Mast Cell Hyperplasia Is Associated With Aldosterone Hypersecretion in a Subset of Aldosterone-Producing Adenomas

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Context: Adrenal mast cells can stimulate aldosterone secretion through the local release of serotonin (5-HT) and activation of the 5-HT_4 receptor (5-HT_4). In aldosterone-producing adenomas (APAs), 5-HT_4 receptor is overexpressed and the administration of 5-HT_4 receptor agonists to patients with APA increases plasma aldosterone levels. These data and the well-documented role of mast cells in tumorigenesis suggest that mast cells may be involved in the pathophysiology of APA.

Objective: The study aimed at investigating the occurrence of mast cells in a series of APA tissues and to examine the influence of mast cells on aldosterone secretion.

Design: The occurrence of mast cells in APAs was investigated by immunohistochemistry. Mast cell densities were compared with clinical data. The influence of mast cells on aldosterone production was studied by using cultures of human mast cell and adrenocortical cell lines.

Results: In APA tissues, the density of mast cells was found to be increased in comparison with normal adrenals. Mast cells were primarily observed in adrenal cortex adjacent to adenomas or in the adenomas themselves, distinguishing two groups of APAs. A subset of adenomas was found to contain a high density of intratumoral mast cells, which was correlated with aldosterone synthase expression and in vivo aldosterone secretory parameters. Administration of conditioned medium from cultures of human mast cell lines to human adrenocortical cells induced a significant increase in aldosterone synthase (*CYP11B2*) mRNA expression and aldosterone production.

Conclusion: APA tissues commonly contain numerous mast cells that may influence aldosterone secretion through the local release of regulatory factors. (***J Clin Endocrinol Metab*** **100: E550–E560, 2015)**

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Abbreviations: ACAA, adrenal cortex adjacent to adenoma; APA, aldosterone-producing adenoma; ARR, aldosterone to renin ratio; CM, conditioned medium; 5-HT, serotonin; 5-HT_4R, 5-HT_4 receptor; LAD2-CM, LAD2-conditioned medium; LDL, low-density lipoprotein; MC, mast cell; NA, normal adrenal; PA, primary aldosteronism; SCF, stem cell growth factor; Star, steroidogenic acute regulatory protein; VLDL, very low-density lipoprotein.

Primary aldosteronism (PA) is the most common cause of secondary hypertension, its prevalence in hypertensive patients reaching up to 5%–10% (1). The two major causes of PA are aldosterone-producing adenomas (APAs) observed in 30%–40% of patients and bilateral idiopathic adrenal hyperplasia detected in 60%–70% of cases. The mechanisms involved in the maintenance of mineralocorticoid secretion despite down-regulation of the renin-angiotensin system by aldosterone excess have long remained unclear. Recent publications have shown that APA tissues frequently harbor somatic mutations of genes encoding the potassium channel KCNJ5 (2, 3), L-type calcium channel (4), and sodium/potassium and calcium ATPases (5), which may play a role in cell proliferation and aldosterone hypersecretion through calcium influx in adrenocortical cells. However, the real impact of somatic mutations on aldosterone production by APA is still debated (6).

On the other hand, numerous studies, mainly performed in vitro, have shown that aldosterone secretion is controlled by intraadrenal regulatory factors that are released in the immediate vicinity of zona glomerulosa cells by chromaffin cells, endothelial cells, nerve terminals, and cells of the immune system (7). In particular, perivascular mast cells (MCs), which are present in the subcapsular region of the human adrenal cortex, are able to stimulate aldosterone production through local release of serotonin (5-HT) and activation of the 5-HT_4 receptor (5-HT_4R) expressed by zona glomerulosa cells (8). The presence of MCs has also been occasionally observed in adrenocortical tumors, including benign and malignant neoplasms (9, 10). In addition, APAs have been shown to overexpress the 5-HT_4R (11–13), and the administration of 5-HT_4R agonists to patients with APA induces an exaggerated aldosterone secretory response in comparison with healthy volunteers (14, 15). It is thus conceivable that MCs may be involved in the pathogenesis of APA. In support of this hypothesis, it is now well established that MCs, which are frequently observed in tumor-associated immune cell infiltrates, play a pivotal role in tissue remodeling and tumor growth through the release of a wide variety of potent angiogenic signals and growth factors (16, 17). In addition, it has been shown in several animal tumor models, including endocrine neoplasms, that the recruitment and activation of MCs by tumor cells is essential for tumor expansion (16, 17).

The aim of the present study was therefore to investigate the occurrence of MCs in a series of APA tissues and to search for correlations between MC density, biological data, and expression of *CYP11B2*-encoding aldosterone synthase. We have also examined the influence of MCs on aldosterone secretion using cultures of human MCs and adrenocortical cell lines.

Subjects and Methods

Patients

Twenty-nine patients with primary aldosteronism were studied. Detection of PA and diagnosis of unilateral APA were in accordance with international guidelines. Differentiation between unilateral and bilateral aldosterone hypersecretion was assessed using thin-section computed tomography scan and/or adrenal venous sampling. Selectivity index for successful adrenal venous sampling (adrenal vein/inferior vena cava cortisol gradient) and lateralization index (aldosterone to cortisol ratio from high to low side) were, respectively, greater than 2 and greater than 4. The clinical, biological and genetic characteristics of the patients are displayed in Supplemental Tables 1 and 2. Nine and six of 19 patients investigated were carriers of somatic *KCNJ5* and *CACNA1D* mutations, respectively. No patient had *ATP1A1* or *ATP2B3* mutation.

Tissue collection

A retrospective series of formalin-fixed and paraffin-embedded adrenals from patients with PA (n = 29) was investigated by immunohistochemistry. The tissues were provided by the French network for the study of adrenocortical tumors (Cortico et Medullosurrénale Tumeurs Endocrines). Normal adrenal (NA) tissues were obtained from patients undergoing nephroadrenalectomy for kidney cancer (n = 16) and brain-dead organ donors (n = 6). Control tissues also included a mastocytoma. Transcriptomic studies were performed in 48 APA tissues. The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committees and the French Agence Nationale de Biomédecine. Written informed consent or consent from patients' relatives was obtained for all subjects.

Immunohistochemical studies

Deparaffinized and rehydrated sections of APA and NA tissues were incubated with different primary antibodies (Supplemental Table 3) and Envision System or Envision G/2 Doublestain System revelation kits (Dako Corp). For the detailed procedure, see the Supplemental Materials and Methods section.

In situ hybridization coupled to immunohistochemistry experiments

In situ hybridization analyses for *CYP11B2* detection coupled to tryptase immunostaining were conducted by using antisense and sense probes specific to the 3′-untranslated region of *CYP11B2* (nucleotides 2727-2931 of *CYP11B2*, GenBank accession number NM_000498), as indicated in the Supplemental Materials and Methods section.

Transcriptome analysis

Transcriptome data from 48 APAs and 11 control adrenal cortices were generated as previously described (3) and analyzed for expression of approximately 22 000 MC transcripts identified by Dahl et al (18). To this end, data were normalized and quality controlled and then subjected to statistical testing using

the Concomitant evaluation of Distinctness and Similarity (CDS) test (19–21).

