Adrenal Cortex Remodeling and Functional Zona Glomerulosa Hyperplasia in Primary Aldosteronism

Sheerazed Boulkroun, Benoit Samson-Couterie, José-Felipe Golib Dzib, Hervé Lefebvre, Estelle Louiset, Laurence Amar, Pierre-François Plouin, Enzo Lalli, Xavier Jeunemaitre, Arndt Benecke, Tchao Meatchi, Maria-Christina Zennaro

Abstract—Primary aldosteronism is the most common form of secondary hypertension with hypokalemia and suppressed renin-angiotensin system caused by autonomous aldosterone production. Our aim was to compare zona glomerulosa (ZG) structure and function between control adrenals and the peritumoral tissue from patients operated on for aldosterone-producing adenoma. ZG morphology and CYP11B1, CYP11B2, and disabled 2 expression were studied in 15 control adrenals and 25 adrenals with aldosterone-producing adenoma. A transcriptome analysis was done using publicly available data sets. In control adrenals, ZG was discontinuous, and CYP11B2 expression was focal or partly continuous and localized to 3 structures, foci, megafoci, and aldosterone-producing cell clusters. CYP11B2 expression was restricted to a limited number of ZG cells expressing Dab2 but not CYP11B1; aldosterone-producing cell clusters were composed of cells with an intermediate phenotype expressing CYP11B2 but not disabled 2 or CYP11B1. In peritumoral tissue, large remodeling of the adrenal cortex was observed with increased nodulation and decreased vascularization that were not correlated with CYP11B2 expression. In 17 out of 25 adrenals, hyperplasia of adjacent ZG was observed with persistent expression of CYP11B2 that was extended to the entire ZG. In all of the adrenals from patients with aldosterone-producing adenoma, CYP11B2 expression was present in foci, megafoci, and aldosterone-producing cell clusters. Transcriptome profiling indicates a close relationship between peritumoral and control adrenal cortex. In conclusion, adrenal cortex remodeling, reduced vascularization, and ZG hyperplasia are major features of adrenals with aldosterone-producing adenoma. Transcriptional phenotyping is not in favor of this being an intermediate step toward the formation of aldosterone-producing adenoma. (Hypertension. 2010;56:885-892.)

Key Words: zona glomerulosa hyperplasia □ aldosterone-producing adenoma □ secondary hypertension □ adrenal cortex remodeling □ aldosterone □ potassium □ transcriptome

Primary aldosteronism (PAL) is the most common form of endocrine hypertension with a prevalence of 7% to 10% of hypertensive patients.1 In PAL, autonomous production of aldosterone is associated with suppressed renin activity, hypokalemia, and hypertension. The two principal forms are unilateral aldosterone-producing adenoma (APA), a benign tumor of the adrenal cortex, and bilateral adrenal hyperplasia, also known as idiopathic hyperaldosteronism. Whereas in APA the adjacent zona glomerulosa (ZG) is believed to be nonfunctional because of negative feedback regulation, bilateral adrenal hyperplasia involves the entire ZG of both adrenal glands.2 Patients with PAL operated on the basis of an APA, and/or a lateralized aldosterone hypersecretion can be cured by unilateral adrenalectomy. However, only ≈70% of patients with lateralized aldosterone secretion are cured by surgery,3 suggesting that, in some cases, residual aldosterone secretion from the contralateral adrenal gland, although within the normal range, may be inappropriate for salt status and blood pressure levels.

Aldosterone is synthesized from cholesterol in the ZG of the adrenal cortex by a series of specific enzymatic reactions.4 Histology of the mammalian adrenal cortex was originally described in 1866.5 The ZG is the outer layer of the cortex adjacent to the capsule composed of small and clear cells. In rodents, 5 to 10 cell layers (≈50 μm) form a continuous zone6,7; in humans, in contrast, the ZG is discontinuous.8,9

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The zona fasciculata (ZF) is the thickest zone of the cortex and is composed of columns of large and clear cells (because of the presence of many intracellular lipid droplets). Finally, the zona reticularis (ZR) is composed of small and eosinophilic cells. The 3 zones present distinct roles in steroid hormone production: ZG produces mineralocorticoids, ZF produces glucocorticoids, and ZR produces androgens. In human and rodents, the functional zonation involves the zone-specific expression of 2 cytochrome p450 isoenzymes: 11β-hydroxylase (encoded by the CYP11B1 gene) is responsible for the final steps of cortisol biosynthesis in human (corticosterone in rodents), whereas aldosterone synthase (CYP11B2) catalyzes the final steps of aldosterone biosynthesis. The expression of CYP11B2 is limited to the ZG, whereas CYP11B1 is expressed in ZF and ZR, although until very recently the exact pattern of expression of the 2 isoymes in humans was still a matter of debate. Recently, Nishimoto et al showed that CYP11B2 was sporadically detected in ZG, whereas CYP11B1 was expressed in ZF and ZR. Interestingly, in addition to conventional zonation, the authors also described a variegated zonation consisting of subcapsular cell clusters expressing CYP11B2 that they named aldosterone-producing cell clusters (APCCs). In samples from patients with APA, the nontumor portions of the adrenals frequently bear 1 or multiple of these APCCs strongly expressing CYP11B2. Based on these findings, the authors proposed 2 patterns of aldosterone production, 1 regulated by the renin-angiotensin system and 1 autonomous in APCCs. They also propose that APCCs eventually develop into APA.

