Online Supplement

The 18-oxocortisol synthesis in aldosterone-producing adrenocortical adenoma and significance of KCNJ5 mutation status

Short title: 18-oxocortisol and KCNJ5 mutation in APA

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Supplemental Methods

Measuement of tissue steroid levels

18-Oxocortisol (18-oxoF) measurement

Sample preparation

Adrenal tissue was homogenized in distilled water. As an internal standard, 18-oxoF-d_4 was added to a part of the adrenal tissue suspension. After the addition of 1 mL of distilled water, 18-oxoF was extracted with 4 mL of ethyl acetate, and the extract was evaporated to dryness, the residue was subjected to ethoxylation. The residue was reacted with 0.25 ml of hydrochloric acid-ethanol (1:5, v/v) at room temperature for 30 minutes. The ethoxylated mixture was treated with 1 ml of 0.6 M sodium acetate. Then the mixture was applied to adande: l PAX cartridge (Osaka Soda, Osaka, Japan) which had been continuously conditioned with methanol and distilled water. The cartridge was washed successively with 1 ml of distilled water, 1 ml of methanol-water-acetic acid (40:60:1, v/v/v) solution and 1% pyridine solution, and then 18-oxoF was eluted with 1 ml of methanol-water-pyridine (80:20:1, v/v/v) solution. After the eluate was evaporated to dryness, the residue was subjected to derivatization. The residue was reacted with 50 μl of derivatizing solution (80 mg of 2-methyl-6-nitorobenzoic anhydride, 20 mg of 4-dimethylaminopyridine, and 40 mg fusaric acid in 1 ml of acetonitrile) and 10 μl of triethylamine for 30 minutes at room temperature. The reaction mixture was diluted with 0.5 ml of hexane-ethyl acetate-acetic acid (35:15:1, v/v/v). The resulting mixture was applied to InertSepSI cartridge (GLsciences, Tokyo, Japan) which had been continuously conditioned with acetone and hexane. The cartridge was washed successively with 1 ml of hexane and 2 ml of hexane-ethyl acetate (35:65, v/v), and then derivatized 18-oxoF was eluted with 2.5 ml of hexane-acetone (1:1, v/v). After the eluate was evaporated to dryness, the residue was dissolved with 0.1 ml of 40% acetonitrile solution and then subjected to LC-MS/MS.

LC-MS/MS

For the measurement of 18-oxoF in adrenal tissue, an API-4000 triple stage quadrupole mass spectrometer (AB Sciex, Mass, USA) connected to LC-30AD, RACKCHANGER Ⅱ(Shimadzu, Kyoto, Japan), ESI ion source device was employed. As an analytical column, a Kinetex C18 (150 mm × 2.1 mm, 1.7 μm, Phenomenex, CA, USA) was used at 50˚. The mobile phase consisting of acetonitrile and 0.1% formic acid was used with a gradient elution at a flow rate of 0.5 ml/minutes. The following

ESI conditions were used: ion spray voltage, 5500 V; Collision Activated Dissociation, 4 psi; Curtain Gas Flow Rate, 15 psi; ion source temperature,450˚C
For quantification of 18-oxoF, transition of m/z 566.3/520.4 and 570.3/524.4 were selected for 18-oxoF and 18-oxoF-d_4, respectively.

Measurement of Aldosterone (Ald), Cortisol (F), Corticosterone (B) and 11-Deoxycortisol (11-DOF)