Cell cultures

The human immature leukemic MC line HMC-1 (22), the MC line LAD2 (23), the adrenocortical cell line NCI-H295R (H295R), and normal adrenocortical cells were cultured as indicated in the Supplemental Materials and Methods section. Degranulation of LAD2 cells was induced by incubating the cells with 20 μg/mL compound 48/80 (Sigma-Aldrich). Incubation experiments of H295R or normal adrenocortical cells were conducted in quadruplicate for 24 hours in culture with MC culture medium and HMC1- or LAD2-conditioned medium (LAD2-CM). The effect of LAD2-CM was similarly tested in the presence of a mixture of 5-HT G protein-coupled receptor antagonists including methiothepine (5-$HT_{1/2/5/6/7}$), methysergide (5-$HT_{1/2}$), GR113808 (5-HT_4), SB258585 (5-HT_6), and SB269970 (5-HT_7) (Sigma-Aldrich), all used at 10^{-7} M. Angiotensin II (Sigma-Aldrich) was used as positive control.

Real-time RT-PCR analysis

Total RNAs extraction from H295R cells and reverse transcriptions were performed as previously described (24). Amplification of cDNA was performed using specific primers (Supplemental Table 4). Quantification of cDNAs was normalized to cyclophilin cDNAs by using the $2^{-\delta\delta Ct}$ method. Samples were analyzed in duplicates in at least three independent experiments.

Statistical analyses

Statistical analyses were performed as described in the Supplemental Materials and Methods section.

Results

Immunohistochemical characterization and localization of mast cells in APA tissues

Immunohistochemistry for tryptase and chymase, two specific markers of MCs, was performed on adrenal tissues from 29 patients with APA and seven NA. Small tryptase-immunopositive cells were detected between clusters of steroidogenic cells in the subcapsular region of all seven NA and 26 APAs (Figure 1, A and B). No tryptase-immunopositive cells could be observed in the tissues removed from patients 3, 5, and 23 (10%). Similarly, no chymase immunoreactivity could be detected in NA and APA tissues (Figure 1, C and D). By contrast, intense chymase immunostaining was present in mastocytoma (positive control; Figure 1E). Tryptase-positive MCs were localized in the adrenal cortex adjacent to adenomas (ACAA) and/or adenoma tissues themselves, near CD34-positive blood microvessels (Figure 1, F and G).

Innervation of mast cells in APAs

Protein S100-positive nerve fibers were detected in ACAAs, especially under the capsule and at the periphery of adenomas (Figure 1, H and I). Conversely, adenoma tissues exhibited a low innervation score ($\leq$1) in the great majority of APAs (27 of 29). The total innervation scores of the subcapsular and inner cortex of ACAAs were similar to NA [6 (5–7) vs 5 (4–7); $P = .43$]. Double immunostaining of APA tissues with tryptase and protein S100 antibodies, occasionally revealed intimate contacts between MCs and nerve terms (Figure 1J).

Mast cell gene expression profiling in APA and NA

Transcriptome analyses of genes known to be expressed by MCs discriminated the APA from NA tissues using unsupervised, hierarchical clustering (Figure 2A). The 60 most differentially expressed genes between APAs and NA are shown in Supplemental Table 5. The 30 most up-regulated genes included those encoding vascular endothelial growth factor (*VEGF*), histidine decarboxylase (*HDC*), histamine N-methyl-transferase (*HNMT*), and diamine oxidase (*DAO*), three enzymes responsible for histamine metabolism (Figure 2B). Both MC immunoreceptor signal transducer (*MIST*) and sialic acid binding Ig-like lectin 6 (*SIGLEC6*) genes, which encode proteins involved in MC degranulation, were up-regulated in APAs. Various genes encoding membrane receptors for bioactive peptides, such as calcitonin gene-related peptide type 1 receptor (*CALCRL*), calcitonin receptor (*CALCR*), and the tachykinin receptors 2 and 1 (*TACR2* and *TACR1*), which are known to regulate MC activity, were overexpressed in APAs. Similarly, *KITLG*, which encodes Kit ligand, also named mast/stem cell growth factor (SCF), *i.e.* the growth factor responsible for activation and proliferation of MCs, was up-regulated in APAs.

Localization of the mast SCF and its receptor in APA and NA

Immunohistochemical studies using antibodies against the mast/SCF kit ligand revealed diffuse and heterogeneous staining of APA tissues, whereas, like MCs, SCF immunoreactivity is mainly detected in the zona glomerulosa of the NA (Figure 2, C and D). Labeling of adrenal tissues with antibodies to CD117, the SCF receptor also known as c-kit, revealed the presence of small immunoreactive cells located near steroidogenic cells in NA and APA tissues (Figure 2, E and F).

Distribution and density of mast cells in APA

Quantitative analysis of tryptase-positive cells in NA and APAs revealed that global density of MCs was higher in APA [159 MC per square centimeter (interquartile range 60–346); n = 29] than in normal tissues [52 MCs per square centimeter (interquartile range 14–118); n = 7; $P = .03$; Figure 2G]. The distribution of tryptase-immunoreactive cells was heterogeneous among APA tissues

