Somatic ATP1A1, ATP2B3 and KCNJ5 mutations in aldosterone-producing adenomas

Tracy Ann Williams¹, Silvia Monticone¹#, Vivien R. Schack²#, Julia Stindl³, Jacopo Burrello¹, Fabrizio Buffolo¹, Laura Annaratone⁴, Isabella Castellano⁴, Felix Beuschlein⁵, Martin Reincke⁵, Barbara Lucatello⁶, Vanessa Ronconi⁷, Francesco Fallo⁸, Giampaolo Bernini⁹, Mauro Maccario⁶, Gilberta Giacchetti⁷, Franco Veglio¹, Richard Warth³, Bente Vilsen², Paolo Mulatero¹.

# equal contributions

Correspondance should be addressed to T.A.W.
Phone: (+39) 011 633 6959/20/17
Fax: (+39) 011 633 6931
Email: tracyann.williams@unito.it

¹ Department of Medical Sciences, Division of Internal Medicine and Hypertension, University of Torino, Torino, Italy.
² Department of Biomedicine, Aarhus University, Aarhus, Denmark.
³ Medical Cell Biology, University of Regensburg, Regensburg, Germany.
⁴ Department of Medical Sciences Division of Pathology, University of Torino, Turin, Italy.
⁵ Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-Universität München, Munich, Germany.
⁶ Department of Medical Sciences, Division of Endocrinology, Diabetes and Metabolism, University of Torino, Turin, Italy.
⁷ Division of Endocrinology, Azienda Ospedaliero-Universtaria Ospedali Riuniti UmbertoI- GM Lancisi-G Salesi, Università Politecnica delle Marche, Ancona, Italy.
⁸ Department of Medicine, Clinica Medica 3, University of Padova, Padova, Italy.
⁹ Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.

Running title: Somatic APA mutations

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Patient selection
Patients were selected as described previously. PA was diagnosed in accordance with endocrine society guidelines. Briefly, all anti-hypertensive drugs were stopped at least 3 weeks before the aldosterone and PRA measurements (diuretics and spironolactone were stopped at least 6 and 8 weeks, respectively). Patients who could not remain untreated received an alpha-blocker (doxazosin) and/or a calcium channel blocker (verapamil or amlodipine) during the entire study period. After a positive screening, patients underwent the intravenous saline load as confirmatory test. Subtype diagnosis was performed by CT scanning with contrast and fine cuts of the adrenal and subsequent AVS. All patients included in the present study displayed an adrenal vein/inferior vena cava cortisol gradient of at least 2 (greater than 3 in most cases) and an aldosterone/cortisol ratio from one adrenal at least 4 times the ratio from the other adrenal gland. A final diagnosis of APA was considered proven, providing that all the following conditions were satisfied: 1) histological demonstration of adenoma, 2) normalization of hypokalemia if present, 3) cure or improvement of hypertension, and 4) normalization of ARR and suppressibility of aldosterone levels less than 5ng/dL under saline load.

The indications for patients’ referral to the different Italian centres were newly diagnosed hypertension, onset of hypertension at a young age, resistance of hypertension or side-effects to conventional antihypertensive therapy, hypertension with spontaneous or diuretic-induced hypokalemia, high plasma aldosterone, low PRA or adrenal incidentaloma.

RNA isolation and RT-PCR
Adrenal tissues were homogenised in 1 mL TRI reagent for RNA extraction and first-strand cDNA was synthesized from total RNA (5 µg) as described. DNA sequencing of KCNJ5, ATP1A1 and ATP2B3
DNA sequencing of KCNJ5 was as described in Boulkroun et al.; PCR primers used to amplify cDNA fragments for direct sequencing of ATP1A1 and ATP2B3 are shown in supplemental table S1. The validity of novel mutations was confirmed by sequencing both strands of an independently amplified PCR fragment. The present cohort constitutes 112 APA from different centers: Torino 1 (n=54, that comprises the 29 samples with 13 KCNJ5 mutations of the Torino group reported in Boulkroun et al. of which 28 were also used for the study of Beuschlein et al. that identified an ATP1A1 mutation (p.Leu104Arg)); Torino 2 (n=19); Ancona (n=21); Padova (n=10, of which 4 samples correspond to the Padova-A group with a single KCNJ5 mutation and the same 4 samples, in which ATP1A1 or ATP2B3 mutations were not present, were used as part of the Padova group reported previously); and Pisa (n=8). No mutations were identified in paired peripheral blood DNA samples (n=43) and mutations were absent from paired peritumoral tissue (n=23 of which 7 carried KCNJ5 mutations in the corresponding APA).

