithromycin treated.

Systolic BP was measured by tail-cuff plethysmography (BP analysis System, BIOSEB in vivo research instruments series 2000) at days 0, 3, 7, and 14 of intervention, and expressed as the average of 10 individual observations for each animal. Measurements were performed at the same time of the day and animals were first adjusted to the measurement device 2-3 times before starting treatment.

Statistical analysis

Results were expressed as mean ± SD, or median and interquartile range; parametric (student t-test) and non-parametric (Wilcoxon test) statistics were used for within-patient comparison of quantitative

variables, as appropriate. To determine results robustness two different scenarios were examined: i) an intention-to-treat analysis in all the patients regardless of their having reached or not effective plasma levels of roxithromycin after dosing; ii) a per-protocol analysis restricted to those who had achieved effective plasma levels of roxithromycin after dosing.

For animal studies, results were expressed as mean ± SEM, and analyzed by two-way ANOVA followed Bonferroni post hoc test.

For data analysis MedCalc (MedCalc Software Ostend Belgium, vers. 15·8), GraphPad Prism (vers. 10·5 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com) and SPSS (ver. 29 for Mac, SPSS Bologna, Italy) were used.

Contributors. GPR and TMS were involved in study conceptualization and development of the methods. GPR and TMS participated to the recruitment; AB, BC, GC contributed to data collection, GPR, TMS, AB to data analysis and visualization. GPR, TMS and AMB were involved in conceptualization of the experimental studies. CB, ABG-R and AMB performed the *ex vivo* and *in vivo* experimental studies. RP contributed to the set up of plasma roxithromycin assay; GZ was involved in modeling of Kir3·4 channel. GC and AG performed NGS analysis. GPR, TMS, and AMB supervised the overall study. GPR was responsible for the study.

All authors provided approved the manuscript and were responsible for the decision to submit for publication.

Data sharing. Data from this study can be requested to the Prof. G.P. Rossi two years after the publication. Participant will be de-identified. The request needs the submission of a proposal with a valuable research question.

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Table S1. Exclusion criteria.

- History of allergy/intolerance to any macrolide;
- Refusal of the patient to undergo to roxithromycin administration;
- Long-QT syndrome (familial and /or drug induced), e.g. a marked baseline prolongation (demonstration of a QTc interval >450 ms);
- Use of concomitant medications that prolong the QT/QTc interval, as domperidone, sulpiride, serotonin reuptake inhibitors (citalopram, escitalopram), and anti-arrhythmic drugs that prolong the QT interval;
- History of additional risk factors for Torsade de Pointes (e.g., heart failure, hypokalemia);
- History of syncope and/resuscitated sudden death;
- Drugs known to affect the renin-angiotensin-aldosterone system (RAAS), as diuretics and RAAS blockers;
- 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.

AVS: adrenal vein sampling; QT: QT interval on ECG; QTc: heart rate-corrected QT interval; RAAS: Renin Angiotensin Aldosterone System.

Table S2. Changes in plasma aldosterone concentration (PAC), direct renin concentration (DRC), plasma cortisol concentration (PCC), systolic blood pressure (SBP) in KCNJ5 mutated and wild-type APA patients before and after roxithromycin challenge.

	KCNJ5 mutated (n=19)			**KCNJ5 wild-type (n=27)**		
	Baseline	After roxithromycin challenge	p	Baseline	After roxithromycin challenge	p
Intention-to-treat Analysis						
PAC, ng/dL	22·6 [17·5-34·9]	21·9 [16·4-34·5]	0·0008	24·3 [17·2-31·6]	23·9 [17·7-34·6]	0·3040
DRC, mIU/L	2·0 [2·0-3·2]	2·0 [2·0-2·9]	0·0625	2·5 [2·0-5·9]	2·0 [2·0-4·3]	0·0542
PCC, nmol/L	254 [198-287]	203 [166-236]	0·0011	255 [171-298]	196 [163-299]	0·1068
SBP, mmHg	150±20	151±22	0·6934	147±15	145±14	0·3163
Per Protocol Analysis						
PAC, ng/dL	26·7 [17·6-38·7]	24·9 [16·4-37·2]	0·0034	24·3 [17·6-38·7]	27·4 [17·7-36·0]	0·7146
DRC, mIU/L	2·0 [2·0-2·9]	2·0 [2·0-2·6]	0·2500	2·5 [2·0-6·0]	2·5 [2·0-4·3]	0·0498
PCC, nmol/L	257 [200-289]	196 [166-237]	0·0034	247 [175-316]	193 [145-302]	0·1090
SBP, mmHg	152±20	152±22	0·9327	146±14	145±13	0·5035

Mean ± SD, or median [interquartile range].