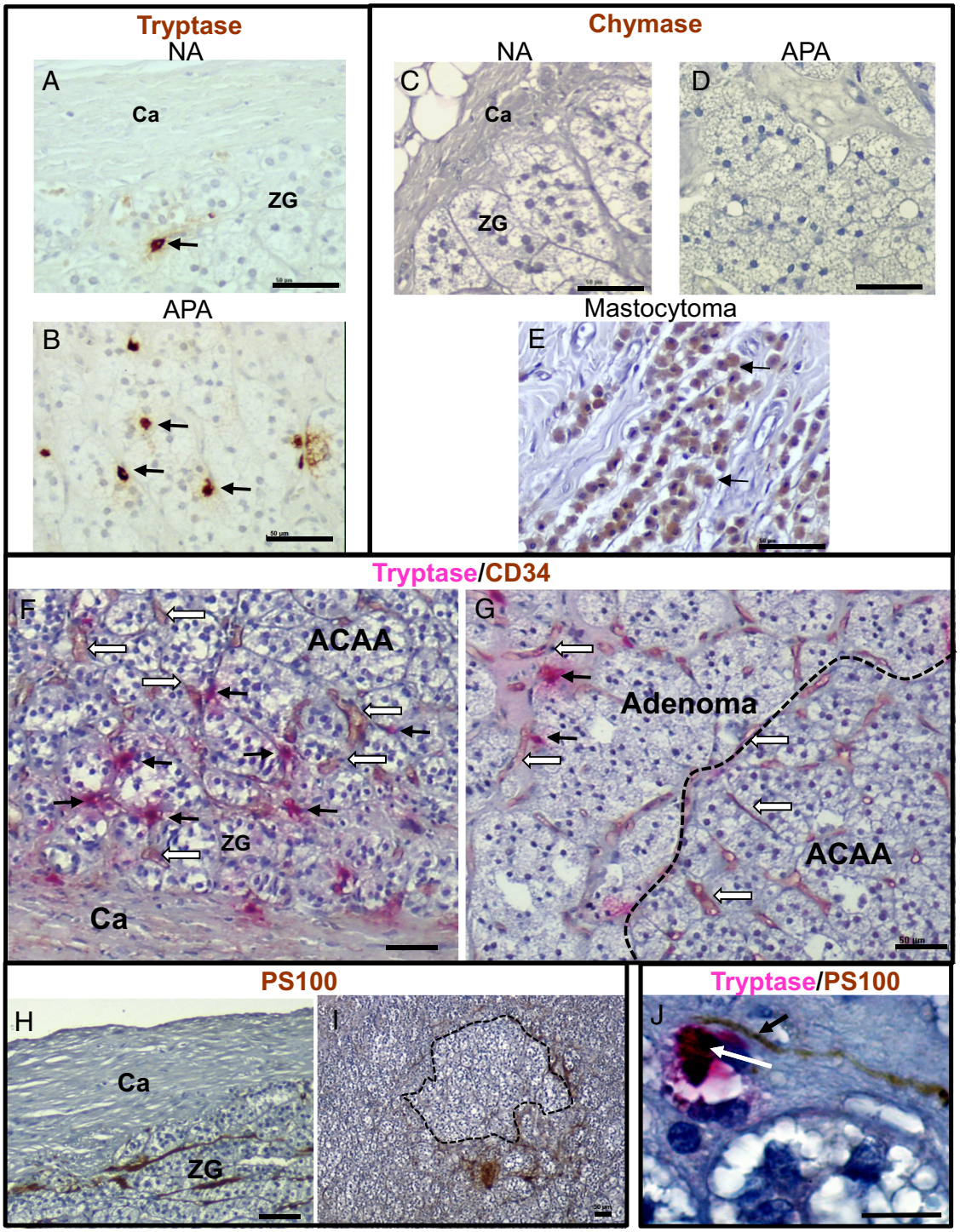
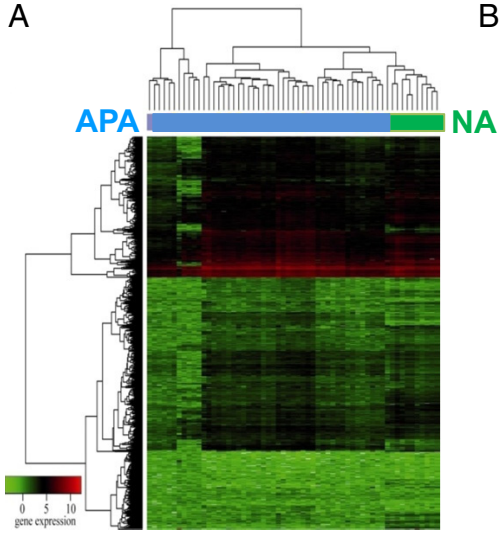


Figure 1. Immunohistochemical characterization of mast cells in APA tissues. A and B, Tryptase-immunopositive cells in NA (A) and APAs (B). Arrows show positive mast cells. C and D, Absence of chymase immunoreactivity in NA (C) and APA (D). E, Visualization of chymase immunoreactivity in a mastocytoma tissue (positive control). Arrows show positive mast cells. F and G, Distribution of tryptase-positive MC (pink; black arrows) and CD34-positive vessels (brown; white arrows) in the subcapsular region of ACAA (F) and in adenoma tissue (G) revealed by double labeling. H and I, Protein S100-positive nerve fibers were essentially detected in ACAA (H) or surrounding adenoma (I). J, Intimate contact between a mast cell (pink; white arrow) and a nerve fiber (brown, black arrow) in APA tissue revealed by double labeling. Ca, capsule; ZG, zona glomerulosa. Scale bars, 50 μm.

both in ACAA and adenomas (Figure 3, A–D). The role of MC in tumorigenesis has been found to depend on their localization, either intra- or peritumoral (16). Intratumoral mast cell densities appeared as a continuous variable among APAs (Figure 3E). In contrast, peritumoral mast cell density was less variable among patients (Figure 3E). Analysis of the distribution of MC density ratio revealed that, in 18 tissues (62%), the density of MC was higher in ACAA than in the adenoma tissues, whereas, in eight APAs (28%), MCs were mainly localized in the ad-



B

30 up-regulated genes	
VEGF	*C1QBP*
HIST1H2BG	*BHLHB2*
NR1D1	*MTHFD1*
MNAB	*HEY1*
CALCRL	*WHSC1*
RAP1GDS1	*TACR1*
GALC	*KHDRBS1*
MIST	*KIF23*
POU6F1	*HDC*
SIGLEC6	*SMYD3*
CALCR	*RPL2*
SUPT6H	*DAO*
TACR2	*HS3ST1*
LOH11CR2A	*KITLG*
HNMT	*PTP4A1*

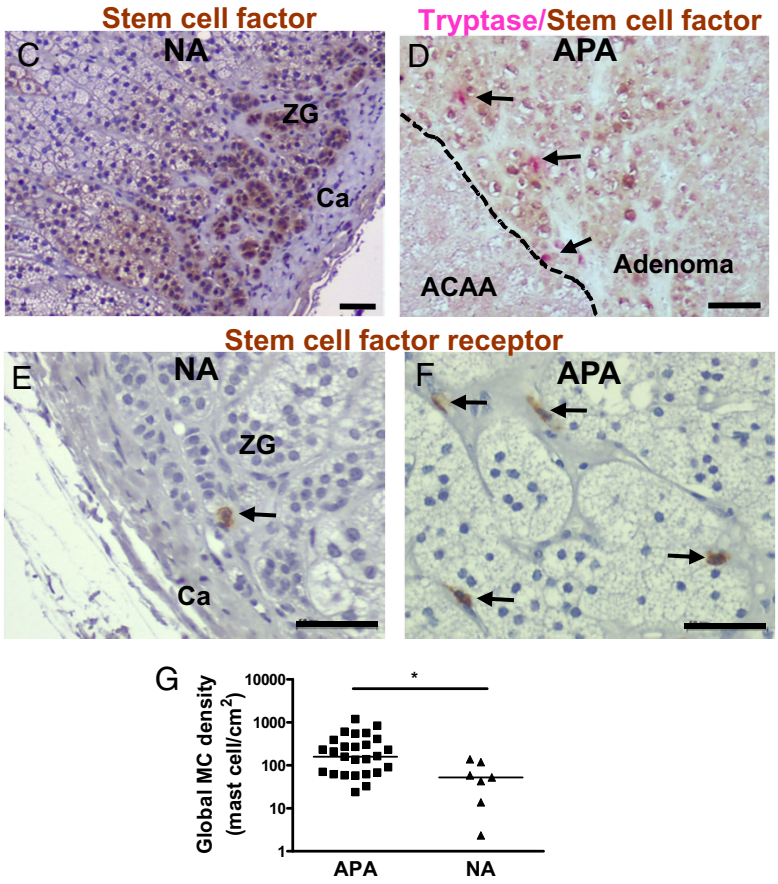


Figure 2. Mast cell gene expression profiling and global mast cell density in APA and NA. A, Transcriptome analysis of genes known to be expressed by mast cells in 48 APA (blue) and 11 NA (green) allowed discrimination of the two types of adrenal tissues using unsupervised clustering. B, List of 30 up-regulated genes in APA vs NA. C and D, SCF (Kit ligand) immunoreactivity (brown) in glomerulosa cells of the NA (C) and adenoma cells near tryptase-positive MC (pink; arrows) in APA tissue (D), revealed by simple (C) or double labeling (D), respectively. E and F, SCF receptor (CD117) immunoreactivity present in some cells in the zona glomerulosa of the NA (E) and between clusters of steroidogenic cells in the adenoma of APA tissue (F). G, Comparison of global mast cell densities in APA vs NA ($P = .03$). *, $P < .05$; Ca, capsule; ZG, zona glomerulosa. Scale bars, 50 μm.