To further investigate the cellular phenotype of aldosterone-producing cells in normal adrenal glands and PAL and to explore the nature of APCCs, we have analyzed the expression of CYP11B2 in relation to specific markers of ZG (disabled 213) and ZF/reticularis (CYP11B1). Furthermore, we investigated the morphology, nodulation, and vascularization of the perinodular adrenal cortex and correlated these findings with CYP11B2 expression. These results were complemented by transcriptome analyses of peritumoral tissue inferred from publicly available data sets.

### Subjects and Methods

#### Patients

We obtained formalin-fixed and paraffin-embedded adrenals through the COMETE (Cortico- et médullo-surrénale, les tumeurs endocrines) Network from 25 patients who had undergone surgery for lateralized PAL at Hôpital Européen Georges Pompidou between 2002 and 2008. Methods for screening and criteria for diagnosing PAL were in accordance with institutional guidelines and have been described recently. The clinical and biological characteristics of the patients are described in Tables 1 and S1 (available in the online Data Supplement at http://hyper.ahajournals.org). Fifteen control adrenals were obtained from enlarged nephrectomies, consisting of removal of one kidney together with the neighboring adrenal gland and neighboring lymph nodes (kindly provided by the Department of Pathology, University Hospital of Rouen, and by Dr M. Sibony, Hôpital Tenon). Two cortisol-producing adenomas were obtained through the COMETE Network. All of the patients gave written informed consent to participate to the study.

#### Histological Examination

Histological annotations were performed on control adrenals, APA, and peritumoral adjacent tissue. Cellular compositions of APA and the ZG hyperplasia were determined on hematoxilin-eosin-safran staining. The main criterion used to determine ZG hyperplasia was the continuous character of the ZG; the second criterion, in case of discontinuity of the ZG, was its thickness (≥200 μm) measured with a micrometer integrated in the microscope. Nodulation was analyzed by Sirius red staining and converted into a semiquantitative score using the formula: nodulation score=∑ni×i, where “i” is the score attributed to each type of nodulation (1=partial nodulation; 2=near complete nodulation; 3=complete nodulation; Figure 4A), and “ni” is the number of nodules of the corresponding type. All of the microscopic examinations were done on a Leica microscope.

#### In Situ Hybridization

In situ hybridization was performed on the entire adrenal gland (APA or nodules and peritumoral adjacent tissue) from PAL patients and from control adrenals. For detailed procedures, please see the online Data Supplement at http://hyper.ahajournals.org.

#### Determination of CYP11B2 Expression Area

CYP11B2 expression area was defined as the percentage of the adrenal gland expressing the enzyme. Analysis was performed at ×25 (ocular lens: ×10.0; objective lens: ×2.5) on all of the adrenal glands. The CYP11B2 expression area was determined on the entire adrenal gland (APA or nodules and peritumoral adjacent tissue) from PAL patients and from control adrenals using ImageJ software. For

### Table 1. Clinical and Biological Characteristics of PAL Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Preadrenalectomy</th>
<th>Postadrenalectomy</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45±7</td>
<td>45±6</td>
<td></td>
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<tr>
<td>Sex (male/female), %</td>
<td>52/48</td>
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</tr>
<tr>
<td>SBP, mm Hg</td>
<td>145.8±15.9</td>
<td>129.4±14.1</td>
<td>0.0008</td>
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<tr>
<td>DBP, mm Hg</td>
<td>90.6±10.4</td>
<td>83.3±9.4</td>
<td>0.0197</td>
</tr>
<tr>
<td>Duration of hypertension, y</td>
<td>7±8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum K⁺ level, mmol/L</td>
<td>3.3±0.5</td>
<td>4.2±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urinary aldosterone, mean (range), pmol/L</td>
<td>124.6 (41.0 to 575.0)</td>
<td>27.8 (10.0 to 58.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Active renin supine, mean (range), mU/L</td>
<td>3.14 (0.00 to 9.40)</td>
<td>16.7 (2.1 to 83.6)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Aldosterone supine, mean (range), pmol/L</td>
<td>891.4 (303.0 to 2193.0)</td>
<td>133.5 (33.0 to 330.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ARR, mean (range)</td>
<td>388.00 (83.85 to 1205.00)</td>
<td>25.4 (2.5 to 101.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumor size, mean (range), mm</td>
<td>14 (9 to 25)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; ARR, aldosterone:renin ratio. Data are mean±SEM unless otherwise specified.
peritumoral adjacent tissues and control adrenals, CYP11B2 expression area was calculated as the percentage of measured adrenal cortex surface. For APA, CYP11B2 expression area was calculated as the percentage of APA surface; this measure was then reported to APA volume calculated using measured nodule dimensions (CYP11B2 expression area = % CYP11B2 expression × APA volume).

**Immunohistochemistry**

Immunohistochemistry was performed on serial sections of the same tissues used for in situ hybridization. For the detailed procedure please see the online Data Supplement.

**Analysis of Vascularization**

Vascularization was analyzed by CD34 immunostaining in control adrenal and adjacent tissues of APA. Microvessel surface analysis was performed at ×100 (ocular lens: ×10; objective lens: ×10), 4 different areas were measured, and the average was then calculated. The vessel surface was determined using ImageJ software.

**Transcriptome Data Sets Used and Nomenclature**

Transcriptome data set form Giordano et al16 (GEO accession No. GSE10927) consists of 4 groups defined according to sample tissue type and clinical annotation (Table S2): (1) G_ACA, adrenal cortex adenomas other than aldosterone-producing adenoma; (2) G_APA, adrenal cortex aldosterone-producing adenoma; (3) G_CA, normal adrenal cortex adjacent to adrenal adenomas other than aldosterone-producing adenoma; and (4) G_ADJ, normal adrenal cortex adjacent to aldosterone-producing adenoma.

