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2   DR ADINA TURCU (Orcid ID : 0000-0001-9831-6190)

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8   **Discordance between Imaging and Immunohistochemistry in Unilateral Primary Aldosteronism**

9   **Running title:** Pitfalls in Primary Aldosteronism Localization

10   Aya T. Nanba, Kazutaka Nanba, James B. Byrd, James J. Shields, Thomas J. Giordano, Barbara S. Miller,

11   William E. Rainey, Richard J. Auchus[*], Adina F. Turcu[*]

12   Division of Metabolism, Endocrinology, and Diabetes (ATN, WER, RJA, AFT), Department of Molecular

13   and Integrative Physiology (KN, WER), Division of Cardiovascular Medicine (JBB), Department of

14   Radiology (JJS), Department of Pathology (TJG), Department of Surgery (BSM), Department of

15   Pharmacology (RJA), University of Michigan, Ann Arbor

16   * RJA and AFT contributed equally to this work.

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28 **Reprints and correspondence:** Adina F. Turcu, MD

29 Division of Metabolism, Endocrinology and Diabetes, University of Michigan,

30 1150 W Medical Center Drive, MSRB II, 5570B,

31 Ann Arbor, MI, 48109

32 Email: aturcu@umich.edu

33 **Summary**

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35 **Objective:** Correct subtyping of primary aldosteronism (PA) is essential for good surgical outcomes.

36 Adrenal vein sampling (AVS) and/or computed tomography (CT) are used for PA sub-classification.

37 Clinical and/or biochemical improvement after surgery, however, is not always achieved in patients with

38 presumed unilateral PA. We aimed to identify the pitfalls in PA sub-classification leading to surgical

39 treatment failures.

40 **Patients and Design:** We retrospectively studied 208 patients who underwent adrenal vein sampling

41 (AVS) for PA sub-classification in a tertiary referral centre, between January 2009 and August 2016.

42 Simultaneous bilateral AVS was performed before and after cosyntropin administration. We implemented

43 immunohistochemistry for aldosterone synthase (CYP11B2) and 17α-hydroxylase/17,20 lyase

44 (CYP17A1) in adrenal glands resected from patients without improvement of PA after surgical treatment

45 and from those with limitations in AVS interpretation.

46 **Results:** Of 55 patients who underwent adrenalectomy, three (5.5%) had no improvement of PA. All

47 three patients underwent partial adrenalectomy to remove a CT-detected nodule present on the same side

48 with AVS lateralization. Immunohistochemistry revealed a CYP11B2-negative nodule in both cases

49 available. All patients who underwent total adrenalectomy based on AVS lateralization benefitted from

50 surgery, including three patients with unilateral unsuccessful AVS and aldosterone suppression in the

51 catheterized side vs. inferior vena cava.

52 **Conclusions:** Radiographically identified adrenal nodules are not always a source of PA, even when

53 ipsilateral with AVS lateralization. These data caution against reliance on imaging findings, either alone

54 or in conjunction with AVS, to guide surgery for PA.

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56 Key words: primary aldosteronism, aldosterone producing adenoma, adrenal vein sampling, CYP11B2

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59 **Introduction**

60   Primary aldosteronism (PA) is the most common identifiable form of secondary hypertension[1].

61   PA is highly prevalent among patients with resistant hypertension and is diagnosed in over 10% of the

62   patients referred to specialized centres[2-4]. PA is associated with increased cardiovascular morbidity and

63   mortality as compared with equivalent degrees of essential hypertension[5-7], and hence early recognition

64   and treatment of PA is imperative[8]. PA is traditionally sub-classified as either bilateral

65   hyperaldosteronism (BHA) or aldosterone producing adenomas (APA)[1], although some patients have

66   features of both subtypes. Correct sub-classification of PA is essential, as patients with unilateral PA can

67   benefit from surgical treatment. Immunostaining for aldosterone synthase (CYP11B2), the key enzyme in

68   aldosterone production, is not widely available; consequently, when postoperative clinical assessment

69   demonstrates surgical cure of hyperaldosteronism, it is assumed that a histologically identified

70   macroscopic adrenal tumor was the primary source of aldosterone. Since the development of highly

71   specific CYP11B2 antibodies, it has been demonstrated that the sub-classification of PA spans a

72   continuum, ranging from single or multiple APA, to small aldosterone producing cell clusters (APCC), to

73   zona glomerulosa hyperplasia[9-12]. The variable immunohistochemistry findings in resected adrenals from

74   patients with PA underscore the limitations of adrenal imaging in identifying the source(s) of aldosterone

75   excess.