Sample preparation

As internal standards, Ald-d_7, F-d_4, B-d_4 and 11-DOF-d_5 were added to a part of the adrenal tissue suspension. After steroids were extracted with 4 mL of t-butyl methyl ether, and the extract was evaporated to dryness, the residue was dissolved in 0.5 ml of methanol and diluted with 1 ml of distilled water. The sample was applied to Oasis MAX (Waters, Mass, USA) cartridge which had been successively conditioned with 3 ml of methanol and 3 ml of distilled water. The cartridge was washed with 1 mL of 1% acetic acid and 1 ml of methanol-water (45:55, v/v) solution, and then steroids were eluted with 1 ml of methanol. After evaporation, the sample was treated with 0.25 ml of hydrochloric acid-ethanol (1:5, v/v) at room temperature for 30 minutes. The reaction mixture was neutralized with 1 mL of 5% sodium hydrogen carbonate solution and was extracted by 4 mL of t-butyl methyl ether. After the extract was evaporated to dryness, the sample was reacted with 50 µl of the mixed solution (40 mg of 2-methyl-6-nitrobenzoic anhydride, 20 mg of 4-dimethylaminopyridine and 40 mg of picolinic acid in 1 ml of acetonitrile) and 10 µl of triethylamine for 30 minutes at room temperature. After the reaction, the sample was diluted with 0.5 ml of hexane-ethyl acetate-acetic acid (35:15:1, v/v/v) and then the sample was applied to InertSep SI cartridge which had been successively conditioned with 3 ml of acetone and 3 ml of hexane. After the cartridge was washed with 1 ml of hexane and 2 ml of ethyl acetate-hexane (3:7, v/v), the sample was eluted with 2.5 ml of acetone-hexane (7:3, v/v). After the eluate was evaporated to dryness, the residue was dissolved with 0.1 ml of 40% acetonitrile solution and then subjected to LC-MS/MS.

LC-MS/MS

For the measurement of Ald, F, B and 11-DOF in adrenal tissue, an API-4000 triple stage quadrupole mass spectrometer connected to LC-30AD, Rack Changer Ⅱ ESI ion source device was employed. As an analytical column, a Kinetex C18 (150 mm × 2.1 mm, 1.7 µm, Phenomenex, CA, USA) was used at 50˚C. The mobile phase consisting of acetonitrile and 0.1% formic acid was used with a gradient elution at a flow rate of 0.5 ml/minutes. The following ESI conditions were used: ion spray voltage, 5500 V; collision activated dissociation, 4 psi; curtain gas flow rate, 15 psi; ion source

temperature,350 ˚C.
For quantification of Ald, F, B and 11-DOF, transition of m/z 494.2/448.3, 501.2/455.2, 468.2/309.2, 472.2/454.3, 452.3/311.3, 456.3/315.3, 452.2/293.1 and 457.3/298.1 were selected for Ald, Ald-d7, F, F-d4, B, B-d4, 11-DOF and 11-DOF-d5, respectively.

Materials

18-oxoF and 18-oxoF-d4 were synthesized by its own. Ald-d7, B-d4 and 11-DOF-d5 was purchased from IsoSciences (PA, USA). Ald, B and 11-DOF was purchased from Sigma-Aldrich (MO, USA). F was purchased from NIST (MD, USA). F-d4 was purchased from CDN Isotopes (QC, Canada).
Fusaric acid, picolinic acid, 4-dimethyl-aminopyridine and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Chemical Industry (Tokyo). Triethylamine was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka). All other reagents and solvents were of analytical grade.

Histopathological Analysis for Expression of Steroidogenic Enzymes

Immunohistochemical staining and image analysis

All resected adrenal specimens were embedded in paraffin for immunohistochemical staining (IHC) and immunofluorescence staining (IF). Ten percent neutral formalin-fixed paraffin-embedded (FFPE) blocks which harbored APA were sectioned into 6 slides at 3μm thick; 3 slides for IHC for CYP11B1, CYP11B2 and 17α-hydroxylase (C17); 3 slides for IF. IHC for CYP11B1, CYP11B2 and C17 was performed as previously reported using rat monoclonal antibody for CYP11B1 (RRID: AB_2650563), mouse monoclonal antibody for CYP11B2 (RRID: AB_2650562) and mouse monoclonal antibody for C17 (RRID: AB_2731997).[1,2] All IHC sections were digitally scanned, and the images were captured by Image Scope AT2 (Leica, Wetzlar, Germany) for digital image analysis (DIA). We evaluated cross-sectional area (CSA) of APA and each immunoreactivity of these enzymes above were quantitatively evaluated using HALO digital image software; Area Quantification ver. 1.0 (Indica Laboratories, Corrales, NM, USA) as previously described.[1] The status of individual steroidogenic enzymes was evaluated by the following two quantitative approaches: One was the ratio of positive area to CSA of APA referred as “immunoreactivity”, the other an area of positive area in APA referred as “immunoreactive area”.