Table S3. Changes in plasma aldosterone concentration (PAC), direct renin concentration (DRC), plasma cortisol concentration (PCC), systolic blood pressure (SBP) in non-PA patients before and after roxithromycin challenge.

	non-PA patients (n=307)		
	Baseline	After roxithromycin	p
Intention-to-treat Analysis			
PAC, ng/dL	7·6 [5·9-9·3]	6·7 [5·4-8·5]	<0·0001
DRC, mIU/L	6·8 [3·6-13·1]	6·0 [3·0-12·2]	<0·0001
PCC, nmol/L	240 [184-295]	197 [150-258]	<0·0001
SBP, mmHg	137±14	135±13	0·0004
Per Protocol Analysis			
PAC, ng/dL	7·5 [6·0-9·3]	6·7 [5·4-8·6]	<0·0001
DRC, mIU/L	6·6 [3·1-12·7]	5·8 [2·5-11·8]	<0·0001
PCC, nmol/L	239 [183-295]	197 [150-258]	<0·0001
SBP, mmHg	138±14	135±13	0·0003

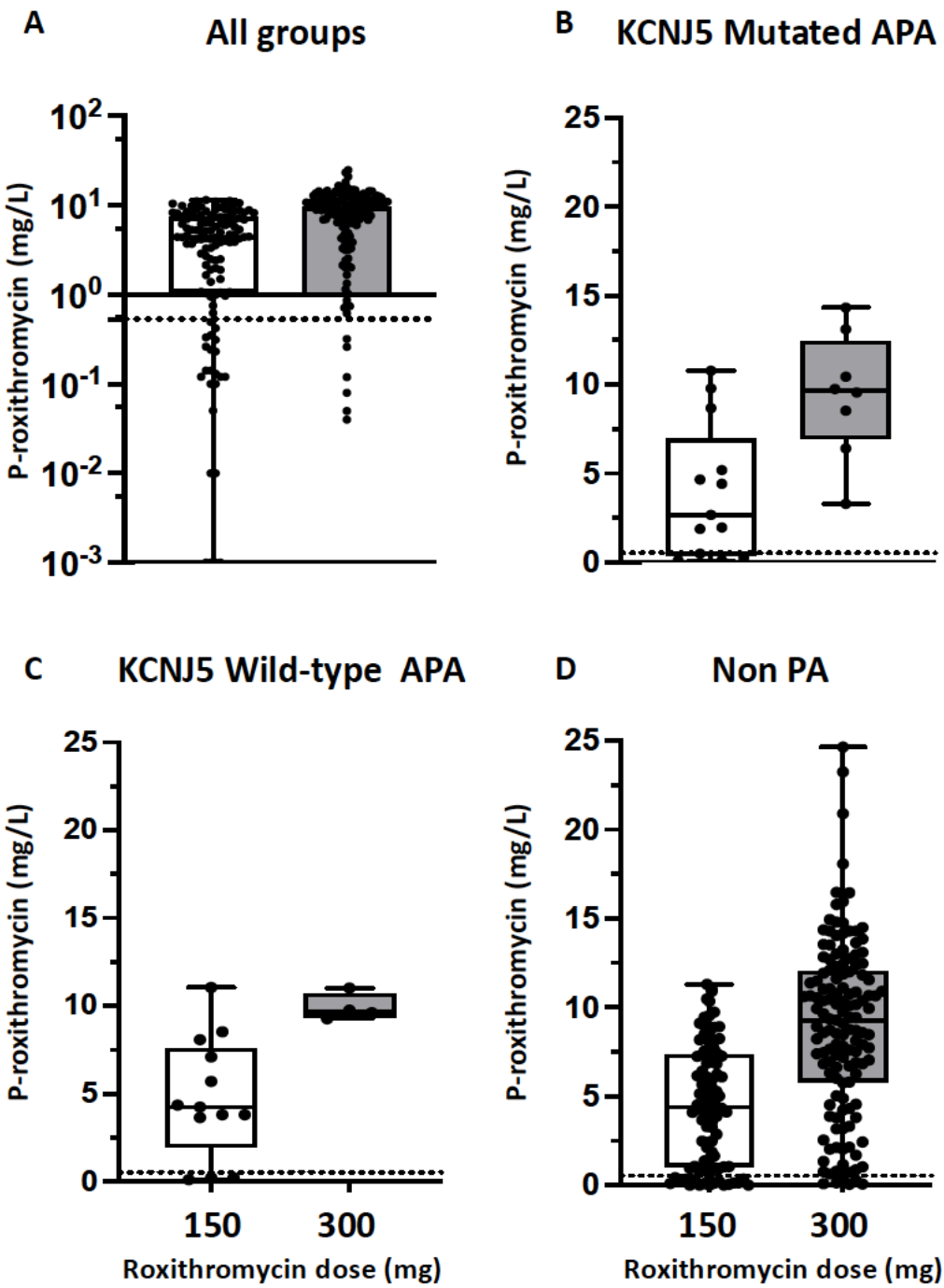

Figure S1. Plasma levels of roxithromycin measured after a single dose of 150 and 300 mg in all patients (Panel A), KCNJ5 mutated (Panel B) and wild type (Panel C) APA, and in non-PA patients (Panel D). With the 150 mg dose, about 13% of the patients did not reach the effective concentration of the drug (above the dotted line). This rate fell to (4%) with the 300 mg dose indicating that 300 mg was the appropriate dose for the study. Log scale allows better visualization of the proportion of cases not achieving plasma levels of roxithromycin higher than IC50 level (Panel A).