76   Adrenal vein sampling (AVS) is considered the most accurate method for determining whether

77   one or both adrenals produce excess aldosterone[8]. Aside from patients younger than 35 years with

78   unequivocal PA[13,14], cross-sectional adrenal imaging findings are frequently discordant with the

79   aldosterone source as lateralized by AVS[15-19]. Nevertheless, resolution of PA after unilateral

80   adrenalectomy is not always achieved, even if the decision is based on AVS lateralization[9,13,20], in part

81   because AVS technique and interpretation vary widely between centres[21]. Moreover, AVS results might

82   be influenced by several antihypertensive agents and by autonomous adrenal cortisol synthesis[1,8]. For

83   these reasons, the supremacy of AVS in PA sub-classification has been questioned[20].

84   Several studies have focused on outcomes after adrenalectomy in PA, but the emphasis has been

85   on success rates rather than on causes of treatment failure. While clinical improvement is achieved in the

86   majority of PA cases treated surgically[9,13,22-24], data regarding contributors to surgical treatment failure

87   have been scarce. In the present study, we aimed to identify the pitfalls in PA sub-classification, leading

88   to absence of PA improvement after surgical treatment.

89

90   **Patients and Methods**

91   *Study participants*

92   In our centre, all patients with PA considered for surgical treatment undergo AVS. We studied

93   patients who underwent AVS at the University of Michigan between January 1st, 2009 and August 31st,

94   2016. Patient demographics, laboratory results, computed tomography (CT) imaging, AVS data,

95   pathology reports, and postoperative follow-up records were retrospectively reviewed. The

96   antihypertensive medications were converted to a standardized daily defined dose (DDD) according to the

97   WHO ATC/DDD Index[25]. Immunohistochemistry studies were conducted on paraffin-embedded adrenal

98   tissues in a subset of patients, as detailed below. All studies were conducted under University of

99   Michigan Internal Review Board (IRB) approved protocols. A waiver of consent was granted for the

100   retrospective studies; adrenal tissue experiments were conducted after obtaining written informed consent

101   from all participants.

102   *Clinical assessment*

103   Plasma aldosterone concentration (PAC) and plasma renin activity (PRA) were measured for case

104   detection of PA, and screening was positive in 164/170 (96.5%) of patients, based on a PAC (ng/L)/PRA

105   (μg/L/h) ratio (ARR) ≥ 200, PAC >100 ng/L and PRA <1 μg/L/h. In 125/170 (73.5%) patients, the

106   diagnosis was confirmed based on oral sodium loading test (followed by a 24 h urinary aldosterone >12

107   μg), saline infusion test (PAC >100 ng/L at 4 hours), or a suppressed PRA (<1.0 μg/L/h) with

108   spontaneous hypokalaemia and PAC >200 ng/L[8]. Six patients with a high clinical index of suspicion did

109   not meet ARR screening criteria but were included due to inability to discontinue interfering medications.

110   PAC was measured by a competitive chemiluminescent immunoassay on the DiaSorin Liaison XL

111   analyzer; the coefficient of variability (CV) was 8.9-9.4 %. PRA was determined using a

112   radioimmunoassay for angiotensin I manufactured by DiaSorin, with a CV between 13.0% (at 2.3 μg/L/h)

113   and 8.6% (at 5.8 μg/L/h). Serum cortisol was measured by a competitive chemiluminescent

114   immunoassay on a Siemens ADVIA Centaur analyzer, with CV of 5.1-7.1%. Urinary aldosterone was

115   measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Mayo Medical

116   Laboratories.

117   Because patients with unilateral dominance of PA might still have milder autonomous

118   aldosterone production from the contralateral side, we focused on patients with lack of PA improvement

119   after surgery, rather than on PA cure. Absence of PA improvement was defined as: postoperative PRA <1

120   μg/L/h and PAC fall by less than 50% as compared with the pre-operative value.

