Primary aldosteronism (PA) is the most common form of secondary hypertension, with a prevalence of 5% to 15% among hypertensive patients and is characterized by the autonomous hypersecretion of aldosterone. Sporadic PA and 3 familial forms (familial hyperaldosteronism types I, II, and III) have been described.1 Sporadic PA accounts for >90% of all cases and is caused by either an aldosterone-producing adenoma (APA), which can be surgically removed, or bilateral adrenal hyperplasia, which is the most common form of PA. Somatic mutations in the \textit{KCNJ5} gene, which encodes the G-protein–activated inward rectifier K\textsuperscript{+} channel 4, GIRK4, account for ~40% of APAs. Additional somatic APA mutations were identified recently in 2 other genes, \textit{ATP1A1} and \textit{ATP2B3}, encoding \textit{Na}/K+/ATPase 1 and \textit{Ca}\textsuperscript{2+}/ATPase 3, respectively, at a combined prevalence of 6.8%. We have screened 112 APAs for mutations in known hotspots for genetic alterations associated with primary aldosteronism. Somatic mutations in \textit{ATP1A1}, \textit{ATP2B3}, and \textit{KCNJ5} were present in 6.3%, 0.9%, and 39.3% of APAs, respectively, and included 2 novel mutations (\textit{Na}/K+/ATPase p.Gly99Arg and GIRK4 p.Trp126Arg). \textit{CYP11B2} gene expression was higher in APAs harboring \textit{ATP1A1} and \textit{ATP2B3} mutations compared with those without these or \textit{KCNJ5} mutations. Overexpression of \textit{Na}/K+/ATPase p.Gly99Arg and GIRK4 p.Trp126Arg in HAC15 adrenal cells resulted in upregulation of \textit{CYP11B2} gene expression and its transcriptional regulator \textit{NR4A2}. Structural modeling of the \textit{Na}/K+/ATPase showed that the Gly99Arg substitution most likely interferes with the gateway to the ion binding pocket. In vitro functional assays demonstrated that Gly99Arg displays severely impaired ATPase activity, a reduced apparent affinity for \textit{Na} activation of phosphorylation and \textit{K} inhibition of phosphorylation that indicate decreased \textit{Na} and \textit{K} binding, respectively. Moreover, whole cell patch-clamp studies established that overexpression of \textit{Na}/K+/ATPase Gly99Arg causes membrane voltage depolarization. In conclusion, somatic mutations are common in APAs that result in an increase in \textit{CYP11B2} gene expression and may account for the dysregulated aldosterone production in a subset of patients with sporadic primary aldosteronism. (\textit{Hypertension}. 2014;63:188-195.) • Online Data Supplement

\textbf{Key Words:} aldosterone • Conn adenoma • hypertension • potassium channels • sodium-potassium-exchanging ATPase

\section*{Genetics}

\textbf{Somatic ATP1A1, ATP2B3, and KCNJ5 Mutations in Aldosterone-Producing Adenomas}

Tracy Ann Williams, Silvia Monticone,* Vivien R. Schack,* Julia Stindl, Jacopo Burrello, Fabrizio Buffolo, Laura Annaratone, Isabella Castellano, Felix Beuschlein, Martin Reincke, Barbara Lucatello, Vanessa Ronconi, Francesco Fallo, Giampaolo Bernini, Mauro Maccario, Gilberta Giacchetti, Franco Veglio, Richard Warth, Bente Vilsen, Paolo Mulatero

See Editorial Commentary, pp 24–26

\textbf{Abstract}—Aldosterone-producing adenomas (APAs) cause a sporadic form of primary aldosteronism and somatic mutations in the \textit{KCNJ5} gene, which encodes the G-protein–activated inward rectifier K\textsuperscript{+} channel 4, GIRK4, account for ~40% of APAs. Additional somatic APA mutations were identified recently in 2 other genes, \textit{ATP1A1} and \textit{ATP2B3}, encoding \textit{Na}/K+/ATPase 1 and \textit{Ca}\textsuperscript{2+}/ATPase 3, respectively, at a combined prevalence of 6.8%. We have screened 112 APAs for mutations in known hotspots for genetic alterations associated with primary aldosteronism. Somatic mutations in \textit{ATP1A1}, \textit{ATP2B3}, and \textit{KCNJ5} were present in 6.3%, 0.9%, and 39.3% of APAs, respectively, and included 2 novel mutations (\textit{Na}/K+/ATPase p.Gly99Arg and GIRK4 p.Trp126Arg). \textit{CYP11B2} gene expression was higher in APAs harboring \textit{ATP1A1} and \textit{ATP2B3} mutations compared with those without these or \textit{KCNJ5} mutations. Overexpression of \textit{Na}/K+/ATPase p.Gly99Arg and GIRK4 p.Trp126Arg in HAC15 adrenal cells resulted in upregulation of \textit{CYP11B2} gene expression and its transcriptional regulator \textit{NR4A2}. Structural modeling of the \textit{Na}/K+/ATPase showed that the Gly99Arg substitution most likely interferes with the gateway to the ion binding pocket. In vitro functional assays demonstrated that Gly99Arg displays severely impaired ATPase activity, a reduced apparent affinity for \textit{Na} activation of phosphorylation and \textit{K} inhibition of phosphorylation that indicate decreased \textit{Na} and \textit{K} binding, respectively. Moreover, whole cell patch-clamp studies established that overexpression of \textit{Na}/K+/ATPase Gly99Arg causes membrane voltage depolarization. In conclusion, somatic mutations are common in APAs that result in an increase in \textit{CYP11B2} gene expression and may account for the dysregulated aldosterone production in a subset of patients with sporadic primary aldosteronism. (\textit{Hypertension}. 2014;63:188-195.) ● Online Data Supplement