Immunofluorescence staining and image analysis

In our present study, we also performed IF for CYP11B1 and CYP11B2 in order to

count the number of B1+B2 cells manually. As previously reported, the same antibodies for CYP11B1 and CYP11B2 were used as primary antibodies with the following secondary antibodies; goat anti-rat IgG conjugated to Alexa flour 488 (112-545-167, Jackson ImmunoResearch, Allentown, PA, USA) and goat anti-mouse IgG conjugated to Alexa flour 594 (A11032, Invitrogen, Carlsbad, CA, USA), respectively.[3] Additionally, we also evaluated CYP11B2 and C17 co-expressing cells (B2+C17 cells) as supplemental data. In the analysis, we use same primary antibodies and the following secondary antibodies; goat anti-rabbit IgG conjugated to Alexa fluor 488 (A11034, Invitrogen, Carlsbad, CA, USA) and goat anti-mouse IgG conjugated to Alexa fluor 594 (A11032, Invitrogen, Carlsbad, CA, USA). The images of IF were obtained as 1024 pixel (512 μm) square pictures using a confocal laser-scanning microscope system (A1R, Nikon, Tokyo) and merged on Adobe Photoshop CC 2017 (Adobe Systems, San Jose, CA, USA). B1+B2 cells was calculated as the average of the numbers at 3 pictures from other lesions in each APA cases and adjusted with CSA. As a control, 7 non-functional adrenocortical adenoma (NFA) cases were analyzed.

Supplemental Results

Evaluation of CYP11B2 and CYP17A1 co-expressing cells

The number of B2+C17 cells was 16.4 [7.6, 27.2]/mm^2 in APA and was significantly higher than that of NFA (6.5 [3.0, 7.6]/mm^2, P <0.01, Supplemental Figure 5). In contrast to B1+B2 cells, the number of B2+C17 cells showed no significant correlation with the size of APA or p18oxoF. There was also no significant difference in the number of B2+C17 cells between KCNJ5 mutated and wild-type groups.

References

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3. Nakamura Y, Kitada M, Satoh F, Maekawa T, Morimoto R, Yamazaki Y, Ise K, Gomez-Sanchez CE, Ito S, Arai Y, Dezawa M, Sasano H. Intratumoral heterogeneity of steroidogenesis in aldosterone-producing adenoma revealed by intensive double- and triple-immunostaining for CYP11B2/B1 and CYP17. Mol Cell Endocrinol. 2016;422:57-63.

Table

	KCNJ5 positive		P value
	G151R	L168R	
Number	21	11	…
Age, y*	52.7 ±11.1	43.9 ±8.0	<0.05*
Sex, M and F	9,12	4, 7	NS
Body mass index, kg/m^2	23.7 ±2.6	25.2 ±2.5	NS
PAC, ng/dL	48.6 [37.2, 64.5]	51.7 [36.2, 55.4]	NS
PRA, ng/mL/h	0.20 [0.10, 0.50]	0.40 [0.15, 0.58]	NS
ARR, ng/dL per ng/mL/h	243.0 [155.7, 425.7]	130.7 [75.0, 308.7]	NS
18-oxocortisol, ng/dL*	12.2 [6.4, 26.8]	26.0 [17.3, 49.4]	<0.05*
Duration of hypertension, y	6.0 [1.0, 12.0]	4.0 [2.3, 11.8]	NS
Blood pressure, mmHg	147.7 ±24.5/92.8 ±18.1	156.8 ±21.9/98.6 ±13.8	NS
The number of AHT, n	2.8 ±1.0	3.4 ±1.2	NS
Decrease in AHT after surgery, y	1.5 ±1.4	1.6 ±2.0	NS
Serum potassium, mM	3.87 ±0.50	3.70 ±0.65	NS
Usage of potassium replacement, %	85.7	72.7	NS
MD of APA, mm	15.1 ±5.4	16.4 ±5.4	NS
CSA of APA, mm^2	56.6 [36.6, 99.2]	79.4 [58.5, 157.5]	NS
Immunoreactivitiy, %			
CYP11B1	18.2 [8.3, 24.6]	13.6 [9.6, 27.2]	NS
CYP11B2	23.8 ±10.3	27.3 ±11.8	NS
C17	16.7 ±8.1	13.5 ±8.9	NS
Positive area, mm^2			
CYP11B1	10.2 [5.1, 16.9]	11.2 [5.1, 28.5]	NS
CYP11B2	13.0 [9.5, 19.1]	19.7 [11.7, 37.1]	NS
C17	8.7 [3.7, 17.7]	11.9 [4.8, 16.1]	NS
B1+B2 cells, n/mm^2	61.0 [38.8, 103.7]	90.4 [34.6, 131.3]	NS
CSA-adjusted B1+B2 cells, n	4041.8 [1634.3, 8173.0]	5039.3 [3597.1, 17054.6]	NS