**Correlation Analysis**

Control adrenal versus APA subtraction profiles was used to define a gene signature of statistically significantly differentially expressed common probes for both data sets with P<0.01. The signature identified through this procedure was used to compute the correlation coefficients between samples. We calculated average Pearson correlation coefficients sample by sample for the control adrenal versus APA differentially expressed genes (P<0.01) with the average expression values of the same genes in the control adrenal and APA subpopulations.

**Statistical Analysis**

For details please see the online Data Supplement.

**Results**

**Functional Zonation of the Human ZG Differs From Rodents**

To characterize more precisely the cellular composition of areas expressing CYP11B2, we investigated expression of CYP11B2, CYP11B1, and Dab2 (disabled 2, a specific marker of the ZG in rodent13)13) in 15 control adrenals. We considered that this large number could give a reliable estimate of CYP11B2 expression if considering variables possibly affecting aldosterone biosynthesis, including sex, age, salt intake, anesthesia, and medication. Adrenals were examined using HES staining (Figures 1A and 1E and S1A and S1E), demonstrating that Dab2 is a specific marker of the ZG not only in rodents13 but also in humans. In situ hybridization showed CYP11B2 expression in a limited number of ZG cells (Figures 1C and 1G and S1C and S1G), as described previously.12 Two distinct patterns of expression were distinguished, focal (Figures 1C and 1G and S1C) or partly continuous (Figure S1G). In both patterns, CYP11B2 expression was less extended than Dab2 expression, indicating that part of the ZG cells was silent in terms of aldosterone production. In situ hybridization of CYP11B1 showed expression in the entire ZF and ZR (Figures 1D and 1H and S1D and S1H), with no signal detected in CYP11B2 and Dab2-positive cells. Assessing the focal expression of CYP11B2 disclosed 2 types of clusters that coexisted in the same adrenal. Clusters of cells morphologically and functionally identified as ZG cells (Figures 2A and 2E, and CYP11B2 and CYP11B1) were defined according to their size as foci (<300 μm) or megafoci (>300 μm). We also found large clusters of cells composed of morphological ZG cells in contact with the capsule and inner columnar ZF-like cells (Figure 2I through 2L), resembling the APCCs described by Nishimoto et al.12 In contrast to megafoci, APCCs did not express Dab2 (Figure 2J). In all

![Figure 1](http://hyper.ahajournals.org/)

Pathology of normal adrenal gland. Data show HES staining, Dab2 immunohistochemistry, and in situ hybridization for CYP11B2 and CYP11B1 performed on normal adrenal glands. A and E, HES staining. B and F, Immunostaining for Dab2. C and G, CYP11B2 in situ hybridization (blue). D and H, CYP11B1 in situ hybridization (blue). Nuclei were counterstained with hematoxylin after immunostaining for Dab2. HES staining showed discontinuity of ZG in normal adrenal gland. Dab2 expression is restricted to ZG cells, whereas expression of CYP11B2 is detected only in some ZG cells. CYP11B1 is expressed in ZF and ZR. Black arrows indicate cells expressing CYP11B2. Bars, 1000 μm (A through D) and 200 μm (E through H).
of the cases, no expression of CYP11B1 was detected in CYP11B2-positive cells (Figure 2D, 2H, and 2L). This result strongly suggests that APCCs contain cells presenting an intermediate phenotype between ZG and ZF cells.

**ZG Adjacent to APA Presents Structural and Functional Hyperplasia**

The morphological examination of 25 APAs revealed a mixed cellular composition, with, in two thirds of cases, a majority of ZF-like cells (Table S3 and Figures 3A and S2A). Despite their different cellular compositions, all of the APAs expressed CYP11B2 in all (Figures 3C and S2C) or in a large number of cells (data not shown). Interestingly, Dab2 was coexpressed with CYP11B2 in APAs (Figures 3B and S2B), indicating that APAs consist of ZG cells that may have acquired morphological characteristics of ZF cells because of increased steroid production. In APAs, CYP11B1 was detected only in cells that did not express CYP11B2 (Figures 3D and S2D). Eleven adrenals presented secondary nodules that were also positively stained for CYP11B2.

Morphological analyses of peritumoral adjacent tissue revealed major changes in ZG, which appeared continuous (Figure 3E) or presented extended focal thickening associated with expression of CYP11B2 (Figure 3G) and Dab2 (Figure 3F) but negative for CYP11B1 (Figure 3H). Quantification of ZG hyperplasia\(^8,9\) showed that these changes were significantly different compared with control adrenals (Table 2). In peritumors that did not present ZG hyperplasia, focal expression of CYP11B2 was detected (Figure S2G and S2K) in all but 1 patient. As in control adrenals, megafoci composed of ZG cells (Figure S2E) expressing Dab2 (Figure S2F) and CYP11B2 (Figure S2G) but not CYP11B1 (Figure S2H), and APCCs formed of a mixed population of cells (Figure S2I), expressing CYP11B2 (Figure S2K) but not Dab2 (Figure S2J) and CYP11B1 (Figure S2L), were present. Foci, megafoci, and APCCs were also detected in peritumoral adjacent tissues with ZG hyperplasia (data not shown). No differences were observed between control adrenals and peritumoral adjacent tissues in terms of number of foci \((P=0.30)\) or APCCs \((P=0.86)\), whereas number of megafoci was significantly increased in peritumoral adjacent tissues compared with control adrenals \((0.45±0.25; peritumoral adjacent tissues: 2.58±0.63; \(P=0.011\))

When considering adrenals presenting ZG hyperplasia, CYP11B2 expression area, as quantified by image analysis, was only slightly and not significantly increased in peritumoral adjacent tissue compared with control adrenal \((1.28±0.3; peritumoral adjacent tissue: 1.92±0.3; \(P=0.083)\). Finally, there was no difference between adrenals with or without ZG hyperplasia in terms of age, sex, size of the APA, systolic and diastolic blood pressure, duration of hypertension, plasma potassium, aldosterone and renin, or urinary aldosterone levels (Table S4).