enoma (Figure 3, E and F). The 26 APA tissues, in which MCs were visualized, were thus classified according to their peritumoral to intratumoral MC density ratio into two subgroups corresponding to IN (ratio < 1) and OUT (ratio > 1), respectively (Figure 3F). *KCNJ5*- or *CACNA1D*-mutated APAs were distributed in the two subgroups. The global densities of MCs in APAs of the two subgroups were significantly higher than that in the seven NA studied (Figure 3G). In addition, *CACNA1D* mutated adenomas displayed higher MC density than non-*CACNA1D*-mutated tumors (Supplemental Figure 1). Accordingly, none of the three APAs devoid of mast cells (patients 3, 5, and 23) were mutated for *CACNA1D*.

Analysis of the clinical and biological profiles of the two subgroups of APA

Patients with APAs of the IN group were characterized by a lower female to male sex ratio and tumor size than those with APAs of the OUT group (Table 1). Adenoma size was positively correlated with plasma aldosterone levels and aldosterone to renin ratio (ARR) in all patients with APA as well as in those of the OUT group (Supplemental Figure 2). Although adenoma sizes were different between the two subgroups, plasma aldosterone concentrations and ARR were not significantly distinct. These data suggest that the capacity of the tissues to produce aldosterone was stronger in patients from the IN group than those of the OUT group. We have thus looked for possible correlations between MC densities, *i.e.* global, intraadenomatous or peritumoral densities, and hormone parameters (Figure 4, A–F). An analysis of data obtained from patients with APAs of the IN group revealed positive correlations between upright plasma aldosterone and global MC density ($r = 0.93$; $P = .002$) or

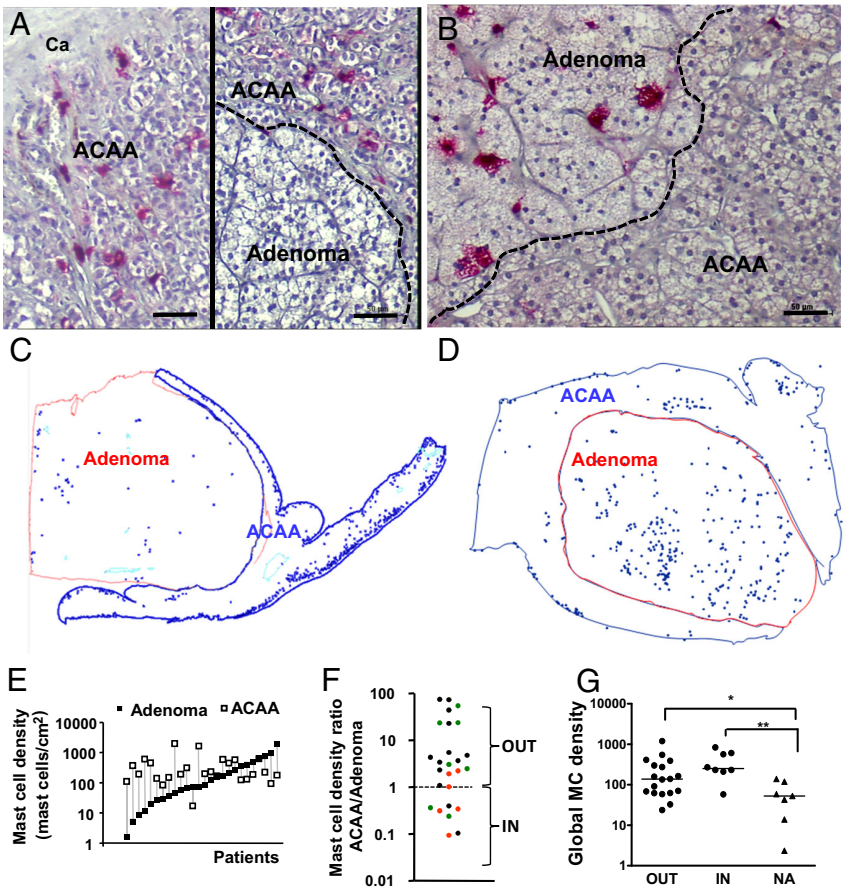


Figure 3. Distribution and density of mast cells in APA subgroups. A and B, Illustrative photographs showing the different distribution profiles of tryptase-positive mast cells. A, In some adenoma specimens, mast cells were essentially located in the ACAA, especially under the capsule (Ca; left) and in the area just surrounding adenoma (right). B, In other adenoma tissues, most mast cells were seen in the adenoma itself. Scale bars, 50 μm. C and D, Example of cartographies used to quantify mast cells density in adenoma (red line) and ACAA (blue line). Blue points represent tryptase-positive mast cells. E, Respective mast cell densities in adenoma (■) and ACAA (□) in APA tissues from the 26 patients. F, Distribution of peritumoral to intratumoral mast cell density ratio values among APA tissues. Two subgroups of APAs were distinguished, *i.e.* the IN group (ratio < 1) and OUT group (ratio > 1). APAs with *KCNJ5* (green) and *CACNA1D* (red) somatic mutations. G, Comparison of global mast cells densities in the two subgroups of APA (OUT and IN) vs NA. *, $P < .05$; **, $P < .01$.

intraadenomatous MC density (r = 0.97; $P = .0002$; Figure 4, A and B). We also observed that the upright plasma ARR was positively correlated to adenoma MC density (r = 0.91; $P = .004$; Figure 4C). Conversely, no correlation could be observed in patients with the APAs of the OUT group after an analysis of the same biological parameters (Figure 4, D–F). However, the upright plasma aldosterone to adenoma size ratio is positively correlated with intraadenomatous MC density in the APAs of the OUT group (Supplemental Figure 3).

Localization of mast cells and *CYP11B2*-positive cells in APA and NA

Combination of immunohistochemistry for tryptase and *in situ* hybridization for *CYP11B2* revealed the presence of clusters of *CYP11B2*-expressing cells, identified as aldosterone-producing cell clusters (25, 26), close to mast cells in the subcapsular region of NA (Figure 4G).

In the APAs of the OUT group, *CYP11B2* expression, at both mRNA and protein levels, was localized both in adenoma and peritumoral tissues (Supplemental Figure 4). In adenoma, *CYP11B2*-positive cells were observed independently of the presence of mast cells, whereas they were organized as clusters intermingled with MC in the subcapsular region of peritumoral tissues (Figure 4H and Supplemental Figure 4).