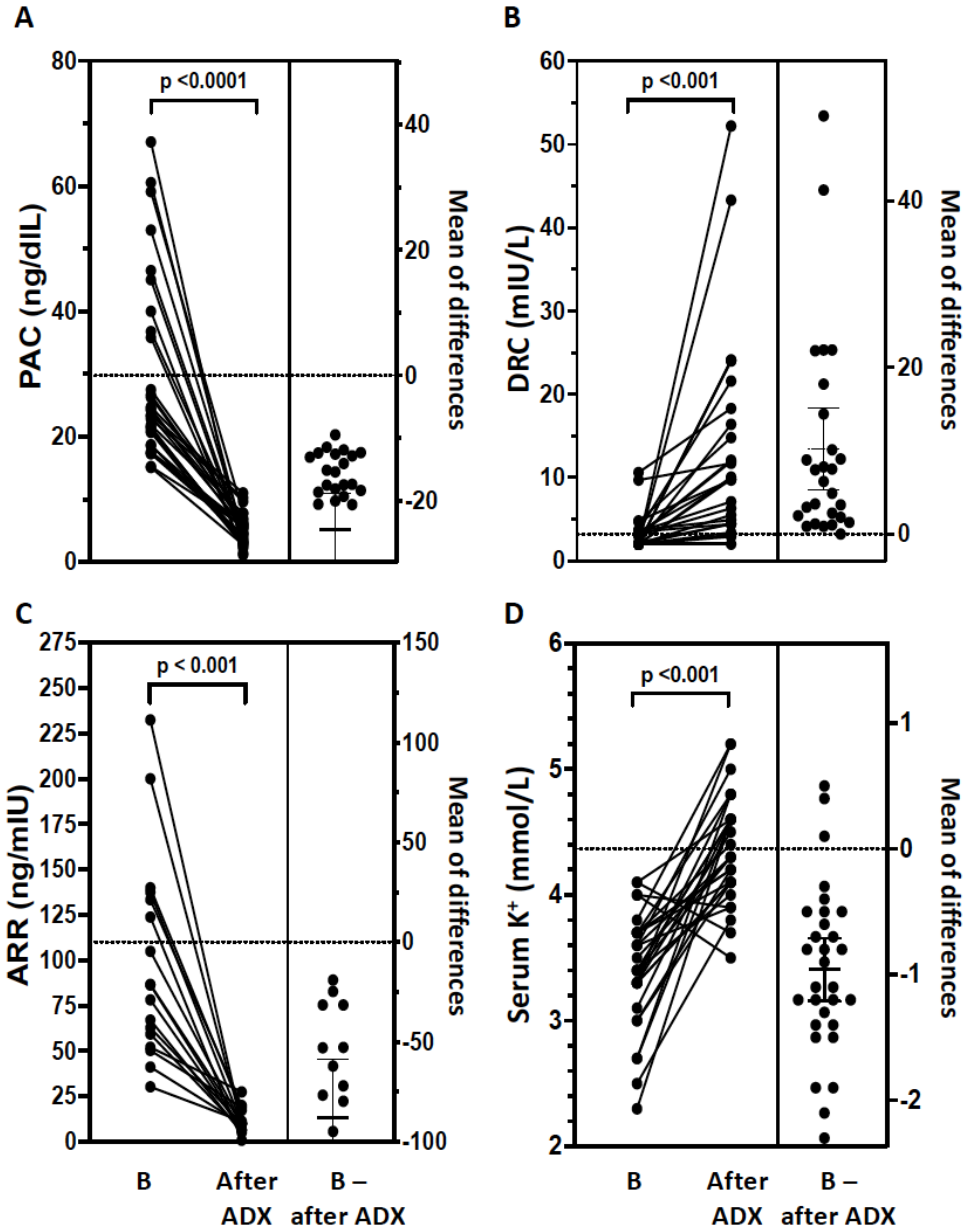

Figure S2. Changes in plasma aldosterone concentration (PAC, Panel A), direct renin concentration (DRC, Panel B), aldosterone to renin ratio (ARR, Panel C) and serum K^+ concentration (Panel D) in APA patients, before and after adrenalectomy (ADX). Surgery, as requested by the by 5-corners criteria,(3) corrected the abnormal values of PAC, DRC, ARR and serum K^+, thus supporting the diagnosis of PA.