**Tissue Remodeling and Decreased Vascularization of the Adrenal Cortex Adjacent to APA**

To evaluate factors possibly promoting APCC and megafoci formation, as well as ZG hyperplasia, we next investigated nodu-
Control adrenals all presented some degree of incomplete or near complete nodulation, and in some adrenals complete nodulation was observed (Figure 4). Interestingly, the peritumoral adjacent tissues presented a major increase of tissue remodeling compared with control adrenals (score of nodulation in control adrenals: 11.19 ± 1.95; peritumoral adjacent tissue: 56.00 ± 8.92; \( P < 0.0001 \); Figure 4B). However, there was no correlation between nodulation and any of the measured parameters related to aldosterone production, including number of foci, megafoci, and APCC; CYP11B2 expression area in peritumoral adjacent tissues and APA; as well as clinical and biological parameters (urinary aldosterone, plasma potassium, systolic and diastolic blood pressure, or duration of hypertension; Table S5).

Because tissue vascularization might be related to nodulation or to the presence of foci, megafoci, or APCC, we analyzed vascular surface in control adrenals and peritumoral adjacent tissues by CD34 immunohistochemistry (Figure 4C and 4D). Vascular architecture was similar in both tissues (Figure 4C). Although there was no difference in vascularization area between APA and control adrenals (not shown), as already described,\(^1\) vascularization surface was significantly decreased ( \( \sim 33\% \) ) in peritumoral adjacent tissues compared with control adrenals (Figure 4D; control adrenals, 9.67 ± 0.94 versus peritumoral adjacent tissues, 6.47 ± 0.42; \( P = 0.0010 \)). Again, no correlation was observed between vascular surface and nodulation score, number of foci, megafoci, APCC, or CYP11B2 expression area in both control adrenals and peritumoral adjacent tissues (Table S5).

### Transcriptional Phenotyping Does Not Suggest Adrenal Cortex Nodulation Being an Intermediate Toward APA Formation

To gain insight into the sequence of events linking adrenal cortex remodeling to APA, we inferred gene expression in the peritumoral adjacent tissue from published data by Giordano et al.\(^1\) To this end we determined differentially expressed genes (\( P < 0.01 \)) between the control adrenals and APA groups compiled from the data set (Table S2). When the covariance of gene expression of those selected genes is used as a distance measure in a principal component analysis of the control adrenals, peritumoral adjacent tissues, ACA, and APA (Figure 5A), the peritumoral adjacent tissue samples are very closely related to the control adrenal samples, even if they are shifted slightly along the first principal component toward the APA samples. The ACA samples are truly intermediate between control adrenals/peritumoral adjacent tissues and APA in their molecular phenotype. To further explore the nature of peritumoral adjacent tissue, we calculated specific gene signatures for control adrenal and APA likeliness. Again, the peritumoral adjacent tissue samples from the data set of Giordano et al.\(^1\) correspond in their molecular phenotype much more to control adrenal than APA (Figure 5B). Hence, the molecular profiling done supports a general close relationship between peritumoral adjacent tissues and control adrenals.

### Correlation of CYP11B2 Expression With Aldosterone Biosynthesis and Function

An important issue in PAL is whether there is a correlation between CYP11B2 expression and aldosterone biosynthesis and function. In PAL, total CYP11B2 expression area, including APA(s) and peritumoral adjacent tissue, was significantly increased as compared with control adrenals (control adrenals: 1.28 ± 0.3; PAL: 39.86 ± 3.5; \( P < 0.0001 \)).

![Figure 3. Morphological hyperplasia in adjacent tissue of APA. HES staining and CYP11B2 expression in APA (A through D) and adjacent tissue (E through H). A and E, HES staining. B and F, Dab2 immunostaining (brown). C and G, CYP11B2 in situ hybridization (blue). D and H, CYP11B1 in situ hybridization (blue). Dab2 and CYP11B2 but not CYP11B1 are expressed in APAs. HES staining reveals continuous ZG hyperplasia, associated with expression of Dab2 and CYP11B2 but not CYP11B1. Bars, 1000 \( \mu \)m.](image)

![Table 2. ZG Hyperplasia in Control Adrenals vs Peritumoral Adjacent Tissues](table)
significant negative correlation was observed between plasma potassium levels and 24-hour urinary aldosterone concentration \((r = -0.75; P < 0.0001; \text{Figure S3A})\) in PAL patients. CYP11B2 expression area in APA was significantly correlated with plasma potassium levels \((r = 0.51; P = 0.016)\), whereas it was not correlated with 24-hour urinary aldosterone levels \((r = 0.32; P = 0.16)\). Interestingly, when APA volume was taken into account for CYP11B2 expression, a significant correlation was observed between CYP11B2 expression*volume and 24-hour urinary aldosterone \((r = 0.52; P = 0.012; \text{Figure S3B})\). CYP11B2 expression*volume in APA was again negatively correlated with plasma potassium concentration, although less significantly \((r = -0.41; P = 0.056)\). When considering CYP11B2 expression area in peritumoral adjacent tissue, a positive, although not significant, correlation with plasma potassium levels was observed \((r = 0.39; P = 0.06; \text{Figure S3C})\).