In the APAs of the IN group, *CYP11B2* mRNA was detected in adenoma tissues near numerous MC (Figure 4I). In addition, aldosterone synthase immunoreactivity was exclusively observed in adenoma tissues (Figure 4, J–O). Quantitative analysis of aldosterone synthase immunoreactivity revealed that MC density in adenomas was correlated with the percentage of aldosterone synthase-immunopositive areas in this subgroup (Figure 4P).

Impact of mast cells on steroidogenic cells in APAs

Influence of MCs on the steroidogenic activity of adrenocortical cells was explored using the human MC lines, HMC1 and LAD2. Incubation of the human adrenocortical cell line H295R with conditioned medium (CM) from HMC1 or LAD2 cell cultures induced significant increases in aldosterone production (+110% ± 9%; $P < .0001$ and +131% ± 24%; $P < .0001$, respectively; Figure 5, A and B). Similarly, application of LAD2-CM to normal adrenocortical cells stimulated aldosterone secretion (+38% ± 14%; $P = .008$; Figure 5C). When LAD2 cells were preincubated with the MC degranulator, compound 48/80 (20 μg/mL), the stimulatory effect of LAD2-CM on aldosterone production by H295R cells increased up to +467% ± 125% ($P = .0003$; Figure 5B). H295R cells and adrenocortical carcinoma tissues are known to express various 5-HT receptor types that are involved in the control of steroidogenesis (10). We have thus investigated the effect of a cocktail of 5-HT receptor antagonists on the H295R

Table 1. Clinical and Biological Characteristics of Patients in the OUT and IN Subgroups of APA

Variable	Group IN	Group OUT	*P* Value
Patients, n	8	18	
Age, y	45 [36–53.5]	43 [36–49.5]	.69
Body mass index, kg/m^2	26.4 [24.1–31.05]	24.6 [22.3–28.2]	.33
Gender (female/male)	2/6	12/6	.049
Systolic blood pressure, mm Hg (n < 140)	154.5 [143.5–173.5]	165 [141–175.5]	.78
Diastolic blood pressure, mm Hg (n < 90)	89.5 [84.5–104]	98 [83.5–112]	.49
Duration of hypertension, y	8 [1.5–15]	4 [2–12]	.76
Serum K^+ level, mmol/L (n > 3.7)	3.25 [3–3.5]	3.0 [2.9–3.4]	.24
Tumor size, mm	10 [7.5–14]	17 [12.5–20]	.0076
Aldosterone supine, pmol/L (n < 500)	677.5 [444.5–1003]	1043 [501–1478]	.23
ARR supine, pmol/mU (n < 64)	121.5 [89–170]	190 [123.5–242.5]	.13
Aldosterone upright, pmol/L	972 [735–1431]	999 [687.5–2024]	1.00
ARR upright (pmol/mU)	176.7 [140.8–269.2]	215.7 [135.8–371.4]	.57
KCNJ5 mutation (yes/no)	2/5	5/4	.28
CACNA1D mutation (yes/no)	4/3	2/7	.15
Global mast cell density	251.4 [221.9–587.6]	137.3 [62.4–346]	.11
Mast cell density in ACAA	150 [105.9–199.9]	264.2[155.5–567.6]	.07
Mast cell density in adenoma	551.9 [364.4–818.3]	50.6 [15.1–138.1]	.0003

Data are median [25th to 75th percentiles]. Group IN, peritumoral to intratumoral MC density ratio is less than 1; group OUT ratio is greater than 1.

aldosterone response to LAD2-CM. We observed that LAD2-CM-induced aldosterone production was reduced to +33% ± 15% ($P = .0005$) by the mixture of 5-HT receptor antagonists.

In addition, LAD2-CM increased expression of mRNAs encoding the scavenger receptor B1 (SRB1), the high density lipoprotein cholesterol receptor, the low-density lipoprotein (LDL) cholesterol receptor, the steroidogenic acute regulatory protein (Star), the transcription factor Nurr1A, and aldosterone synthase in H295R cells. Conversely, LAD2-CM had no influence on expression of the very low-density lipoprotein (VLDL) receptor (Figure 5E).

Discussion

It has been shown in recent years that subsets of APA harbor somatic mutations affecting the *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACN1D* genes, resulting in the activation of the calcium signaling pathway, which is a major stimulator of aldosterone synthase expression and consequently aldosterone secretion (2–4). It is thus considered that the increased secretory activity of APA results in many cases from these somatic genetic events. However, recent data obtained in a large cohort of 474 APAs showed no correlation between the *KCNJ5* and *CACN1D* mutational status and *CYP11B2* expression or preoperative aldosterone levels (27). It seems therefore that an additional pathophysiological mechanism, possibly favored by the somatic mutations, is involved in aldosterone hypersecretion. In the NA, aldosterone secretion by glomerulosa cells is stimulated by subcapsular mast cells through local release of 5-HT and activation of the 5-HT_4 receptor (12). On the other hand, it is now well demonstrated that expansion of tumor clones requests close interactions with MC, even when tumor development is genetically determined (28). In the present study, we report an increased density of MC in APA tissues and show, by use of immunohistochemistry, that adrenal mast cells belong to the MC_T type, *i.e.* being tryptase positive and chymase negative. Abundance of MC in APAs, which was also noticed in one sample of bilateral zona glomerulosa hyperplasia (data not shown) but not in cortisol-producing adenomas (9), suggests that MC may be involved in the control of glomerulosal cell differentiation and/or aldosterone biosynthesis. However, the absence of MC in three tumors of our series indicates that this intercellular communication process is not mandatory for APA development and further illustrates the heterogeneity of the disease.

In agreement with immunohistochemical data, transcriptome analysis of genes known to be expressed by MC allowed the discrimination of APA from NA tissues by the use of unsupervised clustering. The most differentially overexpressed genes included the angiogenic factor VEGF gene, consistently with our finding that MC were visualized close to CD34-positive blood microvessels in both APA and ACAA tissues, genes encoding proteins involved in the control of MC degranulation (*MIST*, *SIGLEC6*), enzymes responsible for histamine synthesis (*HDC*), and degradation (*HNMT*, *DAO*) and neuropeptide membrane receptors known to control the activity of MC, such as tachykinin and calcitonin gene-related peptide receptors. As in other organs, some connections between peritumoral MC and protein S100-positive adrenal nerve fibers could be occasionally seen, but these observations were too rare to support the possibility that the sympa-