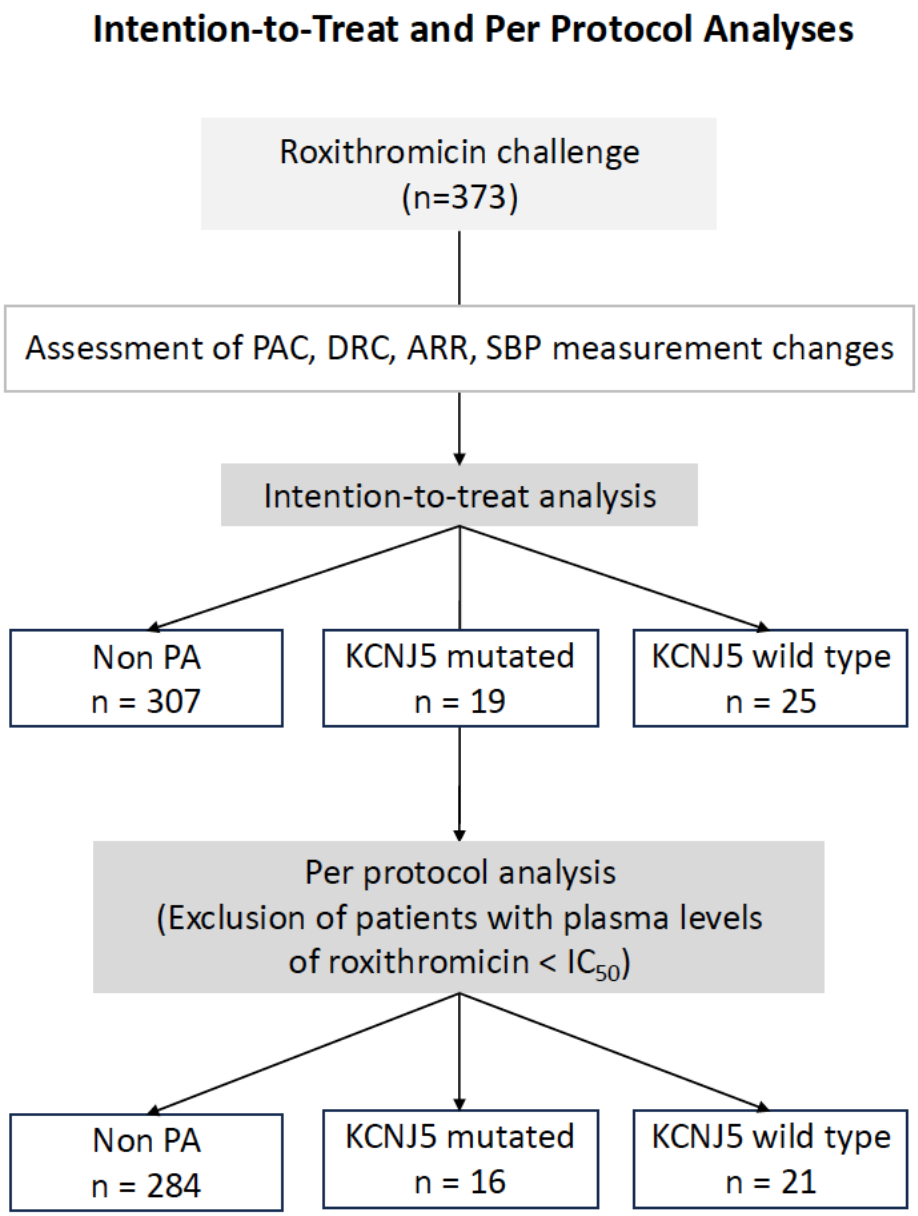

Figure S3. All enrolled patients were exposed to the roxithromycin challenging test, with measurement of PAC, DRC, ARR, PCC and SBP changes before and after the test. Both intention-to-treat and per protocol analyses were performed: all patients were considered for the intention-to-treat analysis, whereas only patients who did achieve effective plasma levels of roxithromycin, i.e. higher than IC50, were included in the per protocol analysis.

PAC: plasma aldosterone concentration; DRC: direct renin concentration; ARR: aldosterone to renin ratio; PCC: plasma cortisol concentration; SBP: systolic blood pressure.