### Discussion

In the present study, we report the occurrence of increased adrenal cortex remodeling and ZG hyperplasia in PAL. We found a dramatic increase in the nodulation of the cortex adjacent to the tumor in a large series of patients operated on for APA, as well as decreased vascularization. The ZG adjacent to APA expressed CYP11B2 mRNA, suggesting that it was functional despite autonomous aldosterone production from APA and suppressed renin-angiotensin system.

We first extensively characterized aldosterone-producing structures in the adrenal ZG. CYP11B2 was detected in all of the normal adrenal glands; however, its expression was restricted to a limited number of ZG cells. Only in rare cases was CYP11B2 expression semicontinuous. In most adrenals, CYP11B2 localized to 3 types of focal structures, the already described APCCs,\(^1\) which we show are specific cell clusters composed of a mixed population of Dab2\(^{-}\) and CYP11B1\(^{-}\) cells.

![Figure 4. Nodulation in control adrenal and in peritumoral adjacent tissue. A, Sirius red staining and semiquantitative scores of nodulation. 0 indicates absence of nodulation; 1, incomplete nodulation; 2, near complete nodulation; 3, complete nodulation; B, Nodulation scores were determined for control adrenal and APAs and indicate a significant increase of nodulation in peritumoral adjacent tissue compared to control adrenal. C, CD34 immunohistochemistry on control adrenal and peritumoral adjacent tissue revealing normal vascular architecture. D, Quantification of vascular surface in control adrenal and peritumoral adjacent tissue showed a significant decrease of vascularization in peritumoral adjacent tissue. Bars, 1000 \(\mu m\).](image1)

![Figure 5. Analysis of peritumoral adjacent tissue in terms of transcriptome patterns and comparison with pathological conditions. A, Principal component analysis of the Giordano et al\(^{16}\) transcriptome profiles. Covariance along the first 2 principal components of differentially expressed genes is shown for the 4 indicated subgroups of patients. B, Likelihood analysis based on Pearson correlation coefficients calculated for the average values of control adrenal versus APA differentially expressed genes \((P < 0.01)\) and the values obtained individually from each patient for the Giordano et al\(^{16}\) data set.](image2)
certain degree of nodularity can be observed, of which the
peritumoral adjacent tissue. Indeed, in most adult adrenals, a
event is the fact that we observe reduced vascularization in
hypertension.14 Accordingly, a certain degree of nodulation
localized ischemic changes attributed to atherosclerosis or
facilitating overgrowth of adrenocortical cells in response to
characteristics, because APCCs do not express the ZG marker Dab2,
in contrast to APA. Furthermore, although persistence of
expression in the ZG or in APCCs has been observed previously in adrenals with APA,12,17 our results
demonstrate that not only is the adjacent ZG not suppressed
in the presence of autonomous aldosterone production, but
that in 50% of patients, the entire ZG has acquired the
capacity to express CYP11B2.

Our study demonstrates that adrenal cortex remodeling,
decreased vascularization, and ZG hyperplasia are major
features of adrenals with APA. A question arising from these
results is whether nodulation of adjacent cortex precedes
development of APA. In favor of nodulation being a primary
event is the fact that we observe reduced vascularization in
peritumoral adjacent tissue. Indeed, in most adult adrenals, a
certain degree of nodularity can be observed, of which the
frequency increases with age and severity of hypertension;19
based on these findings, some authors propose that adreno-
cortical nodules may occur as a result of localized compensatory
overgrowth of adrenocortical cells in response to
localized ischemic changes attributed to atherosclerosis or
hypertension.14 Accordingly, a certain degree of nodulation
was observed in our control adrenals. In our study, the mean
age of cases with PAL was 45±7 years compared with
61±14 years for patients operated with normal adrenals
(P<0.0001), and a majority of control patients were also
hypertensive, excluding age and hypertension as major deter-
minants for the large remodeling observed in peritumoral
adjacent tissue. Furthermore, there was no evidence for any
correlation between vascularization and nodulation of the
adjacent adrenal cortex, as well as between nodulation and
levels or duration of hypertension. Although we could not
perform expression studies on our peritumoral adjacent tis-
sue, data inferred from publicly available transcriptomes
indicate that the molecular phenotype of peritumoral adjacent
tissue is not intermediary between control adrenals and APA,
implying that abnormalities of the cortex adjacent to APA do
not precede APA formation. Nodulation of the adrenal cortex
is a feature specific to APA, because it was absent from
adrenals with cortisol-producing adenomas (Figure S4).
However, the natural history of the development of APA is
unknown; therefore, we cannot exclude that tissue remodel-
ing initiated a long time before detection of the clinical
phenotype of PAL is underlying the formation of an autonom-
ous nodules with autonomous aldosterone production and then
developing into APA.