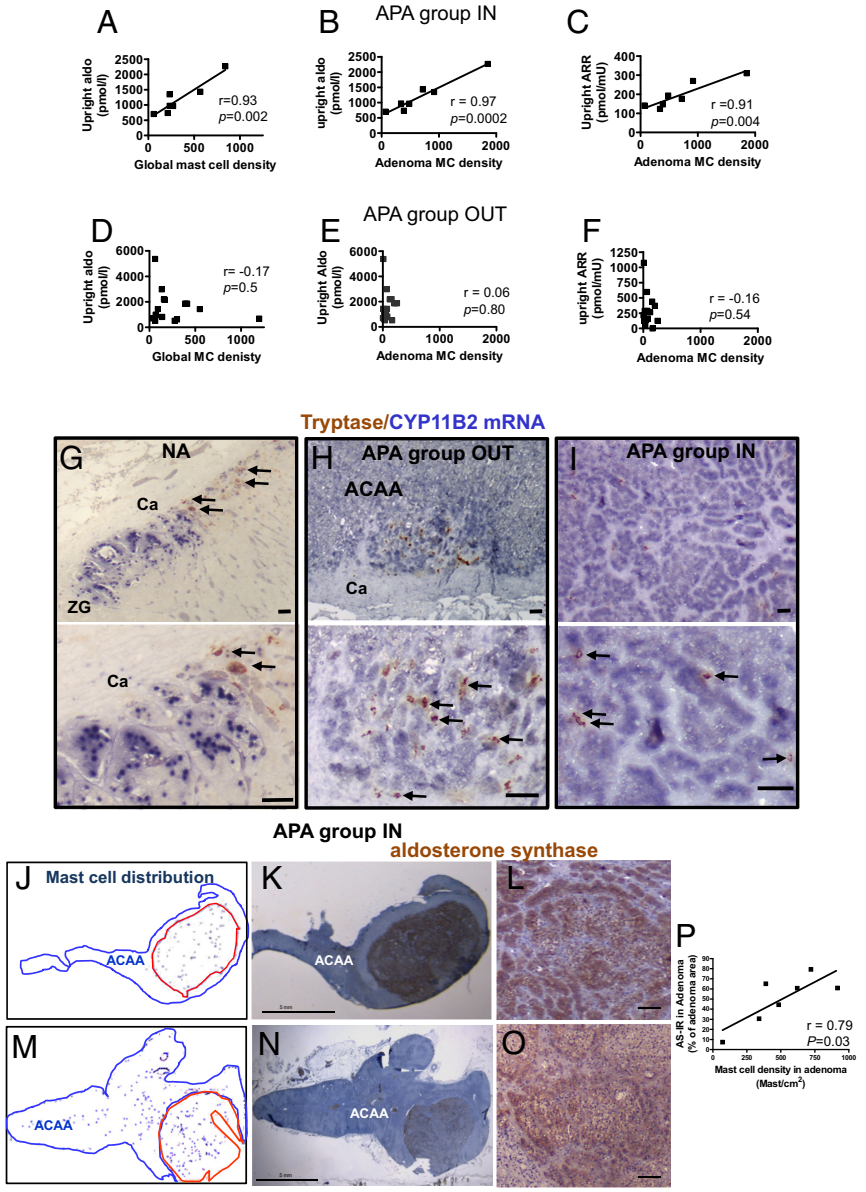


Figure 4. Analysis of aldosterone production in patients of the IN and OUT groups of APA. A–F, Correlations between upright plasma aldosterone (A, B, D, and E) and upright ARR (C and F) with global density of mast cells (A and D) or density of mast cells in adenoma tissue (B, C, E, and F) in the IN (A–C) and OUT (D–F) groups of APAs. r, Pearson coefficient. G–I, Localization of *CYP11B2* mRNA and mast cells in APA and NA. *CYP11B2 in situ* hybridization (blue) and tryptase-positive MC (brown; arrows) in NA (G) and APA of the OUT (H) and IN (I) groups at low (upper panel) and high (lower panel) magnifications. Ca, capsule; ZG, zona glomerulosa. Scale bars, 50 μm. J–O, Localization of aldosterone synthase and mast cells in APA of the IN group. Cartographies of mast cells (blue points) in the ACAA (blue line) and adenoma tissue (red line) of two APAs of the IN group (J and M). Aldosterone synthase immunoreactivity (brown) in the adenoma tissues of the same patients at low (K and N) and high (L and O) magnifications. Hematoxylin staining is shown in blue. Scale bars, 5 mm (K and N) and 100 μm (L and O). P, Correlation between intraadenomatous MC densities and AS-immunoreactive surfaces, expressed as percentages of total adenoma surfaces in APA of the IN group.AS, aldosterone synthase r, Pearson coefficient

thetic system may influence the activity of a subset of APAs through involvement of resident MC.

APAs of the OUT group, which represent the main subpopulation of adenomas in our tumor cohort, were larger than those of the IN group (29, 30), suggesting that peritumoral MC, by stimulating neoangiogenesis and tissue remodeling, may be more potent to promote tumor expansion than intratumoral MC, as shown in previous studies (31). In addition, the positive correlation between tumor diameter and upright plasma aldosterone or ARR indicates that adenoma size is an important determinant of aldosterone production in APA patients, as previously reported (32). However, despite their larger size, adenomas of the OUT group were not associated with higher plasma hormone levels in comparison with APAs of the IN group. This apparent discrepancy may result from differences in the capacity of tumor tissues to produce mineralocorticoid between the two subgroups. In fact, cells of adenomas of the IN group may exhibit more active steroidogenesis than cells of OUT group tumors. We have thus investigated *CYP11B2* expression in relation to the presence of MC in APA tissues.

As in previously published studies, we observed two patterns of distribution of *CYP11B2* mRNA and aldosterone synthase immunoreactivity (26). In APAs of the IN group, the two signals were exclusively detected in the tumor tissues, in adrenocortical cells located near mast cells. In addition, adenoma MC density was strongly correlated with aldosterone synthase immunoreactivity, and upright plasma aldosterone and aldosterone to renin ratio levels, indicating that intratumoral MC may stimulate aldosterone secretion by adenoma cells through a paracrine mechanism in this subgroup.

By contrast, in the APA tissues of the OUT group, *CYP11B2* transcripts and aldosterone synthase immunoreactivity were present in both adenoma and peritumoral tissue as previously reported (33, 34). Aldosterone synthase expression was not associated with the presence of MC in the tumor tissues and plasma aldosterone and aldosterone to renin ratio were not correlated to intratumoral MC density, indicating that the expression of intratumoral aldosterone