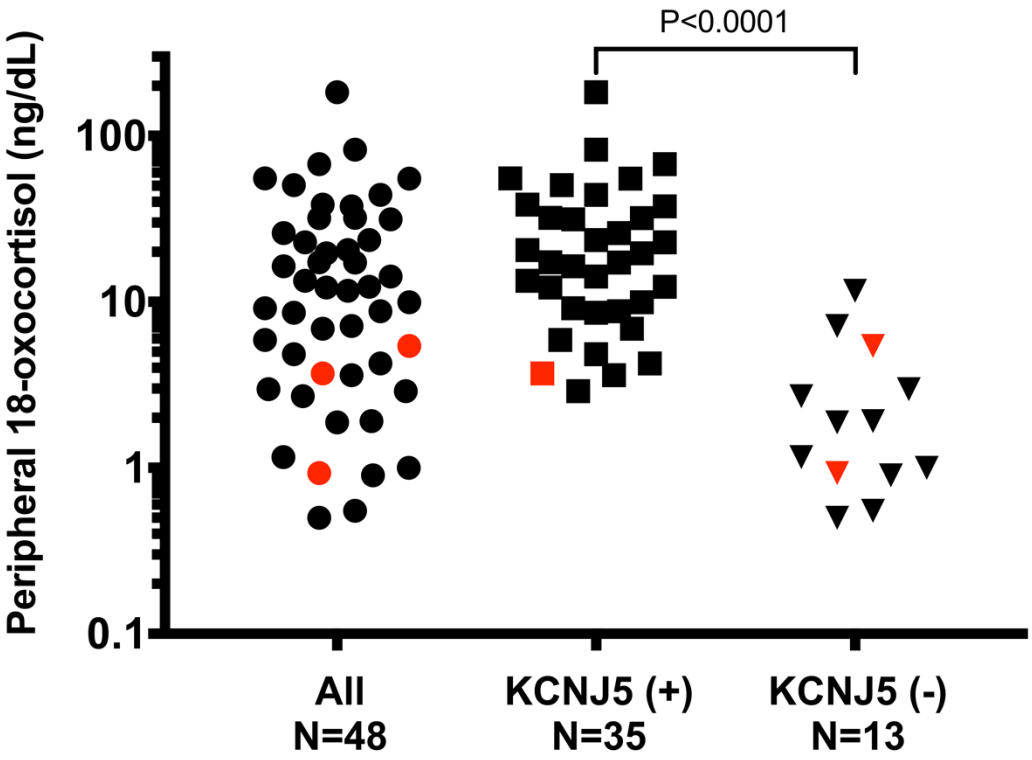
Supplemental Table Comparison between APA cases with and those without *KCNJ5* mutation.

Data are described as mean±standard deviation in normal distribution and median [interquartile range] in log-normal distribution. P values are calculated based on comparisons between *KCNJ5* mutation positive and negative APA cases. NS; not significant, APA; aldosterone-producing adenoma, PAC; plasma aldosterone concentration, PRA; plasma renin activity, ARR; aldosterone-to-renin ratio, AHT; anti-hypertensive agent, MD; maximum diameter, CSA; cross-sectional area, CYP11B2; aldosterone-synthase, CYP11B1; 11β-hydroxylase, C17; 17α-hydroxylase, B1+B2 cells; the number of CYP11B1 and CYP11B2 co-expressed cells. *P<0.05.

There were several kinds of *KCNJ5* mutated aldosterone-producing adenoma; G151R, 21 cases; L168R, 11 cases; T158A, 2 cases; F154C 1 case. Comparing clinicopathological factors between G151R and L168R mutated groups, age and peripheral 18-oxocortisol level were significantly lower and higher in L168R group

than G151R group, respectively. Other influential factors show no significant difference between them.