\textbf{Key Words:} aldosterone • Conn adenoma • hypertension • potassium channels • sodium-potassium-exchanging ATPase

\section*{Primary aldosteronism (PA) is the most common form of secondary hypertension, with a prevalence of 5% to 15% among hypertensive patients and is characterized by the autonomous hypersecretion of aldosterone. Sporadic PA and 3 familial forms (familial hyperaldosteronism types I, II, and III) have been described.1 Sporadic PA accounts for >90% of all cases and is caused by either an aldosterone-producing adenoma (APA), which can be surgically removed, or bilateral adrenal hyperplasia, which is treatable with mineralocorticoid receptor antagonists. Somatic APA mutations in the \textit{KCNJ5} gene, which encodes the G-protein–activated inward rectifier K\textsuperscript{+} channel 4, GIRK4 (also called the inward rectifier K\textsuperscript{+} channel, Kir3.4), were first identified by Choi et al.2 Subsequently, Boulkroun et al3 determined a 34% prevalence of \textit{KCNJ5} mutations in a large European cohort of 380 APA. Intriguingly, the \textit{KCNJ5} mutations were markedly more prevalent in women,1 and this predominance was confirmed by successive studies.4,5 To date, 5 different \textit{KCNJ5} mutations causing sporadic PA have been identified, the majority of which are
the substitutions p.Gly151Arg or p.Leu168Arg or, more rarely, p.Glu145Gln, p Thr158Ala, and p.Ile157del. A common feature of all the mutations is that they are situated in or around the selectivity filter of the K+ channel pore and in several cases have been shown to cause a loss of channel selectivity resulting in sodium entry and membrane depolarization and the opening of voltage-gated Ca2+ channels. The resultant increase in the intracellular Ca2+ concentration determines an increase in the transcription of the Cyp11b2 gene, which encodes aldosterone synthase, and in the production of aldosterone. Furthermore, APAs harboring KCNJ5 mutations have been reported to display higher levels of Cyp11b2 expression compared with those with wild-type KCNJ5, although this was not confirmed in another study.

To identify further genetic determinants of PA, Beuschlein et al performed exome sequencing of APAs from male patients without somatic KCNJ5 mutations. Novel mutations in ATP1A1, encoding the Na+/K+-ATPase α-subunit, and in ATP2B3, encoding the plasma membrane Ca2+-ATPase 3, were identified. In a European cohort of 308 APAs, the prevalence of these mutations in ATP1A1 and ATP2B3 was 5.2% and 1.6%, respectively. The ATP1A1 mutations were primarily c.311T>G (p.Leu104Arg), less frequent was a c.995T>G mutation (p.Val332Gly), as well as 2 different in-frame DNA deletions that result in the same deletion at the amino acid level (p.Phe100_Leu104del). In ATP2B3, 3 different in-frame DNA deletions were identified that result in either p.Leu425_Val426del or p.Val426_Val427del. In all cases, the affected amino acids are highly conserved across species and between different members of the P-type ATPase family. In vitro functional characterization of the substitutions in Na+/K+-ATPase 1 demonstrated that they have a profound effect on K+ binding and ATPase activity and cause membrane depolarization in whole cell patch-clamp studies that in adrenal cells would lead to the opening of voltage-dependent Ca2+-channels as for the KCNJ5 mutations.

In this study, we have analyzed 112 APAs, diagnosed and surgically removed in Italian referral hypertension centers, for mutations in ATP1A1, ATP2B3, and KCNJ5 and searched for new mutations within known hotspots for genetic alterations in these genes.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Patient Selection**

Patients were selected as described previously. PA was diagnosed in accordance with Endocrine Society guidelines. Further details are available in the online-only Data Supplement. The protocol was approved by our local ethics committee, and all participants gave their written informed consent.