Although we cannot exclude that hypertension attributed to
APA is initiating tissue remodeling at some time point,
another mechanism whereby APA may lead to increased
nodulation is a local effect of aldosterone itself. Indeed, it is
now clearly established that aldosterone is able to induce
important tissue remodeling in many organs, including heart,
kidney, and blood vessels.20 A prerequisite for a possible
local effect of aldosterone in the adrenal cortex adjacent to
APA is the expression of the mineralocorticoid receptor.
Indeed, we found strong mineralocorticoid receptor expres-
sion in the ZG of control adrenals, as well as in peritumoral
adjacent tissue (Figure S5). Also, local aldosterone effects have
been described in adrenal H295R cells, where aldosterone
induces, through an autocrine mineralocorticoid receptor–medi-
ated action, the expression of T-type calcium channels.21 Future
studies should address the relationship between increased aldo-
sterone levels in APA and adrenal cortex remodeling.

Perspectives
The present study provides evidence for adrenal cortex
remodeling, reduced vascularization, and ZG hyperplasia
being major features associated with APA. Our results may
have some clinical implications for the treatment of patients
with PAL. Extensive remodeling of the adrenal cortex adja-
cent to APA, whether primary or secondary to APA itself, and
the presence of secondary functional nodules in a large
number of adrenals suggest that, despite a curative effect of
enucleation of the adenoma associated with normalization of
adrenal endocrine function,22 long-term follow-up is required
because of the possibility that buds with autonomous aldoster-
one production, with the potential to develop into APA,
may be present in the remaining adrenal tissue. This work
provides the basis for future studies addressing the sequence
of events leading to the development of APA.

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None.

References
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Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism.

Online Supplement

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Supplementary Materials and Methods

**In situ hybridization**

Paraffin-embedded tissue specimens were cut in serial sections of 4 µm and mounted on Super Frost Plus Slides. The sections were deparaffinized with xylene, rehydrated through graded ethanol and treated with proteinase K (Sigma; St Louis, USA) 8 µg/ml in phosphate-buffered saline (PBS) for 10 min. Pre-hybridization were performed incubating the sections with pre-heated (at 85°C for 5 min) hybridization buffer (1X salt, 50% formamide, 10% dextran sulphate, yeast RNA 10 mg/ml, Denhardt’s solution) for 4 hours at 65°C in a humidified chamber (50% formamide, 2X SSC). Then pre-heated hybridization solution containing hybridization buffer and digoxigenin labelled probe was applied on the sections, covered with parafilm and put in a humidified chamber at 65°C overnight. The slides were rinsed in SSC 5X at 65°C, washed once in a solution containing SSC 2X and 50% formamide for 30 min at 65°C, and then once in TNE (10 mM Tris HCl pH 7.6, 500 mM NaCl, 1 mM EDTA for 10 min at 37°C. slides were treated with RNase A (Roche, 20 µg/ml; Indianapolis, USA) diluted in TNE for 30 min at 37°C, washed once in TNE 10 min at 37°C, once in SSC 2X 20 min at 65°C, twice in SSC 0.2X 20 min at 65°C and twice in MABT [MAB (100 mM maleic acid, 150 mM NaCl, 192 mM NaOH), 1.1% Tween 20] for 10 min at room temperature. The sections were covered with blocking solution (MABT, 20% normal goat serum) at room temperature for 1h30 and subsequently incubated with anti-DIG antibody (Roche) diluted 1:2000 overnight at 4°C. After washing the sections twice in MABT for 5 min and once in NTMT (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM MgCl2, 1% Tween20) for 10 min, they were revealed using BMP purple (Roche; Indianapolis, USA), 0.1% Tween 20 for 3-5 days and mounted in aqueous medium.

The 3’-untranslated region of CYP11B1 (nucleotides 2741-2946 of CYP11B1, Genebank accession number NM_000497) and CYP11B2 (nucleotides 2727-2931 of CYP11B2, Genebank accession number NM_000498) was subcloned into pGEMT-Easy (Promega; Madison, USA). For each gene, antisense and sense probes were synthesized using the DIG RNA labelling kit (Roche) according to the manufacturer’s protocol. No specific binding was observed for CYP11B1 and CYP11B2 sense probes (data not shown).

**Immunohistochemistry**

Sections were deparaffinised in xylene and rehydrated through graded ethanol. To unmask the antigens, the slides were incubated in antigen unmasking solution (Vector Laboratories; Burlingame, USA) for 30 min at 98°C. Then endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide in water for 10 min and nonspecific staining blocked with normal goat serum. Primary antibodies (Dab2 antibody (Santa Cruz; Santa Cruz, USA), 1/1000; CD34 (Clone QBEND 10, Immunotech; Marseille, France), 1/500; MR antibody 1D5, kindly provided by C. Gomez-Sanchez, 1/100) were incubated overnight at 4°C. Sections were washed, incubated 30 min with affinity purified horse anti mouse (1/400, Vector laboratories) or goat anti rabbit (1/400, Vector Laboratories), washed and incubated with an avidin-biotin-peroxydase complex (Vectastain ABC Elite; Vector Laboratories) for 30 min. the slides were developed using either aminobenzidzin (Vector Laboratories) and a counterstaining with hematoxinlin (Sigma) for Dab2 or Histogreen (Abcys; Paris, France) and a counterstaining with nuclear fast red (Biolyon; Lyon, France) for CD34. For MR, an additional amplification step using the TSA Biotin system (Perkin Elmer; Boston, USA) was added to the protocol. In the negative control reactions, the primary antibodies were omitted from the dilution buffer, which in all instances resulted in a complete absence of staining.
Statistical analysis