121   *AVS*

122   AVS was performed by one of two experienced interventional radiologists at the University of

123   Michigan. Samples were obtained simultaneously from the inferior vena cava (IVC) and both adrenal

124   veins (AV) before and 10-30 minutes after 0.25 mg cosyntropin administration (minimum three time

125   points). Cosyntropin was injected as a 0.125 mg bolus followed by continuous infusion (0.75 mg/h prior

126   to November 2014, and 0.125 mg/h thereafter). AV catheterization was considered successful when the

127   selectivity index (SI), defined by the AV/IVC cortisol concentrations, was ≥2 prior to and ≥5 after

128 cosyntropin administration, respectively. The lateralization index (LI), defined as the aldosterone/cortisol

129 ratio between the two AVs, and contralateral index (CI), defined as (aldosterone/cortisol)$_{\text{non-dominant AV}}$ /

130 (aldosterone/cortisol)$_{\text{IVC}}$ were used to assess lateralization. Unilateral PA was diagnosed if the LI was ≥ 2

131 before and ≥ 4 after cosyntropin administration, respectively. A CI <1 defined contralateral suppression.

132 *Immunohistochemistry*

133 We assessed the aldosterone synthetic capacity of the surgically removed adrenal tissue in

134 patients who failed surgical treatment and in patients with unsuccessful access of one AV. Localization of

135 CYP11B2 and 17α-hydroxylase/17,20 lyase (CYP17A1, an enzyme involved in glucocorticoid and

136 androgen synthesis) expressing cells was assessed by immunohistochemistry, using corresponding anti-

137 human antibodies (anti-CYP11B2, mouse; 1:1500, Millipore, Billerica, MA, USA, #MABS1251; anti-

138 CYP17A1, rabbit; 1:1000, kindly provided by Dr. Michael R. Waterman, Vanderbilt University School of

139 Medicine, Nashville, TN, USA), as previously described[10,26].

140 *Statistical analysis*

141 Statistical differences in measured parameters between groups were evaluated using the Mann-

142 Whitney *U* test. A *p* value *<0.05* was considered significant.

143

144 **Results**

145 *Demographic and diagnostic data*

146 Between January 2009 and August 2016, 208 patients underwent AVS in our centre. Of these, 38

147 patients referred directly to AVS, without clinical records available for review, were excluded (Figure 1).

148 Of the remaining 170 patients, 101 (59%) were men. The median age of all participants was 54 (range,

149 30-79). AVS was considered successful (based on SI criteria defined in Methods) in 122 (72%) and 159

150 patients (94%) before and after cosyntropin administration, respectively (Figure 1). Unilateral PA was

151 diagnosed in 66/122 (54%) and 87/159 (55%) patients before and after cosyntropin administration,

152 respectively. Contralateral suppression was present in 50/66 (75%) patients and 82/87 (94%) patients with

153 unilateral PA before and after cosyntropin administration, respectively. In total, 16 of the 159 (10%)

154 successfully catheterized patients had discordant lateralization before vs. after cosyntropin administration.

155 In five patients, unilateral PA was apparent only before cosyntropin, while 11 other patients lateralized

156 only after cosyntropin administration (Figure 1). No patients had opposite lateralization based on pre- vs.

157 post-cosyntropin LI.

158 Because catheterization is less often successful without cosyntropin administration, AVS data

159 after cosytropin administration have been preferentially used for clinical decisions in our centre, and PA

160 will be further referred to as unilateral or bilateral based on these results, unless otherwise specified. In

161 unilateral PA, median PAC (330 ng/L vs. 210 ng/L, *p <0.001*) and ARR (1970 vs. 870, *p <0.001*) were

162 higher, and PRA was lower (0.1 μg/L/h vs. 0.3 μg/L/h, *p=0.007*) than in bilateral PA (Table 1).

163 Hypokalaemia was more frequent in patients with unilateral PA (97%) than in those with bilateral PA

164 (75%).

165   Adrenal CT was performed in 158 patients (Figure 2). Unilateral adrenal abnormalities were

166 diagnosed in 114/158 (72%) patients: single nodule in 93 patients; multiple nodules in two patients;

167 nodules and underlying hyperplasia in four patients; unilateral thickening alone in 15 patients. Bilateral

168 adrenal nodules and/or hyperplasia were observed in 24 patients (15%). CT findings coincided with the

169 lateralization determined by AVS in 80/158 (51%) patients (Figure 2). Of 117 patients with adrenal

170 nodules, 51 patients were evaluated for autonomous cortisol synthesis; five patients had a cortisol >50

171 nmol/l after 1 mg dexamethasone suppression test. Of these five patients, two lateralized on the same side

172 with the nodule, suggesting cortisol co-secretion from the ipsilateral side; two had bilateral PA, and in one

173 patient AVS was unsuccessful.