**RNA Isolation and Reverse Transcription Polymerase Chain Reaction**

Adrenal tissues were homogenized 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**

Polymerase chain reaction (PCR) primers used to amplify cDNA fragments for direct sequencing of ATP1A1 and ATP2B3 are shown in Table S1 in the online-only Data Supplement. The validity of novel mutations was confirmed by sequencing both strands of an independently amplified PCR fragment. The present cohort constitutes 112 APAs from different centers and included 32 samples from the Beuschlein study. A full description of these samples is included in the online-only Data Supplement. 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**

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. For clarity, throughout this article, when referring to either rat or human ATP1A1, the numbering used to describe mutations follows the human cDNA or amino acid numbering.

**Modeling of Protein Structures**

Structural models of Na+/K+-ATPase 1 (PDB code 2ZXE) were prepared using PyMOL software (www.pymol.org).

**Cell Transfection and Gene Silencing**

HAC15 cells were grown in culture and transfected with plasmids and siRNAs by Amaxa nucleofection using program X005. Equivalent transfection rates of rat cDNAs and silencing of HAC15 ATP1A1 were confirmed by real-time TaqMan PCR.

**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 using GAPDH as the endogenous reference gene. Gene expression levels in APA were compared with those in corresponding surrounding tissue (peri-APA) for 7 of 8 APA with ATP1A1 or ATP2B3 mutations and for 11 APA without mutations in KCNJ5, ATP1A1, or ATP2B3.

**Na+/K+-ATPase Functional Assays**

COS cells were transiently transfected with rat wild-type or mutated Atp1a1 encoding Na+/K+-ATPase p.Gly99Arg together with a siRNA to interfere specifically with the expression of the endogenous COS cell Atp1a1. ATPase activity assays, Na+ dependence of phosphorylation by MgATP and K+ inhibition of phosphorylation by MgATP, were as described.

**Electrophysiological Characterization of Cells Expressing Na+/K+-ATPase Gly99Arg**

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

**Statistical Analyses**

All data are expressed as means±SEM for normally distributed variables and as median (25th–75th percentile) for non-normally distributed variables. The Student t test or the Kruskal–Wallis test was used for quantitative variables, and the χ2 test or the Fisher exact test was applied for qualitative variables. Confidence intervals were calculated for frequency data using Wilson’s method.
Results

Prevalence of Somatic APA Mutations in ATPases

Sequencing analysis of 112 APA DNA samples collected from 5 different Italian centers demonstrated a prevalence of \( ATP1A1 \) mutations of 6.3% (Table 1). A novel \( ATP1A1 \) c.295G>A mutation (p.Gly99Arg) was identified in a single sample, and the \( ATP1A1 \) c.311T>G mutation (p.Leu104Arg)\(^{11} \) was present in 6 samples (Figure 1A and B; Table 1). The c.995T>G mutation (p.Val332Gly) and the deletion mutations (p.Phe100_Leu104del) described previously\(^{11} \) were not detected. However, 1 APA with an \( ATP2B3 \) mutation (c.1272-1277delGCTGGT corresponding to p.Leu425-Val426del) was identified (Figure 1C; Table 1). Together, the mutations in these 2 ATPases were present in 7.2% of APAs and were absent from the corresponding peripheral DNA, thereby demonstrating their somatic nature. This prevalence is in close agreement with the 6.9% frequency reported by Beuschlein et al.,\(^{11} \) but there was no correlation with increased preoperative aldosterone concentrations or with reduced serum K\(^+ \) levels (Table S2).

Novel Somatic APA Mutation in \( ATP1A1 \)

The novel APA mutation in \( ATP1A1 \) was identified in the Torino 1 group of samples, \( ATP1A1 \) c.295G>A (p.Gly99Arg). The patient harboring the p.Gly99Arg mutation displayed a phenotype of PA with very low serum potassium (1.4 mEq/L), and the phenotype of this patient is described in the online-only Data Supplement.

Novel Somatic APA Mutation in \( KCNJ5 \)

Sequence analysis of \( KCNJ5 \) demonstrated the presence of 4 different somatic APA mutations in this sample set: the frequently reported c.451G>A/C (p.Gly151Arg) and c.503T>G (p.Leu168Arg) substitutions,\(^{2,3} \) c.472A>G (p.Thr158Ala)\(^{6} \) and a novel c.376T>C (p.Trp126Arg) mutation that affects a highly conserved residue (Figure 1D; Table 2; and Figure S1). In agreement with other reports,\(^{3,5} \) the p.Gly151Arg substitution was identified more frequently than p.Leu168Arg (31.5% versus 9.0%; \( P<0.02 \)). A wide variation in prevalence was observed between different centers (20.0%–47.6%), but the overall prevalence was 39.3%. There was no correlation between different centers (20.0%–47.6%), but the overall prevalence was 39.3%.