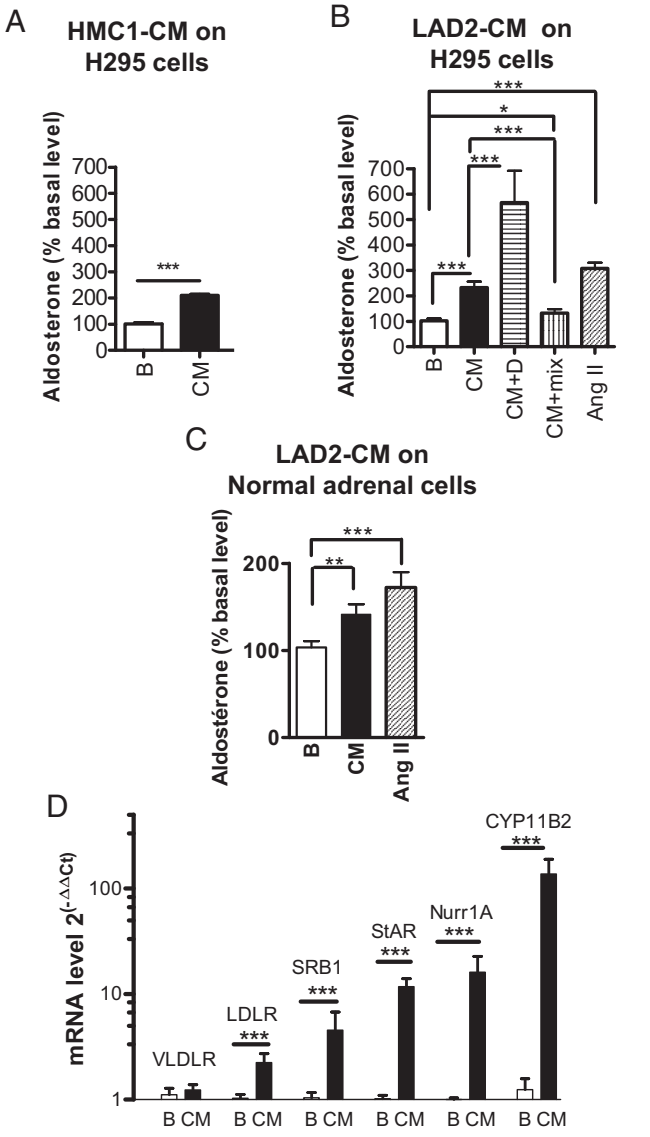


Figure 5. Mast cell impact on steroidogenic cells. A–C, Incubation of H295R cells (A and B) or normal adrenal cells (C) with CM from cultured HMC1 (A) or LAD2 (B and C) cell lines induced significant increases in aldosterone production. Stimulatory effect of LAD2-CM on H295R cells was potentiated by the preincubation of LAD2 cells during 30 minutes with the mast cell degranulator compound 48/80 (CM+D) but reduced by coapplication with the mixture (CM+mix) of 5-HT receptor antagonists methiothepine (10^{-7} M), methysergide (10^{-7} M), GR113808 (10^{-7} M), and SB269970 (10^{-7} M) (D). Angiotensin II (Ang II; 10^{-6} M) was used as a positive control. B, Basal level corresponds to 11.7 ± 0.8 pg per 100 μL (A), 12.32 ± 1.38 pg per 100μL (B) and 48.1 ± 13.9 pg per 10 0μL (C) normalized to 100%. D, Influence of LAD2-CM compared with the basal level (B) on the expression of mRNAs encoding VLDL receptor (VLDLR), LDL receptor (LDLR), scavenger receptor B1 (SRB1), steroidogenic acute regulatory protein (StAR), nuclear receptor related 1 protein (Nurr1A), and aldosterone synthase in H295R adrenocortical cells. Aldosterone secretion and gene expression by H295 cells are expressed as mean ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

synthase is not affected by MC in adenomas of the OUT group. Conversely, clusters of aldosterone synthase-positive cells intermingled with MC were observed in the zona glomerulosa of the peritumoral tissues, as previously noticed in NA. This finding suggests a role of MC in enhancing aldosterone synthesis in the peritumoral tissues. In fact, MC may stimulate aldosterone secretion by steroidogenic cells through a paracrine mechanism similar to the physiological process formerly described in the zona glomerulosa of the NA (8).

These data prompted us to investigate the functional interactions between MC and adrenocortical cells. Our experiments conducted on the human mast cell and H295R adrenocortical cell lines show that MC release secretory signals, including 5-HT, which are able to stimulate aldosterone secretion. The activation of aldosterone production by MC line-conditioned medium involves an increase in expression of transcripts for scavenger receptor B1 and LDL-R, which play an important role in the cholesterol supply to adrenocortical cells, the mitochondrial cholesterol transporter Star, Nurr1, and *CYP11B2*. The elevation of *CYP11B2* expression is likely to be the result of the increase in Nurr1 expression, a transcriptional factor that is known to up-regulate the synthesis of aldosterone synthase mRNA (35, 36). Conversely, the expression of the receptor for VLDL, which is also a significant supplier of cholesterol for adrenocortical cells (37), is not influenced by MC secretory products.

Collectively these data show, in some APA tissues, a paracrine amplification of aldosterone synthesis by MC resulting from MC proliferation. It is conceivable that MC proliferation may represent a pathophysiological link between the previously reported somatic mutations and both aldosterone synthase overexpression and aldosterone hypersecretion. In agreement with this hypothesis, *CACNA1D*-mutated tissues, which are found at a relatively high frequency in our cohort [about 31% vs 10% in a large European cohort of tumors (28)], harbored a higher density of MC than non-*CACNA1D*-mutated adenomas. Consistently, the L-type calcium channel blocker nifedipine is known to decrease MC proliferation in some tissues (38). The way by which *CACNA1D* mutations may influence the proliferation of MC in APAs is unknown. It may involve SCF, which is known to play a major role in MC differentiation and proliferation (31). However, we did not find any significant correlation between SCF mRNA levels and MC densities (data not shown). It is thus possible that adenoma cells may influence MC proliferation through the release of other bioactive signals such as IL-8 or MCP-1, as shown in various tumors models (39, 40). It is also conceivable that aldosterone itself may activate MCs, which have been shown to express the mineralocorticoid receptor (41, 42). This process may thus lead to formation of an intraadrenal amplification loop, resulting in high aldosterone secretory levels.

In conclusion, the increased density of MC in some APAs, together with the recently reported somatic muta-

tions of *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D*, may play a role in the pathophysiology of aldosterone hypersecretion associated with Conn's adenomas and may be a promising target for future pharmacological treatment of primary aldosteronism. Characterization of the mechanisms by which somatic mutations may influence MC proliferation as well as the molecular signals involved in MC proliferation will necessitate additional investigations on large cohorts of tissues.