Figure

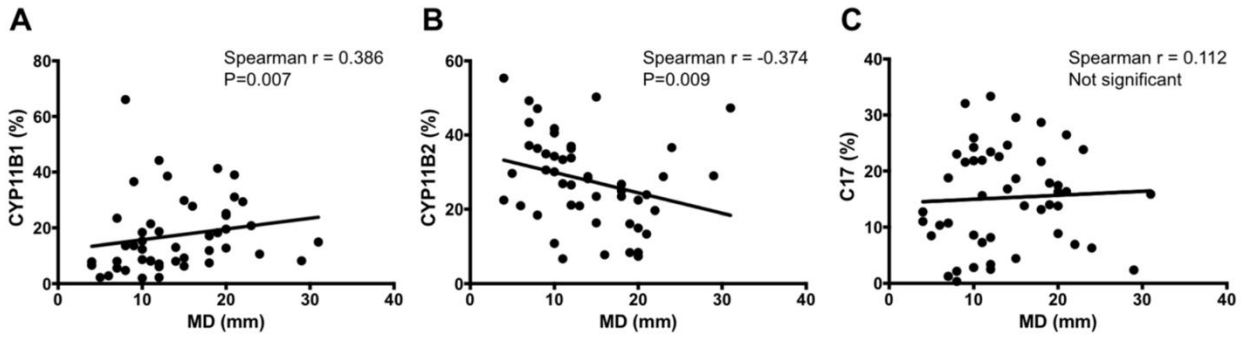


Supplemental Figure S1

The peripheral 18-oxocortisol concentration was widely distributed from 0.50 ng/dL to 183.13 ng/dL (with a median of 11.92 ng/dL). As for the comparison of p18oxoF peripheral 18-oxocortisol concentration among APA cases with or without *KCNJ5* mutation, APA cases bearing the *KCNJ5* mutation had higher concentrations of p18oxoF than those without the mutation. Three cases of microadenoma are shown with red symbols.

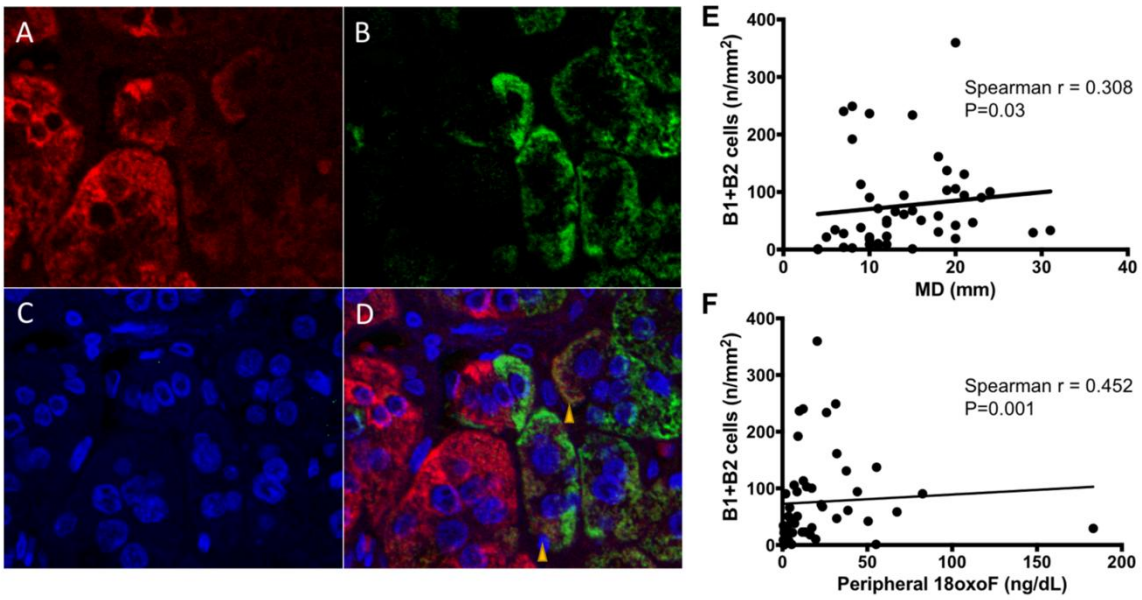
There was an APA case with an extremely high concentration of p18oxoF (183.13 ng/dL). The case corresponded to a 38-year-old female with a left APA (29 mm). Her baseline characteristics were as follows: blood pressure, 139/89 mmHg treated with 5 anti-hypertensive agents; plasma aldosterone, 328.7 ng/dl; plasma renin activity, 0.7 ng/ml/h; ACTH 14.8 pg/ml; and cortisol 5.5 μg/dl. The patient required more than 100 mmol/day of potassium replacement therapy to keep her level of potassium within normal limits. Adrenal venous sampling revealed a significantly higher aldosterone-to-cortisol ratio in the venous sample from the left adrenal vein (18461 ng/dl per 475.5 μg/dl) compared with the right adrenal vein (482.3 ng/dl per 456.2 μg/dl). She subsequently underwent left adrenalectomy resulting in improvement of