Prevalence of APA Mutations According to Sex

In this study, the ATPase mutations were distributed equally between men and women (\( P=0.5 \)), rather than predominantly in men.\(^{4} \) In accordance with previous reports,\(^{3,5} \) the \( KCNJ5 \) mutations were more prevalent in women than in men (50.0% APA women carried \( KCNJ5 \) mutations versus 29.3% men; \( P=0.02 \); Table 3).

Structural Effects of the Na\(^+\)/K\(^+\)-ATPase Gly99Arg Mutation

Gly99 resides in the transmembrane domain M1 (Figure 1E and F) in close proximity to residues Ile292 and Glu334 in domains M3 and M4, respectively (Figure 1F). The Gly99-Ile292 pair functions as a pivot for the movement of M1 leading to the opening of the gate at the entrance to the cation binding pocket, whereas Glu334 is part of the gate\(^{17} \) (Figure 1F). Introduction of the large positively charged arginine side-chain is likely to cause a severe structural alteration affecting surrounding residues including Glu334 that binds K\(^+ \) (Figure 1G).

APAs Carrying \( ATP1A1 \) and \( ATP2B3 \) Mutations Display Increased \( CYP11B2 \) Gene Expression

Analysis of gene expression levels by TaqMan real-time PCR demonstrated that the expression of \( CYP11B2 \) in APAs with ATPase mutations (in either \( ATP1A1 \) or \( ATP2B3 \)) compared with corresponding peri-APA tissue was significantly higher than that in APAs without mutations in either \( KCNJ5 \), \( ATP1A1 \), or \( ATP2B3 \) (51.6- versus 4.0-fold; \( P<0.027 \)). In contrast, there was no significant difference in \( CYP11B1 \) gene expression (Figure 2).

Table 1. Prevalence of Somatic APA Mutations in \( ATP1A1 \) and \( ATP2B3 \) in Different Italian Centers

<table>
<thead>
<tr>
<th>Center</th>
<th>No. of Samples</th>
<th>ATP1A1 c.311T&gt;G (p.Leu104Arg)</th>
<th>ATP1A1 c.295G&gt;A (p.Gly99Arg)</th>
<th>Total No. of ATP1A1 Mutations (%)</th>
<th>ATP2B3 c.1272-1277delGCTGGT (p.Leu425-Val426del)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torino 1</td>
<td>54</td>
<td>4</td>
<td>1</td>
<td>5 (8.3%, 4.0–19.9)</td>
<td>1 (1.9%, 0.3–9.8)</td>
</tr>
<tr>
<td>Torino 2</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ancona</td>
<td>21</td>
<td>2</td>
<td>0</td>
<td>2 (9.5%, 2.7–28.9)</td>
<td>0</td>
</tr>
<tr>
<td>Padova</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pisa</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>6</td>
<td>1</td>
<td>7 (6.3%, 3.1–12.3)</td>
<td>1 (0.9%, 0.2–4.9)</td>
</tr>
</tbody>
</table>

APA indicates aldosterone-producing adenoma; and CI, confidence interval.
A novel ATP1A1 c.295G>A mutation (p.Gly99Arg) was identified in the APA of 1 patient but not in the peripheral blood DNA (A); the somatic ATP1A1 c.311T>G mutation (p.Leu104Arg)\(^{11}\) was identified in 6 APAs (B); a single APA sample carried an ATP2B3 c.1272-1277delGCTGGT (p.Leu426-Val426del) mutation that was absent from peripheral DNA (C); a novel KCNJ5 c.376T>C mutation (p.Trp126Arg) was identified in the APA of 1 patient but not in the patient’s peripheral DNA (D); structural overview of Na’/K’-ATPase (PDB code 2ZXE). The backbone carbons of Gly99 in transmembrane helix M1 are highlighted in orange. The A-domain, N-domain, P-domain, and transmembrane regions are shown in green, cyan, yellow, and grey, respectively. Violet spheres indicate K+ ions (E); close up of the relevant region in E shows residue Gly99 in close proximity to Ile292 in M3 and Glu334 in M4. The Gly99-Ile292 pair functions as a pivot for the movement of M1 leading to opening of the gate at the entrance to the cation binding pocket, whereas Glu334 is part of the gate\(^{16}\) (F); introduction of a large, positively charged arginine residue in the position of Gly99 is likely to cause a steric clash with an impact on the surrounding residues, including Glu334 that binds K+ (G).