KCNJ5 and ATP1A1 expression vectors
Expression vectors containing cDNAs encoding human wild-type KCNJ5 or KCNJ5 c.451G>A (p.Gly151Arg) have been reported previously and rat wild-type Atp1a1 and Atp1a1 c.311T>G (p.Leu104Arg) plasmids were as described. The rat Atp1a1 cDNA was used for this study so that endogenous human ATP1A1 in the HAC15 cell line could be inactivated by RNA interference using the strategy described. The Atp1a1 c.295G>A (p.Gly99Arg) and KCNJ5 c.376T>C (p.Trp126Arg) plasmids were constructed by PCR-mediated site-directed mutagenesis. For clarity, throughout this manuscript, when referring to either rat or human ATP1A1, the numbering used to describe mutations follows the human cDNA or amino acid numbering.

Modelling of protein structures
Structural models of Na⁺/K⁺-ATPase 1 (PDB code 2ZXE) were prepared using PyMOL software.

Cell transfection and gene silencing
HAC15 cells were grown in culture as described. Cells (1 x 10⁶) were transfected with plasmid DNA (1 µg) and for transfections using rat Atp1a1 cDNA, with 2 µL 100 µM siRNA to specifically
interfere with the expression of the endogenous human \( \text{ATP1A1} \) by Amxa nucleofection using programme X005\(^9\). Equivalent transfection rates for each \( \text{Atp1a1} \) cDNA were confirmed by TaqMan real-time PCR by measuring rat \( \text{Atp1a1} \) gene expression in each transfection compared to empty vector controls using GAPDH as the endogenous reference gene. The fold changes in \( \text{Atp1a1} \) gene expression were: wild-type, 53.77±2.62; L104R, 51.93±3.29; G99R, 56.46±4.20.

Silencing of endogenous HAC15 \( \text{ATP1A1} \) mRNA and constant levels of silencing between transfections with wild-type or mutated rat \( \text{Atp1a1} \) constructs was confirmed by TaqMan real-time PCR: \( \text{ATP1A1} \) mRNA was decreased by 87.1% ±0.9, 87.4%±1.0 and 88.0%±1.0 in co-transfections of si\( \text{ATP1A1} \) with wild-type \( \text{Atp1a1} \), Leu104Arg or Gly99Arg, respectively (n=6).

**TaqMan gene expression assays**

Gene expression levels in transfected cells and in adrenal tissues were determined by real-time PCR using TaqMan gene expression assays and expression levels were calculated by the 2\(^{-\Delta\Delta C_t}\) relative quantification method as described previously using GAPDH as the endogenous reference gene\(^6\). The TaqMan gene expression assays used were: \( \text{CYP11B2} \), Hs1597732_m1; \( \text{CYP11B1} \), Hs01596404_m1; \( \text{NR4A2} \), Hs00428691_m1; \( \text{GAPDH} \), Hs9999905_m1 (Applied Biosystems, Life Technologies, Carlsbad, CA). Gene expression levels in APA were compared to those in corresponding surrounding tissue (peri-APA) for 7 of 8 APA with \( \text{ATP1A1} \) or \( \text{ATP2B3} \) mutations (surrounding tumor tissue was not available for one APA with an \( \text{ATP1A1} \) mutation) and for 11 APA without mutations in \( \text{KCNJ5} \), \( \text{ATP1A1} \) or \( \text{ATP2B3} \).

**Na\(^+\)/K\(^+\)-ATPase functional assays**

For in vitro functional assays, COS cells were transiently transfected with rat wild-type or mutated \( \text{Atp1a1} \) encoding Na\(^+\)/K\(^+\)-ATPase p.Gly99Arg together with a siRNA to specifically interfere with the expression of the endogenous COS cell \( \text{Atp1a1} \)\(^8\). ATPase activity assays were performed at 37°C as described\(^8\). For measurements of Na\(^+\) dependence of phosphorylation by MgATP, assays were performed under the conditions described at 0°C for 10s\(^8\) in the presence of varying concentrations of Na\(^+\) (0-50 mM) and N-methyl-D-glucamine to maintain the ionic strength at 150 mM. Assays of K\(^+\) inhibition of phosphorylation by MgATP were as described\(^8\).