blood pressure (120/86 mmHg) and a lower number of anti-hypertensive agents (2 agents). The left adrenal tumor was histopathologically diagnosed as adrenal cortical adenoma (Weiss criteria of 0 point).



Supplemental Figure S2

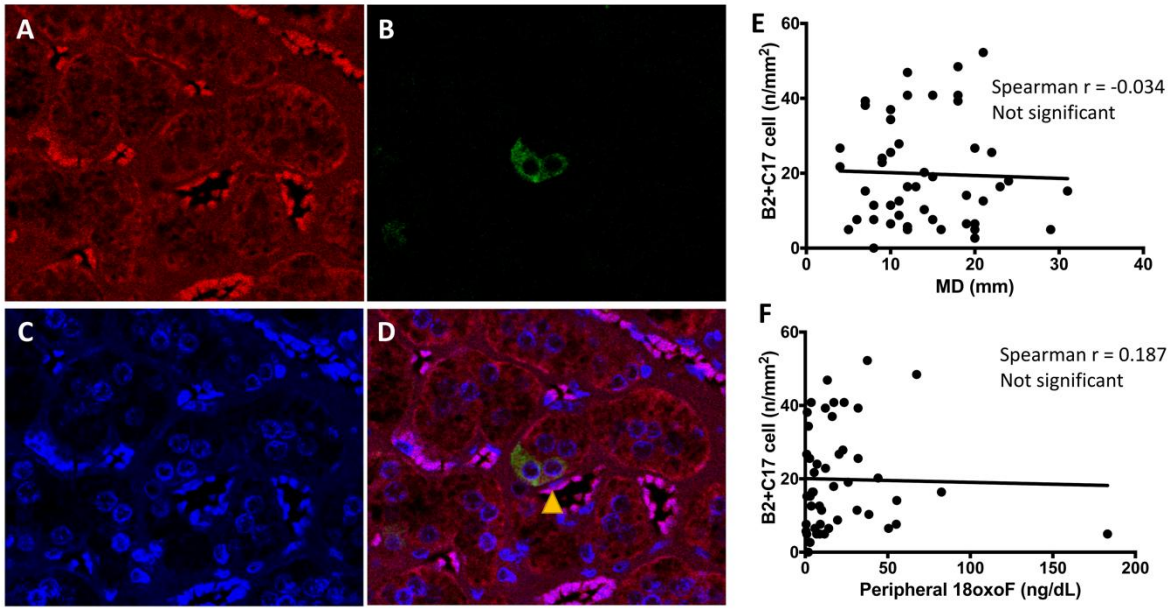
The ratio of CYP11B1 and CYP11B2 was significantly positively and negatively correlated with that of MD, while no significant correlation was observed with that of C17.



Supplemental Figure S3

Images of aldosterone synthase (CYP11B2, A), 11β-hydroxylase (CYP11B1, B), DAPI (C) and merged one (D). Arrow heads indicate B1+B2 cells co-expressing CYP11B1 and CYP11B2. The number of B1+B2 cells was significantly and positively correlated with the maximum diameter (MD) of APA (E) and peripheral concentration of

18-oxocortisol (18oxoF, F).



Supplemental Figure S4

Images of aldosterone synthase (CYP11B2, A), CYP17A1 (C17, B), DAPI (C) and merged one (D). Arrow heads indicate CYP11B2 and C17 co-expressing cells (B2+C17 cells). The number of B2+C17 cells did not show a significant correlation with the maximum diameter (MD) of APA (E) and peripheral concentration of 18-oxocortisol (18oxoF, F).