**Figure 1.** Somatic aldosterone-producing adenoma (APA) mutations in ATP1A1, ATP2B3, and KCNJ5. A novel ATP1A1 c.295G>A mutation (p.Gly99Arg) was identified in the APA of 1 patient but not in the peripheral blood DNA (A); the somatic ATP1A1 c.311T>G mutation (p.Leu104Arg)\(^{11}\) was identified in 6 APAs (B); a single APA sample carried an ATP2B3 c.1272-1277delGCTGGT (p.Leu426-Val426del) mutation that was absent from peripheral DNA (C); a novel KCNJ5 c.376T>C mutation (p.Trp126Arg) was identified in the APA of 1 patient but not in the patient’s peripheral DNA (D); structural overview of Na’/K’-ATPase (PDB code 2ZXE). The backbone carbons of Gly99 in transmembrane helix M1 are highlighted in orange. The A-domain, N-domain, P-domain, and transmembrane regions are shown in green, cyan, yellow, and grey, respectively. Violet spheres indicate K+ ions (E); close up of the relevant region in E shows residue Gly99 in close proximity to Ile292 in M3 and Glu334 in M4. The Gly99-Ile292 pair functions as a pivot for the movement of M1 leading to opening of the gate at the entrance to the cation binding pocket, whereas Glu334 is part of the gate\(^{16}\) (F); introduction of a large, positively charged arginine residue in the position of Gly99 is likely to cause a steric clash with an impact on the surrounding residues, including Glu334 that binds K+ (G).

**GIRK4 p.Trp126Arg Mutation Increases CYP11B2 and NR4A2 Expression In Vitro**

Expression of GIRK4 p.Trp126Arg (KCNJ5 c.376T>C) in HAC15 cells resulted in a 1.90±0.18-fold increase in the expression of CYP11B2 compared with control cells and a 4.70±0.73-fold increase in the expression of NR4A2 (P<0.01; Figure 3B). Expression of the most frequent GIRK4 mutation in this study, Gly151Arg, resulted in 5.19±0.61- and 16.78±2.39-fold increases (P<0.01) in CYP11B2 and NR4A2 gene expression, respectively (Figure 3B).

**Na’/K’-ATPase Gly99Arg Mutation Displays Altered Functional Activity**

The maximal Na’/K’-ATPase activity of Gly99Arg was compared with both the wild-type and a nonphosphorylatable inactive mutant (Asp376Asn). The ATPase activity of Gly99Arg was impaired severely and displayed levels of activity comparable with those of the inactive mutant, which represent background detection (Figure 4A). In addition, the

**Table 2. Prevalence of Somatic APA Mutations in KCNJ5 in Different Italian Centers**

<table>
<thead>
<tr>
<th>Center</th>
<th>No. of Samples</th>
<th>c.451G&gt;A/C (p.Gly151Arg)</th>
<th>c.503T&gt;G (p.Leu168Arg)</th>
<th>c.472A&gt;G (p.Thr158Ala)</th>
<th>c.376T&gt;C (p.Trp126Arg)</th>
<th>No. of Mutations (%), CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torino 1</td>
<td>54</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>22 (40.7%, 28.7–54.0)</td>
</tr>
<tr>
<td>Torino 2</td>
<td>19</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>7 (36.8%, 19.2–59.0)</td>
</tr>
<tr>
<td>Ancona</td>
<td>21</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10 (47.6%, 28.3–67.6)</td>
</tr>
<tr>
<td>Padova</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (20%, 5.7–51.0)</td>
</tr>
<tr>
<td>Pisa</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3 (37.5%, 13.7–59.4)</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>35</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>44 (39.3%, 30.7–48.5)</td>
</tr>
</tbody>
</table>

APA indicates aldosterone-producing adenoma; and CI, confidence interval.
Gly99Arg mutant displayed a reduced affinity for Na⁺ activation of phosphorylation by MgATP with a half maximal activation at 1.19 mmol/L Na⁺ compared with 0.58 mmol/L for the wild-type Na⁺/K⁺-ATPase (Figure 4B). In the Na⁺/K⁺ exchange mechanism, the ATPase is dephosphorylated after K⁺ binding. Therefore, ATPase phosphorylation is inhibited by K⁺, which in vitro can be used for measuring K⁺ affinity. Accordingly, the K⁺ inhibition of Gly99Arg phosphorylation by MgATP was compared with that of wild-type Na⁺/K⁺-ATPase. Gly99Arg was markedly less sensitive to inhibition of phosphorylation by K⁺ with half maximal inhibition occurring at 0.62 mmol/L K⁺ compared with 0.074 mmol/L K⁺ of the wild-type ATPase (Figure 4C).

Na⁺/K⁺-ATPase Gly99Arg Mutation Causes Membrane Voltage Depolarization

The effect of the Na⁺/K⁺-ATPase Gly99Arg mutation on the membrane voltage of transfected human embryonic kidney cells was measured by whole cell patch-clamp. The membrane voltage was depolarized in cells expressing the wild-type (WT) or the novel p.Gly99Arg mutation compared with those expressing the wild-type Na⁺/K⁺-ATPase (Figure 4C).