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References

1. **Hannemann A, Wallaschofski H.** Prevalence of primary aldosteronism in patient's cohorts and in population-based studies—a review of the current literature. *Horm Metab Res*. 2012;44(3):157–162.
2. **Choi M, Scholl UI, Yue P, et al.** K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011;331(6018):768–772.
3. **Boulkroun S, Beuschlein F, Rossi G-P, et al.** Prevalence, clinical, and molecular correlates of KCNJ5 mutations in primary aldosteronism. Hypertension 2012;59(3):592–598.
4. **Scholl UI, Goh G, Stölting G, et al.** Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet*. 2013;45(9):1050–1054.
5. **Beuschlein F, Boulkroun S, Osswald A, et al.** Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45(4):440–444, 444e1–2.
6. **Oβwald A, Fischer E, Degenhart C, et al.** Lack of influence of somatic mutations on steroid gradients during adrenal vein sampling in aldosterone-producing adenoma patients. *Eur J Endocrinol*. 2013;169(5):657–663.
7. **Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP.** Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocr Rev*. 1998;19(2):101–143.
8. **Lefebvre H, Compagnon P, Contesse V, et al.** Production and metabolism of serotonin (5-HT) by the human adrenal cortex: paracrine stimulation of aldosterone secretion by 5-HT. *J Clin Endocrinol Metab*. 2001;86(10):5001–5007.
9. **Aiba M, Iri H, Suzuki H, et al.** Numerous mast cells in an 11-deoxycorticosterone-producing adrenocortical tumor. Histologic evaluation of benignancy and comparison with mast cell distribution in adrenal glands and neoplastic counterparts of 67 surgical specimens. *Arch Pathol Lab Med*. 1985;109(4):357–360.
10. **Louiset E, Isvi K, Gasc JM, et al.** Ectopic expression of serotonin 7 receptors in an adrenocortical carcinoma co-secreting renin and cortisol. *Endocr Relat Cancer*. 2008;15(4):1025–1034.
11. **Cartier D, Jégou S, Parmentier F, et al.** Expression profile of serotonin4 (5-HT4) receptors in adrenocortical aldosterone-producing adenomas. *Eur J Endocrinol*. 2005;153(6):939–947.
12. **Lefebvre H, Cartier D, Duparc C, et al.** Characterization of serotonin(4) receptors in adrenocortical aldosterone-producing adenomas: in vivo and in vitro studies. *J Clin Endocrinol Metab*. 2002; 87(3):1211–1216.
13. **Ye P, Mariniello B, Mantero F, Shibata H, Rainey WE.** G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism. *J Endocrinol*. 2007;195(1):39–48.
14. **Lampron A, Bourdeau I, Oble S, et al.** Regulation of aldosterone secretion by several aberrant receptors including for glucose-dependent insulinotropic peptide in a patient with an aldosteronoma. *J Clin Endocrinol Metab*. 2009;94(3):750–756.
15. **Zwermann O, Suttmann Y, Bidlingmaier M, Beuschlein F, Reincke M.** Screening for membrane hormone receptor expression in primary aldosteronism. *Eur J Endocrinol*. 2009;160(3):443–451.
16. **Maltby S, Khazaie K, McNagny KM.** Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. *Biochim Biophys Acta*. 2009;1796(1):19–26.
17. **Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI.** Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med*. 2007;13(10):1211–1218.
18. **Dahl C, Saito H, Kruhøffer M, Schiøtz PO.** Identification of tryptase- and chymase-related gene clusters in human mast cells using microarrays. *Allergy*. 2006;61(3):276–280.
19. **Bécavin C, Tchitchek N, Mintsa-Eya C, Lesne A, Benecke A.** Improving the efficiency of multidimensional scaling in the analysis of high-dimensional data using singular value decomposition. *Bioinformatics*. 2011;27(10):1413–1421.
20. **Brysbaert G, Pellay F-X, Noth S, Benecke A.** Quality assessment of transcriptome data using intrinsic statistical properties. *Genomics Proteomics Bioinformatics*. 2010;8(1):57–71.
21. **Tchitchek N, Dzib JFG, Targat B, Noth S, Benecke A, Lesne A.** CDS: a fold-change based statistical test for concomitant identification of distinctness and similarity in gene expression analysis. *Genomics Proteomics Bioinformatics*. 2012;10(3):127–135.
22. **Butterfield JH, Weiler D, Dewald G, Gleich GJ.** Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res*. 1988;12(4):345–355.
23. **Kirshenbaum AS, Akin C, Wu Y, et al.** Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 es-

tablished from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res*. 2003;27(8):677–682.
24. **Louiset E, Duparc C, Young J, et al.** Intraadrenal corticotropin in bilateral macronodular adrenal hyperplasia. *N Engl J Med*. 2013;369(22):2115–2125.
25. **Boulkroun S, Samson-Couterie B, Dzib J-FG, et al.** Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. *Hypertension*. 2010;56(5):885–892.
26. **Nishimoto K, Nakagawa K, Li D, et al.** Adrenocortical zonation in humans under normal and pathological conditions. *J Clin Endocrinol Metab*. 2010;95(5):2296–2305.
27. **Fernandes-Rosa FL, Williams TA, Riester A, et al.** Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension*. 2014;64(2):354–361.
28. **Yang F-C, Ingram DA, Chen S, et al.** Nf1-dependent tumors require a microenvironment containing Nf1+/− and c-kit-dependent bone marrow. *Cell*. 2008;135(3):437–448.
29. **Amini R-M, Aaltonen K, Nevanlinna H, et al.** Mast cells and eosinophils in invasive breast carcinoma. *BMC Cancer*. 2007;7:165.
30. **Yano H, Kinuta M, Tateishi H, et al.** Mast cell infiltration around gastric cancer cells correlates with tumor angiogenesis and metastasis. *Gastric Cancer*. 1999;2(1):26–32.
31. **Marichal T, Tsai M, Galli SJ.** Mast cells: potential positive and negative roles in tumor biology. *Cancer Immunol. Res*. 2013;1(5):269–279.
32. **Ono Y, Nakamura Y, Maekawa T, et al.** Different expression of 11β-hydroxylase and aldosterone synthase between aldosterone-producing microadenomas and macroadenomas. *Hypertension*. 2014;64(2):438–444.
33. **Nanba K, Tsuiki M, Sawai K, et al.** Histopathological diagnosis of primary aldosteronism using CYP11B2 immunohistochemistry. *J Clin Endocrinol Metab*. 2013;98(4):1567–1574.
34. **Nakamura Y, Maekawa T, Felizola SJA, et al.** Adrenal CYP11B1/2 expression in primary aldosteronism: immunohistochemical analysis using novel monoclonal antibodies. *Mol Cell Endocrinol*. 2014;392(1–2):73–79.
35. **Bassett MH, White PC, Rainey WE.** A role for the NGFI-B family in adrenal zonation and adrenocortical disease. *Endocr Res*. 2004;30(4):567–574.
36. **Hattangady NG, Olala LO, Bollag WB, Rainey WE.** Acute and chronic regulation of aldosterone production. *Mol Cell Endocrinol*. 2012;350(2):151–162.
37. **Xing Y, Rainey WE, Apolzan JW, Francone OL, Harris RBS, Bollag WB.** Adrenal cell aldosterone production is stimulated by very low-density lipoprotein (VLDL). *Endocrinology*. 2012;153(2):721–731.
38. **Liu W, Shimada M, Xiao J, Hu D, Matsumori A.** Nifedipine inhibits the activation of inflammatory and immune reactions in viral myocarditis. *Life Sci*. 2009;85(5–6):235–240.
39. **Conti P, Castellani ML, Kempuraj D, et al.** Role of mast cells in tumor growth. *Ann Clin Lab Sci*. 2007;37(4):315–322.
40. **Halova I, Draberova L, Draber P.** Mast cell chemotaxis— chemoattractants and signaling pathways. *Front Immunol*. 2012;3:119.
41. **Schjerning J, Uhrenholt TR, Svenningsen P, et al.** Histamine-dependent prolongation by aldosterone of vasoconstriction in isolated small mesenteric arteries of the mouse. *Am J Physiol Heart Circ Physiol*. 2013;304(8):H1094–H1102.
42. **Xiao J, Shimada M, Liu W, Hu D, Matsumori A.** Anti-inflammatory effects of eplerenone on viral myocarditis. *Eur J Heart Fail*. 2009;11(4):349–353.