Pairwise comparisons were done using Student’s unpaired t-test or non-parametric Mann-Whitney test. All analyses were performed using the Graphpad Prism version 5.01 Software (Graphpad Software). Pearson correlation coefficients were calculated and using a T-test, p-values of less than 0.05 were considered to be significant. Results are presented as mean ± S.E.M.
Table S1. Clinical and biological characteristics of control patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61 ± 14*</td>
</tr>
<tr>
<td>Gender (male/female), %</td>
<td>76/24</td>
</tr>
<tr>
<td>Hypertensives (Yes/No), %</td>
<td>78/22†</td>
</tr>
</tbody>
</table>

*, data available for 14 out of 15 patients.
†, data available for 9 out of 15 patients
Table S2. Classification and nomenclature of samples selected for analysis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Biological Samples – Microarrays</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSM277092_CAC069, GSM277097_CAC088, GSM277098_CAC092, GSM277090_CAC006.</td>
<td>G_CA</td>
</tr>
<tr>
<td></td>
<td>GSM277091_CAC063, GSM277093_CAC076, GSM277096_CAC087.</td>
<td>G_ADJ</td>
</tr>
</tbody>
</table>

APA: adrenal cortex aldosterone-producing adenoma, ACA: adrenal cortex adenoma, CA: control adrenal cortex not adjacent to APA, ADJ: adrenal cortex adjacent to APA.
Table S3. Cellular composition of APA.

<table>
<thead>
<tr>
<th></th>
<th>ZG-like cells</th>
<th>Majority of ZF-like cells</th>
<th>ZR-like cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td>16% (4/25)</td>
<td>68% (17/25)</td>
<td>16% (4/25)</td>
</tr>
</tbody>
</table>
Table S4: Clinical and biological data of patients with or without ZG hyperplasia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with ZG hyperplasia</th>
<th>Patients without ZG hyperplasia</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>46±2</td>
<td>45±3</td>
<td>0.6688</td>
</tr>
<tr>
<td>Gender (male/female), %</td>
<td>47/53</td>
<td>50/50</td>
<td>1.0000</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>143.5±4.6</td>
<td>152.5±5.1</td>
<td>0.2422</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>89.1±2.6</td>
<td>95.0±3.4</td>
<td>0.2377</td>
</tr>
<tr>
<td>Duration of hypertension, year</td>
<td>7±2</td>
<td>7±3</td>
<td>0.8054</td>
</tr>
<tr>
<td>Serum K⁺ level, mmol/l</td>
<td>3.4±0.1</td>
<td>3.3±0.3</td>
<td>0.6055</td>
</tr>
<tr>
<td>Urinary aldosterone, mean (range) nmol/day</td>
<td>105.2 (53-285)</td>
<td>166.0 (41-575)</td>
<td>0.7400</td>
</tr>
<tr>
<td>Renine supine, mean (range), mU/l</td>
<td>3.14 (0-9.4)</td>
<td>3.13 (0-8.7)</td>
<td>0.9934</td>
</tr>
<tr>
<td>Aldosterone supine, mean (range), pmol/l</td>
<td>851.14 (303-1786)</td>
<td>1006.0 (362-2193)</td>
<td>0.5385</td>
</tr>
<tr>
<td>ARR, mean (range)</td>
<td>358.5 (83.8-1116)</td>
<td>476.7 (154.2-1205)</td>
<td>0.2949</td>
</tr>
<tr>
<td>APA size, mean (range), mm</td>
<td>14 (9-20)</td>
<td>17 (10-25)</td>
<td>0.0708</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP diastolic blood pressure, NS not significant, ARR aldosterone renin ratio. Data are means±SEM unless otherwise specified.
## Table S5. Correlation analysis between biological and morphological data