**Table 3. Prevalence of APA Mutations According to Sex**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutations in Women/Total</th>
<th>Mutations in Men/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNJ5</td>
<td>27/54 (50.0%, 37.1–62.9)</td>
<td>17/58 (29.3%, 19.2–42.0)</td>
</tr>
<tr>
<td>ATP1A1+ATP2B3</td>
<td>4/54 (7.4%, 2.9–17.6)</td>
<td>4/58 (6.9%, 2.7–16.4)</td>
</tr>
</tbody>
</table>

APA indicates aldosterone-producing adenoma; and CI, confidence interval.

**Discussion**

Mutations in the KCNJ5 gene, which encodes the GIRQ4 K⁺ channel, have been identified that cause both sporadic and familial forms of PA. The mutations are located around the selectivity filter of the K⁺ channel and cause a loss of ion selectivity and cell depolarization that in adrenal glomerulosa cells result in the opening of voltage-gated Ca²⁺ channels.
channels,\textsuperscript{2,8} The Ca\textsuperscript{2+} influx stimulates both CYP11B2 and NR4A2 (a transcriptional regulator of CYP11B2) gene expression, and subsequently aldosterone secretion is increased.\textsuperscript{4,8} In this study, we report a novel somatic mutation in \textit{KCNJ5} (GIRK4 p.Trp126Arg), and, therefore, a total of 6 different somatic \textit{KCNJ5} mutations have been identified to date with the most prevalent being p.Gly151Arg and p.Leu168Arg. The p.Trp126Arg mutation affects a tryptophan residue that is conserved across species and in close proximity to residues Pro128 and Cys129 that form hydrogen bonds with Thr158,\textsuperscript{2} a residue mutated to an Ala in both familial hyperaldosteronism type III\textsuperscript{2} and in sporadic APA.\textsuperscript{6} We show that overexpression of GIRK4 p.Trp126Arg in adrenal cells results in an increase in \textit{CYP11B2} and \textit{NR4A2} gene expression as described for other GIRK4 mutations.\textsuperscript{4,8}

Beuschlein et al\textsuperscript{11} recently reported several newly described somatic APA mutations in 2 different genes, \textit{ATP1A1} and \textit{ATP2B3}, that encode Na\textsuperscript{+}/K\textsuperscript{+}-ATPase 1 and Ca\textsuperscript{2+}-ATPase 3, members of the P-type family of ATPases. In their sample set of 308 APAs, a combined prevalence of 6.8\% \textit{ATP1A1} and \textit{ATP2B3} mutations and 38.3\% \textit{KCNJ5} mutations was reported. The data from the present study are in close agreement with these observations: in our 112 tumor samples, which included 32 samples from the Beuschlein study,\textsuperscript{11} we describe a prevalence of 7.2\% \textit{ATP1A1} and \textit{ATP2B3} mutations and 39.3\% \textit{KCNJ5} mutations. In both studies, concomitant mutations within the same tumor were not observed. However, we found that the ATPase mutations were distributed equally between men and women, although our sample size of 8 APAs with ATPase mutations is smaller than that of the previous study,\textsuperscript{11} which included 21 ATPase mutations.

Furthermore, we did not find a correlation of \textit{KCNJ5} or \textit{ATP1A1}/\textit{ATP2B3} mutational status with clinical characteristics related to adrenoma size or disease severity (higher preoperative aldosterone levels and lower serum potassium levels) that has been observed in some studies\textsuperscript{11,19} but not in others.\textsuperscript{2,3,5,20} Differences in clinical and biochemical characteristics of patients with or without somatic APA mutations, as well as the prevalence of mutations in different cohorts, could be determined by ethnicity and by patient selection and diagnostic strategies.

For each ATP hydrolyzed, the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase couples the extrusion and uptake of 3 cytoplasmic Na\textsuperscript{+} for 2 extracellular K\textsuperscript{+} ions across the plasma membranes of most higher eukaryotes.\textsuperscript{21,22} This ion transport pathway comprises a channel on each side of the lipid bilayer that access the ion binding pocket.\textsuperscript{22} The Na\textsuperscript{+}/K\textsuperscript{+}-ATPase Leu104 residue is located in transmembrane domain M1 and positions Glu334 that functions in the binding and occlusion of potassium ions.\textsuperscript{23} The p.Leu104Arg substitution identified in APAs causes a severe disturbance of K\textsuperscript{+} binding and ATPase activity resulting in depolarization of the cell membrane.\textsuperscript{11} In glomerulosa cells, the inactivation of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase \( \alpha \)-subunit by the inhibitor ouabain has been reported to cause a nifedipine-sensitive elevation in the cytoplasmic Ca\textsuperscript{2+} concentration and a stimulation of aldosterone production.\textsuperscript{24,25} The novel Na\textsuperscript{+}/K\textsuperscript{+}-ATPase p.Gly99Arg mutation identified in this study is located in transmembrane \( \alpha \)-helix M1, intriguingly in the same region as the p.Leu104Arg mutation, in close proximity to residues Ile292 and Glu334, which function in gating entry to the cation binding pocket. The p.Gly99Arg mutation is likely to cause a steric clash with an impact on surrounding residues, including Glu334 that binds K\textsuperscript{+}. Consistently, we show that the p.Gly99Arg mutant displayed minimal ATPase activity, reduced Na\textsuperscript{+} affinity, and markedly reduced K\textsuperscript{+} affinity when compared with the wild-type ATPase as observed in phosphorylation studies. These results are in agreement with a disturbed gating mechanism, in particular, affecting K\textsuperscript{+} binding to the mutated pump. Adrenal zona