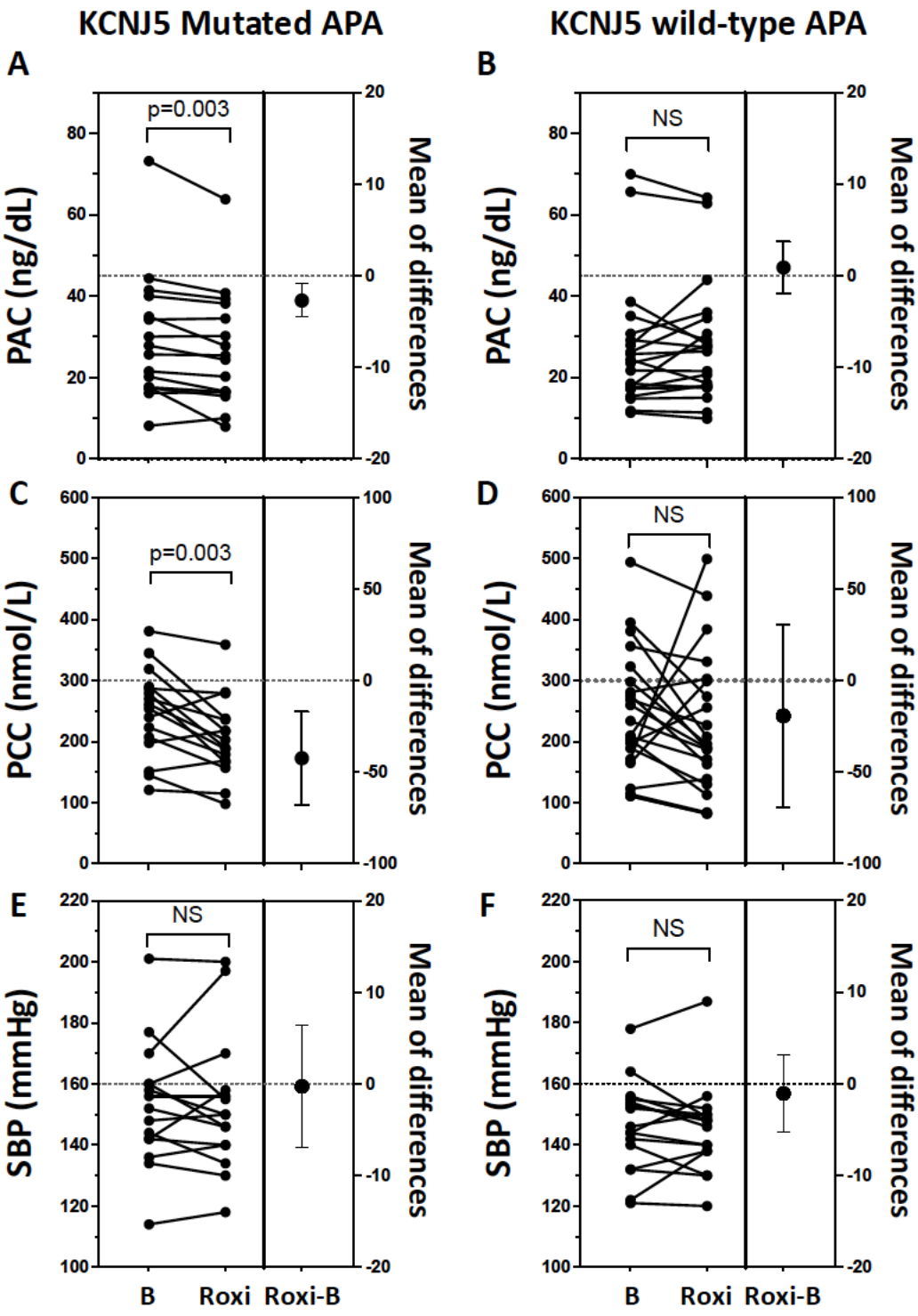

Figure S4. Changes in PAC (Panels A and B), PCC (Panels C and D), and SBP (Panels E and F) in KCNJ5 mutated and wild type APA, after roxithromycin challenging test in APA patients achieving roxithromycin plasma levels > IC50 (per-protocol analysis). As observed in the intention-to-treat analysis, a significant decrease in PAC was seen in the KCNJ5 mutated, but not in the wild-type APA, and SBP did not significantly decrease in either group. The estimation plots (right parts of the plots) visually illustrate the size of the change and the significance level.

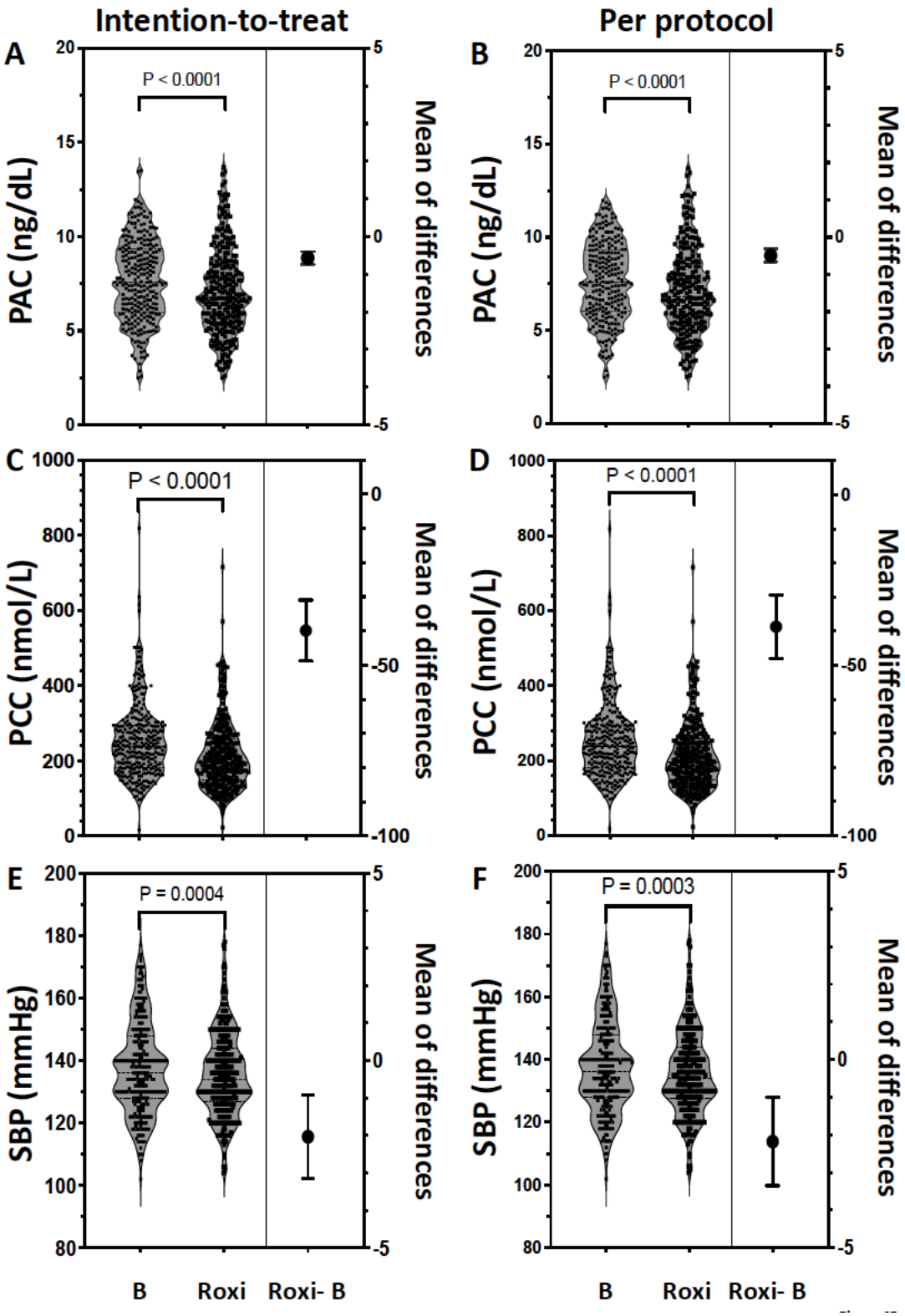

Figure S5. Changes in plasma aldosterone concentration (PAC), plasma cortisol concentration (PCC) and systolic blood pressure (SBP) in non-PA patients, before and after roxithromycin challenging test. A small but statistically significant fall of PAC (Panels A and B), PCC (Panels C and D), and BP (Panels E and F) was found at both per protocol and intention-to-treat analyses. The estimation plots (right parts of the plots) visually illustrate the size of the change and the significance level.