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Correlation (r)</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodulation score vs Vascularization</td>
<td>-0.04</td>
<td>8.69E-1</td>
</tr>
<tr>
<td>Nodulation score vs Urinary aldosterone concentration</td>
<td>0.23</td>
<td>3.50E-1</td>
</tr>
<tr>
<td>Nodulation score vs Plasmatic potassium concentration</td>
<td>-0.26</td>
<td>2.78E-1</td>
</tr>
<tr>
<td>Nodulation score vs ADJ CYP11B2 expression area</td>
<td>0.36</td>
<td>1.29E-1</td>
</tr>
<tr>
<td>Nodulation score vs Number of foci</td>
<td>0.12</td>
<td>6.28E-1</td>
</tr>
<tr>
<td>Nodulation score vs Number of megafoci</td>
<td>-0.16</td>
<td>5.13E-1</td>
</tr>
<tr>
<td>Nodulation score vs Number of APCC</td>
<td>0.21</td>
<td>3.72E-1</td>
</tr>
<tr>
<td>Nodulation score vs APA CYP11B2 expression area</td>
<td>0.31</td>
<td>2.15E-1</td>
</tr>
<tr>
<td>Nodulation score vs Systolic blood pressure</td>
<td>0.30</td>
<td>2.06E-1</td>
</tr>
<tr>
<td>Nodulation score vs Diastolic blood pressure</td>
<td>0.33</td>
<td>1.63E-1</td>
</tr>
<tr>
<td>Nodulation score vs Duration of hypertension</td>
<td>0.23</td>
<td>3.39E-1</td>
</tr>
<tr>
<td>Vascularization vs Urinary aldosterone concentration</td>
<td>-0.20</td>
<td>4.00E-1</td>
</tr>
<tr>
<td>Vascularization vs Plasmatic potassium concentration</td>
<td>0.20</td>
<td>3.88E-1</td>
</tr>
<tr>
<td>Vascularization vs ADJ CYP11B2 expression area</td>
<td>-0.20</td>
<td>3.76E-1</td>
</tr>
<tr>
<td>Vascularization vs Number of foci</td>
<td>0.21</td>
<td>3.62E-1</td>
</tr>
<tr>
<td>Vascularization vs Number of megafoci</td>
<td>-0.16</td>
<td>4.91E-1</td>
</tr>
<tr>
<td>Vascularization vs number of APCC</td>
<td>0.17</td>
<td>8.15E-1</td>
</tr>
<tr>
<td>Vascularization vs APA CYP11B2 expression area</td>
<td>-0.20</td>
<td>4.02E-1</td>
</tr>
<tr>
<td>Vascularization vs Systolic blood pressure</td>
<td>0.00</td>
<td>9.81E-1</td>
</tr>
<tr>
<td>Vascularization vs Diastolic blood pressure</td>
<td>0.05</td>
<td>8.30E-1</td>
</tr>
<tr>
<td>Vascularization vs Duration of hypertension</td>
<td>0.14</td>
<td>5.68E-1</td>
</tr>
<tr>
<td>Urinary aldosterone concentration vs Plasmatic potassium concentration</td>
<td>-0.75</td>
<td>5.40E-5</td>
</tr>
<tr>
<td>Urinary aldosterone concentration vs ADJ CYP11B2 expression area</td>
<td>-0.07</td>
<td>7.41E-1</td>
</tr>
<tr>
<td>Urinary aldosterone concentration vs Number of megafoci</td>
<td>-0.04</td>
<td>8.60E-1</td>
</tr>
<tr>
<td>Urinary aldosterone concentration vs APA CYP11B2 expression area</td>
<td>0.32</td>
<td>1.63E-1</td>
</tr>
<tr>
<td>Plasmatic potassium concentration vs ADJ CYP11B2 expression area</td>
<td>0.39</td>
<td>6.08E-2</td>
</tr>
<tr>
<td>Plasmatic potassium concentration vs Number of megafoci</td>
<td>0.26</td>
<td>2.30E-1</td>
</tr>
<tr>
<td>Plasmatic potassium concentration vs APA CYP11B2 expression area</td>
<td>-0.51</td>
<td>1.58E-2</td>
</tr>
<tr>
<td>ADJ CYP11B2 expression area vs Number of megafoci</td>
<td>0.38</td>
<td>6.56E-2</td>
</tr>
<tr>
<td>ADJ CYP11B2 expression area vs APA CYP11B2 expression area</td>
<td>-0.06</td>
<td>7.79E-1</td>
</tr>
<tr>
<td>Number of megafoci vs APA CYP11B2 expression area</td>
<td>-0.02</td>
<td>9.36E-1</td>
</tr>
</tbody>
</table>

ADJ: peritumoral adjacent tissue
**Figure S1: Pathology of normal adrenal gland.** Exemplary results of HES staining, immunohistochemistry for Dab2 and *in situ* hybridization experiments for CYP11B2 and CYP11B1 obtained for sixteen normal adrenal glands. Two different normal adrenal gland examples are presented (a-d, case 1; e-h, case 2). a, and e, HES staining. b and f, immunostaining for Dab2 (brown). c and g, CYP11B2 *in situ* hybridization (blue). d and h, CYP11B1 *in situ* hybridization (blue). Nuclei were counterstained with hematoxilin after immunostaining for Dab2. HES staining showed discontinuity of ZG in normal adrenal gland. Dab2 expression is restricted to ZG cells, whereas expression of CYP11B2 is detected only in some ZG cells. CYP11B1 is expressed in ZF and ZR. Black arrows indicate cells expressing CYP11B2. Bars 1000 µm.
Figure S2: Expression of CYP11B2 in megafoci and APCC in peritumoral adjacent tissue. HES staining, Dab2, CYP11B2 and CYP11B1 expression in APA (a-d), megafoci (e-h) and APCC (i-l). a, e and i, HES staining, b, f and j, immunostaining for Dab2 (brown). c, g and k, CYP11B2 in situ hybridization (blue). d, h and l, CYP11B1 in situ hybridization (blue). Nuclei were counterstained with hematoxilin after immunostaining for Dab2. CYP11B2 and Dab2 are expressed in the principal nodule, whereas CYP11B1 is not. Megafoci are composed of ZG cells expressing Dab2 and CYP11B2 but not CYP11B1. APCC are composed of a mixed population of cells, expressing CYP11B2, but not Dab2 and CYP11B1. Bars 1000 µm (a-d), 200 µm (e-l).
Figure S3: Correlation of plasma potassium concentrations with urinary aldosterone concentrations and total CYP11B2 expression area. a, analysis of plasmatic potassium concentration and urinary aldosterone concentration shows an anti-correlation between these two parameters. b, positive correlation between urinary aldosterone concentration and APA CYP11B2 expression. c, positive correlation between plasma potassium concentration and peritumoral adjacent tissue CYP11B2 expression area.
Figure S4: Nodulation in control adrenal, peritumoral adjacent tissue and adjacent tissue of cortisol producing adenoma (CPA). Nodulation scores were determined in control adrenals, adrenal cortex adjacent to APA and adrenal cortex adjacent to CPA. A significant increase in nodulation, compared to control adrenals, was observed in adrenal cortex adjacent to APA but not to CPA.
Figure S5: MR expression in control and pathological tissue. MR immunohistochemistry (brown) performed on control adrenal (a and b), peritumoral adjacent tissue (c and d) and APA (e and f). Nuclei were counterstained with hematoxilin (blue). MR is expressed in the cortex of control adrenal and peritumoral adjacent tissue, as well as in APA.