174 *Postsurgical outcomes*

175   In total, 55 (32%) patients underwent unilateral adrenalectomy, either total (52) or partial (3). Of

176 the remaining 37 patients with unilateral PA, 24 were subsequently managed at other institutions, and in

177 the other 13 patients surgery was postponed for various reasons, including physician's recommendations

178 or personal preference. Postoperative follow up ranged from 5 days to 20 months (median 20 days).

179 Postoperative clinical assessment and information regarding antihypertensive medications was available

180 in all but two patients, while hormonal follow up was performed in 38 (69%) patients.

181   Of 55 patients treated surgically, three had no improvement of PA (Table 2). All three patients

182 demonstrated lateralization of PA based on AVS. CT showed one adrenal nodule on the dominant side in

183 all three patients; an additional subcentimetre nodule and lentiform thickening of the contralateral gland

184 was observed in one patient. Intraoperative ultrasound was performed in two of the patients and detected

185 a single nodule on the side with AVS lateralization. All three patients underwent partial adrenalectomy,

186 removing the area of the gland containing the nodule identified on imaging. The clinical pathology report

187 noted an adrenocortical adenoma in all three cases.

188   Adrenalectomy was performed in four patients in whom AVS had failed on one side, three with

189 an aldosterone/cortisol ratio lower in the successfully catheterized side than in IVC. All four patients

190 experienced resolution of PA and a decline in the ATC/DDD index.

191 *Immunohistochemistry*

192   To understand the pathophysiology of poor clinical outcomes after adrenalectomy, as well as

193 successful outcomes in cases with incomplete AVS data, we performed immunohistochemical staining for

194 CYP11B2 on the adrenal glands from patients who failed surgical treatment (two patients; for the third,

195 pathology specimen was not available) and patients in whom AVS failed unilaterally (four patients).

196   In the two patients with available pathology specimens who had failed surgical treatment, the

197   cortical adenomas removed were negative for CYP11B2, while the entire tissue was diffusely positive for

198   CYP17A1 (Figure 3, A and B, Cases 2 and 3 in Table 2). These results demonstrated that despite AVS

199   lateralization, the largest nodule was not the cause of PA in these patients. One of these patients

200   underwent a second AVS, which again demonstrated lateralization of PA to the partial adrenalectomy

201   side. CT –guided radiofrequency ablation of the remnant adrenal tissue was performed, with subsequent

202   resolution of PA (PAC fell from 336 ng/L to 56 ng/L), decline of ATC/DDD index and resolution of

203   hypokalaemia.

204   Of the four patients with unilateral AVS failure who underwent unilateral adrenalectomy,

205   CYP11B2 staining localized to the macroscopic nodule in three. In the fourth patient, however, the CT-

206   detected nodule was negative for CYP11B2 and positive for CYP17A1, while another, smaller, nodular

207   structure showed positive CYP11B2 and weak CYP17A1 expression, suggesting that the source of excess

208   aldosterone production was the smaller nodule rather than the one identified on CT (Figure 3C).

209

210   **Discussion**

211   AVS has been widely accepted as the "gold standard" for lateralization of PA[8,16,27]. The basis for

212   recommending AVS in all patients with PA older than 35 who might consider surgery[8] has emerged from

213   numerous studies demonstrating poor correlation of AVS with cross-sectional imaging[13,14,16]. Previous

214   studies have largely focused on success rates after adrenalectomy, but the cases with absent clinical

215   benefit after surgery have been poorly studied. Published data suggest that both CT and AVS are

216   imperfect tools for PA sub-classification[13,20]. In the present study, we sought to identify parameters

217   associated with poor outcomes after surgical treatment in PA patients guided with both AVS and CT data.