![Figure 4](https://hyper.ahajournals.org/) Functional characterization of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase Gly99Arg. Maximal Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity of the wild-type (WT) and the mutants Gly99Arg and Asp376Asn. Mutation of Asn376 prevents phosphorylation and indicates background activity. Columns represent the mean of 4 to 7 independent experiments. Error bars indicate SEM (A). Na\textsuperscript{+} dependence of phosphorylation by MgATP. Each value corresponds to the mean of 5 to 12 independent experiments. Error bars indicate SEM (B). K\textsuperscript{+} inhibition of phosphorylation by MgATP. Each value corresponds to the mean of 5 to 12 independent experiments. Error bars indicate SEM (C).

![Figure 5](https://hyper.ahajournals.org/) Electrophysiological characterization of human embryonic kidney (HEK) cells expressing Na\textsuperscript{+}/K\textsuperscript{+}-ATPase Gly99Arg. Membrane voltages of HEK cells expressing the WT Na\textsuperscript{+}/K\textsuperscript{+}-ATPase Gly99Arg mutant were depolarized compared with cells expressing the WT Na\textsuperscript{+}/K\textsuperscript{+}-ATPase under control conditions (left). After removal of bath Na\textsuperscript{+}, the difference of the membrane voltage was preserved suggesting that the depolarization was caused by disturbed intracellular ion composition and/or loss of net charge transport by the mutant pump (right). Numbers of cells are shown in parentheses. *P<0.05 vs WT.
glomerulosa cells display a distinctive high resting K⁺ conductance that maintains the high negative membrane potential (~80 mV) of these cells.26 We demonstrate by whole cell patch-clamp studies that the p.Gly99Arg mutation results in the depolarization of the membrane voltage. In adrenal glomerulosa cells, this depolarization results in the opening of voltage-gated Ca²⁺ channels.28 In accordance with an increase in Ca²⁺ influx, we show that the overexpression of p.Gly99Arg or p.Leu104Arg in adrenal cells results in an increase in CYP11B2 gene expression and also, for the p.Gly99Arg mutation, NR4A2. Finally, the expression of CYP11B2 in APAs harboring ATPase mutations was significantly higher compared with those without these or GIRK4 mutations; an effect of heterozygous ATPase mutations on glomerulosa cells is consistent with the unique sensitivity of these cells to small changes in intracellular K⁺ and membrane potential.26,27 Taken together, these observations are in agreement with a pathophysiologic link between the presence of these mutations and the dysregulated aldosterone secretion in patients with APA.

Perspectives
Genetic alterations that lead to cell depolarization and increased transcription of the CYP11B2 gene in adrenal cells account for a large proportion of APAs (47%). The majority of these mutations affect the GIRK4 K⁺ channel, whereas a far smaller number alter Na⁺/K⁺-ATPase 1 and Ca²⁺-ATPase 3. In general, the mutations cluster around hotspot regions and in this study we report a rare Na⁺/K⁺-ATPase p.Gly99Arg mutation that affects the same protein domain as the more frequent p.Leu104Arg mutation involved in gating of the ion transport pathway. The understanding of new molecular pathways responsible for the dysregulation of aldosterone production may lead to the development of novel therapeutic targets aimed at treating the most common form of secondary hypertension.

Sources of Funding
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Disclosures
None.

References


\section*{Novelty and Significance}

\textbf{What Is New?}

\begin{itemize}
  \item Somatic mutations in the \textit{KCNJ5}, \textit{ATP1A1}, and \textit{ATP2B3} genes are present in aldosterone-producing adenomas (APAs).
  \item We report a high prevalence (47\%) of somatic APA mutations in Italian referral centers for hypertension.
  \item Two novel somatic APA mutations, in \textit{KCNJ5} (p.Trp126Arg) and in \textit{ATP1A1} (p.Gly99Arg), were identified.
\end{itemize}

\textbf{What Is Relevant?}

\begin{itemize}
  \item APA-harboring \textit{ATP1A1} or \textit{ATP2B3} mutations express higher levels of \textit{CYP11B2} compared with those without these or \textit{KCNJ5} mutations.
\end{itemize}