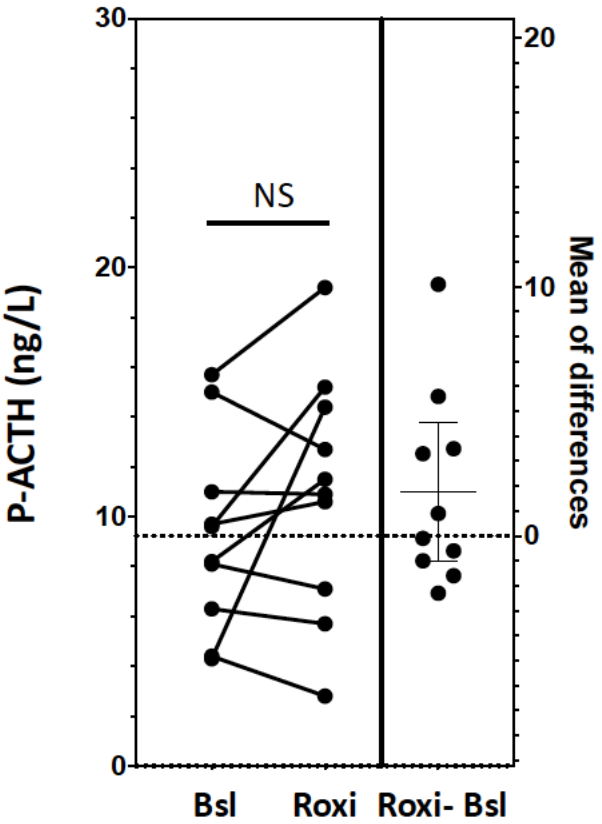

Figure S6. The response of plasma ACTH levels to the roxithromycin challenging test in a small group of non-PA patients was heterogeneous, with not significant change of ACTH values.