218   Of all surgically treated cases, the only ones without PA improvement were three patients who

219   underwent partial adrenalectomy to remove an adrenal nodule demonstrated by CT imaging on the

220   ipsilateral side with AVS lateralization. Staining for CYP11B2 was negative in both patients with

221   available pathology specimens, emphasizing that a prominent nodule is not always the source of

222   aldosterone, even when present on the ipsilateral side with AVS lateralization. One of the patients

223   experienced PA resolution after radiofrequency ablation of the remnant adrenal gland, consistent with the

224   AVS results. Failure of PA resolution has been previously reported in 2/29 patients with partial

225   adrenalectomy[24], although other groups reported no difference in clinical outcomes between patients who

226   underwent total or partial adrenalectomy[23,28]. Using CYP11B2 immunostaining, we further show that in

227   some patients with unilateral PA, the source of aldosterone excess includes neoplastic cells outside of a

228   dominant nodule, as other investigators have reported[9,29]. Moreover, adrenal CT abnormalities can be

229   contralateral to the side suggested by AVS as the source of autonomous aldosterone production[13,16].

230 Taken together, these results suggest that while macroscopic APAs are more common than other types of

231 unilateral PA, surgical planning cannot be reliably based on CT findings.

232 Next, we investigated potential sources of error in AVS interpretation. AVS technique and

233 criteria for successful catheterization and lateralization vary widely among centres[21,30,31]. In our

234 institution, samples are routinely obtained both before and after cosyntropin stimulation. The rate of

235 successful catheterization in our institution using the stated SI criteria is considerably higher when

236 cosyntropin is used (94% vs 72%), even in the hands of experienced interventional radiologists, in

237 agreement with the experiences reported by other centres[32,33]. The proportion of uni- vs. bilateral PA was

238 comparable before and after cosyntropin administration, although 10% of AVS studies yielded discrepant

239 results. In total, 6/16 patients with discordant pre- vs. post-cosyntropin AVS lateralization had

240 adrenalectomy: one who lateralized only before and five who lateralized only after cosyntropin. Of these,

241 only one patient – whose surgery was a partial adrenalectomy – did not have improvement of PA after

242 surgery. In a recent study of 175 patients who underwent AVS at another tertiary referral centre, the

243 lateralization index was discordant in 28% of cases, the majority of which lateralized only prior to

244 cosyntropin administration[32]. Taken together, these data suggest that lateralization under any AVS

245 protocol is a strong predictor of clinical improvement after surgery, even if lateralization is not present

246 under all tested conditions.

247 Adrenalectomy was performed in three patients with unilateral AVS failure but who

248 demonstrated suppression of aldosterone production in the successfully catheterized side as compared to

249 IVC. All three patients experienced resolution of PA and reduction of their antihypertensive regimen.

250 These outcomes suggest that unilateral aldosterone suppression alone is a strong indicator of contralateral

251 dominance in PA and that AV/IVC aldosterone suppression can be utilized as a decision tool when AVS

252 fails unilaterally. Our results substantiate previous reports of favourable outcomes in patients with

253 unilateral PA and contralateral suppression[34-36]. As was previously observed[37], we found that contralateral

254 suppression was more frequent post- as compared with pre- cosyntropin administration. These results,

255 along with the favourable surgical outcomes in such patients[35-37], suggest that cosyntropin does not often

256 disproportionately stimulate aldosterone production from the contralateral adrenal in unilateral PA.

257 Nonetheless, this aspect requires further elucidation by both *in vitro* experiments and prospective clinical

258 trials.

259 While several caveats in AVS technique and interpretation[13,15,21,38,39] require further clarification,

260 we have not identified any cases with absent clinical benefit after AVS-guided total adrenalectomy. The

261 superiority of AVS in PA has been recently questioned by Dekkers and colleagues, who randomly

262 assigned 200 patients with PA to undergo sub-classification by either CT or AVS[20]. At one year follow

263 up, there was no statistical difference in the antihypertensive treatment intensity between the two groups,

264 despite the fact that the agreement between AVS and CT findings was only 50%. Of the 92 patients (46 in

265 each group) who underwent surgery, PA was persistent in nine (20%) versus five (11%) patients with CT-

266 or AVS-guided surgery, respectively. These failure rates are much higher than in other reports[11,13,22].

267 Nonetheless, the study was underpowered to detect a difference in failure rates after AVS or CT-guide

268 adrenalectomy. Furthermore, MRA use was permitted postoperatively, allowing blood pressure control in

269 uncured patients from both groups[40].