\textbf{Summary}

Somatic APA mutations result in cell depolarization and an increase in \textit{CYP11B2} gene expression.

\begin{itemize}
  \item A novel \textit{ATP1A1} mutation (p.Gly99Arg) displays markedly reduced K$^+$ binding and causes cell depolarization.
  \item Overexpression of \textit{ATP1A1} mutations (p.Gly99Arg and p.Leu104Arg) in adrenal cells result in \textit{CYP11B2} upregulation.
\end{itemize}
Somatic ATP1A1, ATP2B3, and KCNJ5 Mutations in Aldosterone-Producing Adenomas
Tracy Ann Williams, Silvia Monticone, Vivien R. Schack, Julia Stindl, Jacopo Burrello, Fabrizio Buffolo, Laura Annaratone, Isabella Castellano, Felix Beuschlein, Martin Reincke, Barbara Lucatello, Vanessa Ronconi, Francesco Fallo, Giampaolo Bernini, Mauro Maccario, Gilberta Giacchetti, Franco Veglio, Richard Warth, Bente Vilsen and Paolo Mulatero

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

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#equal contributions

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Running title: Somatic APA mutations

Key words: Aldosterone, aldosterone-producing adenoma, adrenal gland, secondary hypertension, potassium channel, sodium pump.
Expanded Methods Section

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 Atpla1 and Atpla1 c.311T>G (p.Leu104Arg) plasmids were as described. The rat Atpla1 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 Atpla1 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 Atpla1 cDNA, with 2 µL 100 µM siRNA to specifically
interfere with the expression of the endogenous human ATP1A1 by Amxa nucleofection using programme X005. Equivalent transfection rates for each Atplal cDNA were confirmed by TaqMan real-time PCR by measuring rat Atplal gene expression in each transfection compared to empty vector controls using GAPDH as the endogenous reference gene. The fold changes in Atplal gene expression were: wild-type, 53.77±2.62; L104R, 51.93±3.29; G99R, 56.46±4.20. Silencing of endogenous HAC15 ATP1A1 mRNA and constant levels of silencing between transfections with wild-type or mutated rat Atplal constructs was confirmed by TaqMan real-time PCR: ATP1A1 mRNA was decreased by 87.1%±0.9, 87.4%±1.0 and 88.0%±1.0 in co-transfections of siATP1A1 with wild-type Atplal, 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\DeltaCt relative quantification method as described previously using GAPDH as the endogenous reference gene. The TaqMan gene expression assays used were: CYP11B2, Hs1597732_m1; CYP11B1, Hs01596404_m1; NR4A2, Hs00428691_m1; 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 ATP1A1 or ATP2B3 mutations (surrounding tumor tissue was not available for one APA with an ATP1A1 mutation) and for 11 APA without mutations in KCNJ5, ATP1A1 or ATP2B3.

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

For in vitro functional assays, COS cells were transiently transfected with rat wild-type or mutated Atplal encoding Na\(^+\)/K\(^-\)-ATPase p.Gly99Arg together with a siRNA to specifically interfere with the expression of the endogenous COS cell Atplal. ATPase activity assays were performed at 37°C as described. For measurements of Na\(^+\) dependence of phosphorylation by MgATP, assays were performed under the conditions described at 0°C for 10s 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.

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

The full-length cDNA encoding wild-type rat Atplal and the mutated Atplal c.295G>A (p.Gly99Arg) were subcloned into the bicistronic pIRES-CD8 expression vector. 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.

**Statistical analyses**

All data are expressed as mean ± standard error of the mean (SEM) for normally distributed variables and as median [25th-75th 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.

**Results**

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

The patient carrying the 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**


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<th>Gene</th>
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<td>p.Thr158Ala; p.Ile157del; p.Trp126Arg</td>
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<td></td>
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<td></td>
<td></td>
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<td>CGTCATCATCCTGGTCTCT</td>
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Table S1 Primer pairs used for PCR amplifications
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<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>
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<td>Age at PA diagnosis (years)</td>
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<td>Preop. SBP (mmHg)</td>
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<td>Number preop. AH drugs</td>
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<td>n.s. (0.201)</td>
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<td>Preop. plasma aldo (ng/dL)</td>
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<td>48 [32-66]*</td>
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<td>Preop. PRA (ng/mL/h)</td>
<td>0.20 [0.20-0.39]*</td>
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<td>0.25 [0.20-0.75]*</td>
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<td>Adenoma size (mm)</td>
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<td>18 [12-25]*</td>
<td>15 [9-19]*</td>
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<td>Postop. SBP (mmHg)</td>
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<td>2.1 [1.1-2.6]*</td>
<td>2.7 [2.3-5.7]*</td>
<td>n.s. (0.117)</td>
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</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].
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).