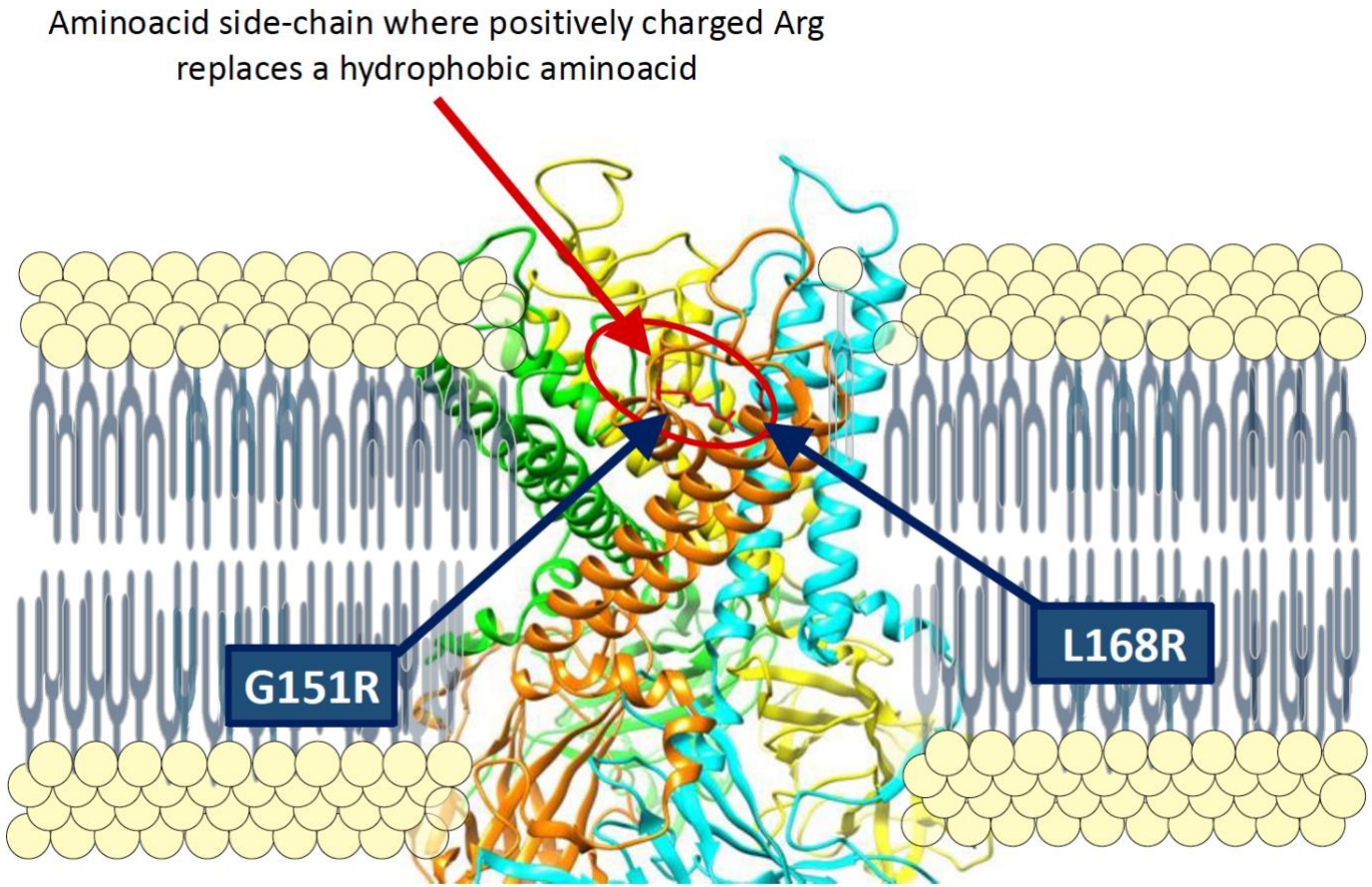

Figure S7. The sketch reproduces the structure of the potassium channel Kir3.4 (coded by KCNJ5 gene). The most common mutations G151R and L168R (blue arrows) entail a replacement of a hydrophobic amino acid with a positively charged Arg at 2 positions where the amino acid side-chain (shown in red) are internal in the model. It can be anticipated that either mutations can cause a global rearrangement of the channel structure causing the loss of selectivity of the channel and/or its selectivity filter.

G151R involves the first portion of the channel and, even though it is not directed toward the pore, the presence of a bulky positively charged residue likely perturbates the structure. L168R is located in the following alpha helix.

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Personalized Management of Aldosterone-Producing Adenoma with Macrolides (MAPA Study)
		(*b*) Background. Primary aldosteronism (PA) is the most common curable cause of arterial hypertension, often due to aldosterone-producing adenoma (APA) with mutations in the KCNJ5 potassium channel in up to 70% of the patients. The altered function of the most common KCNJ5 mutations was corrected in vitro by macrolides, suggesting their usefulness for personalized management of KCNJ5-mutated APA patients. We therefore investigated if roxithromycin could lower plasma aldosterone and blood pressure (BP) in KCNJ5-mutated APA patients. Methods. We prospectively assessed the within-patient changes of plasma aldosterone, active renin, cortisol, and BP, induced by roxithromycin in consecutive hypertensive patients, and investigated the haemodynamic effects of macrolides in mice. Findings. 373 consecutive hypertensive patients were submitted to a roxithromycin challenge: 19 had G151R- or L168R-mutated APA, 25 KCNJ5 wild-type APA; the rest had no identifiable cause of hypertension. Roxithromycin lowered plasma aldosterone concentration ($p<0.0008$) in the G151R- and L168R-mutated APA patients and lowered BP in the hypertensive patients without PA. A significant BP-lowering effect of macrolides and an endothelium-NO-dependent vasodilation was revealed in mice studies. Interpretation. The roxithromycin-induced lowering of plasma aldosterone concentration in vivo, which confirms our findings in KCNJ5-mutated APA-cells ex vivo, represents a marker of APA carrying these mutations. We also found that three macrolides exert an endothelium- and NO-dependent vasodilation that could account for BP lowering effect. Funding. FOundation for advanced Research In hypertension and Cardiovascular disease (FORICA), International PhD in Hypertension and Vascular Biology (ARHYVAB) of the University of Padua European Union's Horizon 2020 research grant agreement 954798.
Introduction		
Background/rationale	2	Primary aldosteronism (PA) is the most common curable cause of arterial hypertension, which is often due to aldosterone-producing adenoma (APA) that have mutations in the KCNJ5 potassium channel in up to 70% of the patients. The altered function of the most common KCNJ5 mutations was found to be corrected in vitro by macrolides, suggesting that their use could be useful for personalized management of KCNJ5-mutated APA patients.
Objectives	3	(a) To investigate if the macrolide roxithromycin affects plasma aldosterone concentration (PAC) and BP in humans. (b) To evaluate if macrolides exert an anti-hypertensive effect in mice and to clarify if this effect is endothelium-NO-dependent, or not.
Methods		