**Electrophysiological characterization of cells expressing Na\(^+\)/K\(^+\)-ATPase Gly99Arg**

The full-length cDNA encoding wild-type rat \( \text{Atp1a1} \) and the mutated \( \text{Atp1a1} \) c.295G>A (p.Gly99Arg) were subcloned into the bicistronic pIRES-CD8 expression vector\(^11\). Human embryonic kidney (HEK) cells were transiently transfected with Lipofectamine and anti-CD8-labelled Dynabeads were used to identify transfected cells and patch-clamp whole cell recordings were performed using an EPC-10 amplifier without leak subtraction\(^8\).

**Statistical analyses**

All data are expressed as mean ± standard error of the mean (SEM) for normally distributed variables and as median [25\(^{th}\)-75\(^{th}\) percentile] for non-normally distributed variables. The Student \( t \) test or the Kruskal-Wallis test were used for quantitative variables and the chi-square test or the Fisher exact test were applied for qualitative variables. Confidence intervals were calculated for frequency data using Wilson’s method\(^12\).

**Results**

**Phenotype of subject with Na\(^+\)/K\(^+\)-ATPase 1 p.Gly99Arg mutation**

The patient carrying the \( \text{ATP1A1} \) c.295G>A (p.Gly99Arg) somatic mutation, diagnosed in the Torino 1 unit, is a female of Philippine origin born in 1957. Diagnosis of hypertension was made in 2001 (44 y.o.); she presented at the emergency department for a paralysis of the legs and a recent history of myalgia. She was unaware of being hypertensive and she was untreated. Serum potassium was very low (1.4 mEq/L), and blood pressure was 180/120. PRA was 0.2 ng/mL/h and aldosterone 45.3 ng/dL. Suspect PA was confirmed by i.v. saline load. CT scan showed a nodule of 20 mm in the left adrenal and a micro-nodule of 6 mm in the right adrenal gland. She underwent adrenal vein sampling that showed an aldosterone/cortisol ratio on the left adrenal five times that of the right.
adrenal. After adrenalectomy, blood pressure and hormonal levels returned to normal and she was still normotensive 1 year after surgery.

**Phenotype of subject with GIRK 4 p.Trp126Arg mutation**

The patient carrying the *KCNJ5* c.376T>C (GIRK4 p.Trp126Arg) mutation, diagnosed in the Torino 2 unit, is a male of Italian origin, born in 1976. Diagnosis of hypertension was made in 2006 (30 y.o.); at diagnosis blood pressure levels were 155/100 under treatment with doxazosin 4 mg/day and the patient had been hypertensive for 10 months. Serum potassium was 3.6 mEq/L, PRA was 0.2 ng/mL/h and aldosterone 34 ng/dL. Suspect PA was confirmed by i.v. saline load. CT scan showed a nodule of 13 mm in the left adrenal and a normal contralateral gland. He underwent adrenal vein sampling that showed an aldosterone/cortisol ratio on the left adrenal six times the same ratio in the right adrenal. After adrenalectomy, blood pressure and hormonal levels returned to normal and was still normotensive 1 year after surgery.

**References**


domain K⁺ channel stimulated by arachidonic acid and polyunsaturated fatty acids. *EMBO J*.
1998;17:3297-3308.
12) Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Fragment size (bp)</th>
<th>Mutations in amplified fragment</th>
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<tbody>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTGGCAGGTCATGCCTGTGGC</td>
<td></td>
<td></td>
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<tr>
<td><strong>ATP1A1-ex4</strong></td>
<td>Forward</td>
<td>TGCTCGTGCAGCTGAGATCC</td>
<td>171</td>
<td>p.Leu104Arg; p.Gly99Arg; p.Phe100_Leu104del</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TTCTGTAGCAGCTTGGATGC</td>
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<td></td>
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<tr>
<td><strong>ATP1A1-ex8</strong></td>
<td>Forward</td>
<td>CGCACGTGGTATTGGTGCTCT</td>
<td>407</td>
<td>p.Val332Gly</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CACTGTCATCCGGTTCTGAG</td>
<td></td>
<td></td>
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<tr>
<td><strong>ATP2B3</strong></td>
<td>Forward</td>
<td>CGCACCGGTGTGTTGTCTTT</td>
<td>221</td>
<td>p.Leu425_Val426del; p.Val426_Val427del</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CGTCATCATCCTGGTCTCCT</td>
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<td></td>
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</table>