Study design	4	The Macrolides for Personalized Management of Aldosterone-Producing Adenoma (MAPA) study was set out as an interventional, within-patient, proof-of-principle study. We investigated the effects of a single oral dose of roxithromycin, a macrolide that potently inhibited aldosterone production in vitro in Kir3.4 channel-mutated cells, in consecutive hypertensive patients of both sexes undergoing screening for PA. The baseline and the post-roxithromycin values of PAC, direct active renin concentration (DRC), cortisol concentration (PCC), ACTH, and systolic and diastolic BP were compared within-patient, with the patient in semi-recumbent position after 60 min of quiet resting.
Setting	5	Setting and location; data collection: Arterial Hypertension Unit of the University of Padua, Italy, a European Society of Hypertension Excellence centre. Periods of recruitment: From April 2018 to November 2021. Exposure: 150 mg (or 300 mg, see later) roxithromycin. Follow-up was needed to have a conclusive diagnosis of APA (5-corner criteria). Diagnosis of APA included the 5 corners criteria: (1) lateralization of aldosterone secretion at AVS, (2) surgery, (3) pathology, (4) outcome of adrenalectomy at follow-up, and demonstration of a CYP11B2-positive adenoma at pathology. Biochemical evidence of PA correction at the follow-up included normalization of hypokalemia without K+ supplementation and the aldosterone-to-renin ratio, along with correction of renin suppression and decrease of plasma aldosterone concentration (PAC) after removal of the responsible adrenal gland.
Participants	6	Inclusion criteria: arterial hypertension on repeated office or home BP measurements, and/or 24-hour BP monitoring, or received antihypertensive agents. Exclusion criteria: treatment with drugs affecting the renin-angiotensin-aldosterone system, or prolonging the QT interval.
Variables	7	The primary endpoint was the change of PAC in peripheral blood from baseline to post-roxithromycin. Secondary endpoints were the changes of DRC, PCC, and systolic and diastolic BP values.
Data sources/ measurement	8*	All biochemical measurements were performed in the ISO 9001-certified central laboratory of the University Hospital of Padua. DRC and PAC were measured shortly after blood sampling in the ad hoc collected samples using an automated system (DiaSorin, LIAISON. XL instrument), the LIAISON. Direct Renin kit (DiaSorin, Saluggia, Italy) and the LIAISON. XL Aldosterone kit.
Bias	9	Measurement of plasma roxithromycin concentration by high performance liquid chromatography (HPLC) was performed in all patients. An interim auditing in 2019 revealed plasma levels of the drug below the IC50 with 150 mg roxithromycin dosing in some patients, which led to double the dose to 300 mg, and to examine the results in all patients ('intention-to-treat' analysis), and in those with plasma concentration of roxithromycin above the drug IC50 ('per protocol' analysis). Hence, the following analyses were performed: 1. Intention-to-Treat (ITT) analysis: All the patients, regardless of having reached or not effective plasma levels of roxithromycin after dosing;

		2. Per-Protocol (PP) analysis: Patients who achieved effective plasma levels of roxithromycin after dosing.
Study size	10	Twelve patients per group would furnish to the study a 94% power to detect a mean fall of PAC values of 4.0 ng/dL after roxithromycin (with a SD of 2.0 ng/dL), at α=0.05 using Wilcoxon test for comparison.
Quantitative variables	11	Comparisons between groups was performed with parametric (t test) or non-parametric test (Mann-Whitney U) for normally or not distributed variables. A p value <0.05 was considered statistically significant.
Statistical methods	12	Describe all statistical methods: see above.
		Missing data. Only 8 of 307 BP values were missing in non-PA patients. No replacement was done. No data lacked in KCNJ5-mutated or wild-type APA patients
		Patients lost at follow-up were excluded from the analysis because no conclusive diagnosis of APA can be performed with no follow-up (5 corner criteria, see above).
		No sensitivity analysis was done
Results		
Participants	13*	(a) As reported in Figure 1: n. 425 patients screened. Excluded n=52 because of lack of consent (n=43), renal artery stenosis (n=6), fibromuscular dysplasia (n=1), isolated systolic hypertension (n=1), hyperthyroidism (n=1). Hence 373 patients were exposed to roxithromycin challenge. Of them, 66 received the diagnosis of PA (21 were found to be bilateral PA, 1 aldosterone producing carcinoma and 44 unilateral PA). Of the 44 unilateral PA, 19 were found to be KCNJ5 mutated and 25 were wild-type.
		(b) Give reasons for non-participation at each stage: see above (only unilateral PA were finally considered).
		(c) Flow diagram: see Figure 1
Descriptive data	14*	(a) General characteristics of KCNJ5 mutated and wild-type APA patients and the hypertensive patients without PA (non-PA) are shown in Table 1.
		(b) As in (12) above, only 8 of 307 BP values were missing in non-PA patients. No replacement was done. No other data lacked in KCNJ5-mutated or wild-type APA patients.
		(c) N.A The follow-up was needed only to have the conclusive diagnosis of unilateral PA.
Outcome data	15*	All outcomes were shown for 19 KCNJ5 mutated and 25 wild-type APA patients.
Main results	16	(*a*) N.A.
		(*b*) No boundaries were used for blood pressure. Age > 18 years.

		(*c*) N.A.
Other analyses	17	Comparison between groups (before and after roxithromycin challenge). No sensitivity analyses.
Discussion		
Key results	18	Roxithromycin lowered plasma aldosterone concentration ($p<0.0008$) in the G151R- and L168R-mutated APA patients and lowered BP in the hypertensive patients without PA.
Limitations	19	A possible limitation could comprise the small number of APA patients that 'survived' our strict inclusion protocol. For example, we excluded the PA patients with long QT because we know that macrolide could further lengthen QT interval. However, this did not impact on the generalizability of our findings given that these excluded patients were very few. The facts that we detected a highly significant fall of PAC after roxithromycin, and that we could recruit more APA patients than initially estimated to be needed in sample size calculation, should reassure about the possibility of a type II error when assessing the effect of the macrolide on PAC.
Interpretation	20	A limitation could be use of only one macrolide in humans, restricting the effects to roxithromycin.
Generalisability	21	There are major strengths supporting the robustness of our findings: i) the prospective design with patients' painstaking phenotyping; ii) the use of unambiguous diagnostic criteria for APA as gold-standard diagnostic reference;30 iii) the measurement of plasma roxithromycin in all patients; iv) the replication of the results in intention-to-treat in and per-protocol analyses. These strengths suggest generalisability of the study results.
Other information		
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