Table S1 Primer pairs used for PCR amplifications
<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild type (n=60)</th>
<th>KCNJ5 mutated (n=44)</th>
<th>ATP1A1/ATP2B3 mutated (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at PA diagnosis (years)</td>
<td>49±11</td>
<td>48±12</td>
<td>48±13</td>
<td>n.s. (0.908)</td>
</tr>
<tr>
<td>Preop. SBP (mmHg)</td>
<td>174±25</td>
<td>171±21</td>
<td>165±24</td>
<td>n.s. (0.497)</td>
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<tr>
<td>Preop. DBP (mmHg)</td>
<td>105±12</td>
<td>103±11</td>
<td>100±10</td>
<td>n.s. (0.554)</td>
</tr>
<tr>
<td>Number preop. AH drugs</td>
<td>2.6±1.2</td>
<td>2.4±1.1</td>
<td>1.9±1.1</td>
<td>n.s. (0.181)</td>
</tr>
<tr>
<td>Lowest recorded K+ (mmol/L)</td>
<td>3.1±0.7</td>
<td>2.87±0.7</td>
<td>2.7±0.8</td>
<td>n.s. (0.201)</td>
</tr>
<tr>
<td>Preop. plasma aldo (ng/dL)</td>
<td>47 [35-60]*</td>
<td>48 [32-66]*</td>
<td>46 [29-71]*</td>
<td>n.s. (0.945)</td>
</tr>
<tr>
<td>Preop. PRA (ng/mL/h)</td>
<td>0.20 [0.20-0.39]*</td>
<td>0.20 [0.10-0.30]*</td>
<td>0.25 [0.20-0.75]*</td>
<td>n.s. (0.288)</td>
</tr>
<tr>
<td>Adenoma size (mm)</td>
<td>15 [10-20]*</td>
<td>18 [12-25]*</td>
<td>15 [9-19]*</td>
<td>n.s. (0.159)</td>
</tr>
<tr>
<td>Postop. SBP (mmHg)</td>
<td>131±13</td>
<td>128±11</td>
<td>121±13</td>
<td>n.s. (0.084)</td>
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<tr>
<td>Postop. DBP (mmHg)</td>
<td>82±8</td>
<td>80±6</td>
<td>74±9</td>
<td>0.024</td>
</tr>
<tr>
<td>Number postop. AH drugs</td>
<td>1.1±1.3</td>
<td>0.6±8</td>
<td>0.6±8</td>
<td>n.s. (0.102)</td>
</tr>
<tr>
<td>Postop. serum K+ (mmol/L)</td>
<td>4.6±0.5</td>
<td>4.6±0.3</td>
<td>4.9±0.4</td>
<td>n.s. (0.292)</td>
</tr>
<tr>
<td>Postop. plasma aldo (ng/dL)</td>
<td>14 [7-19]*</td>
<td>11 [7-17]</td>
<td>9.50[8-10]*</td>
<td>n.s. (0.282)</td>
</tr>
<tr>
<td>Postop. PRA (ng/mL/h)</td>
<td>2.1 [1.2-3]*</td>
<td>2.1 [1.1-2.6]*</td>
<td>2.7 [2.3-5.7]*</td>
<td>n.s. (0.117)</td>
</tr>
</tbody>
</table>

Table S2. Clinical characteristics of Italian population as a function of mutational status.

Values represent mean ± s.d. unless otherwise specified. Wild-type refers to an absence of mutations in KCNJ5, ATP1A1 and ATP2B3. PA, primary aldosteronism; preop., pre-operative; postop, post-operative; DBP, diastolic blood pressure; SBP, systolic blood pressure; AH, antihypertensive; PRA, plasma renin activity; aldo, aldosterone; n, number of subjects for each group; n.s., not significant.

*Data are shown as the median [interquartile range].
Figure S1

Multiple sequence alignment of Na\(^+/K^+\)-ATPase, encoded by *ATP1A1*, primary sequences (A) shows conservation of Gly99 and Leu104 across species (highlighted in yellow and blue, respectively); multiple sequence alignment of GIRK4, encoded by *KCNJ5*, primary sequences shows the conservation of Trp126 (W126, highlighted in yellow) across species as well as of other amino acids found mutated in APA (highlighted in blue). Multiple sequence alignments were performed using CLUSTLW (www.genome.jp/tools/clustlw).