270   Similar to previous reports, the concordance between AVS and CT findings in our study was only

271 53% and highest in cases with single adrenal nodules. Moreover, unilateral PA was diagnosed by AVS in

272 35% of patients with no detectable adrenal abnormalities by CT, and all of these patients benefited

273 clinically from adrenalectomy. These results reinforce that, in contrast with cases of hypercortisolism,

274 autonomous aldosterone synthesis from a small group of highly efficient cells might be sufficient to cause

275 disease. Multiple adrenocortical micronodules or diffuse hyperplasia of the zona glomerulosa has been

276 described in such patients[12]. Taken together, these results suggest that over a third of patients with PA

277 could be denied the opportunity of surgical treatment if subtyping was based on cross-imaging studies.

278   In summary, we explored the reasons behind surgical failure and the pitfalls of the commonly

279 used PA sub-classification tools, CT and AVS. Among the strengths of our study are the large numbers of

280 patients captured within a tertiary referral adrenal centre and the routine collection of serum both before

281 and after cosyntropin administration during AVS. Most importantly, implementing CYP11B2

282 immunostaining, we demonstrated that autonomous aldosterone synthesis does not always correspond

283 with radiological findings, even when ipsilateral AVS lateralization is present. Consequently, our data

284 caution against reliance on cross-sectional imaging as a criterion for PA lateralization or guide for

285 adrenalectomy. The limitations to our data include the retrospective design and the number of patients

286 who did not undergo surgery. In addition, postsurgical evaluation was heterogeneous and relatively

287 limited, making it unfeasible to assess a range of outcomes. Carefully planned prospective studies,

288 incorporating uniform criteria for postoperative follow up, remain necessary to clarify the optimal

289 approach to PA lateralization in cases amenable to surgical treatment.

290

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415 **Table 1**. Clinical and biochemical characteristics of study participants

	Unilateral PA N=87	Bilateral PA N=72
Sex(M/F)	55 (63%) /32 (37%)	40 (56%) /32 (44%)
Age	53 (30-78)	56 (31-79)

BMI	34 (20-54)	33 (23-56)
Hypokalaemia (%)	97%	75%
No. of antihypertensive agents (0/1/2/3/4/5+)	1/4/19/11/8/44	2/10/9/17/18/16
Basal PAC (ng/L)*	330[+] [210 – 460]	220 [160 – 290]
Basal PRA (µg/L/h)*	0.1[++] [0.1 – 0.4]	0.3 [0.1 – 0.6]

416 Classification based on post-cosyntropin data. BMI, body mass index; **,** Data are expressed as medians

417 [interquartile range]; [+] *p <0.001* vs. bilateral PA; [++] *p <0.01* vs. bilateral PA; PAC, plasma aldosterone

418 concentration; PRA, plasma renin activity.

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428 **Table 2.** Characteristics of patients without clinical benefit after surgery

	PAC (ng/L)		PRA (µg/L/h)		ARR		HC	AVS LI (Dominant Side)		CI	CT findings	Surgery	Pathology
	Pre Op	Post Op	Pre Op	Post Op	Pre Op	Post Op		Before ACTH	After ACTH				
Case 1	417	466	0.5	<0.1	834	4660	Negative	4.5 (R)	4.3 (R)	2.6	1.9 cm R nodule; 0.9 cm L nodule and diffuse thickening	R nodule resection	ACA
Case 2	382	203	0.1	<0.1	3820	2030	-	4.5 (L)	4.4 (L)	0.3	1.3 cm L nodule	L nodule resection	ACA
Case 3	269	336	0.1	0.1	2690	3360	Negative	Failed	5.9 (L)	0.1	1.2 cm L nodule	L nodule resection	ACA

429 PAC, plasma aldosterone concentration; PRA, plasma renin activity; ARR, PAC/PRA ratio; PreOp, before surgery; PostOp, after surgery; HC,

430 hypercortisolism; -, not tested; AVS, adrenal vein sampling; LI, lateralized index; CI, contralateral index, after cosyntropin; R, right; L, left; ACA,

431 adrenocortical adenoma.

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439 **Figure legends**

440   **Figure 1.** Adrenal vein sampling (AVS) data.

441   Adrenal vein catheterization was considered successful when selectivity index (SI) was ≥2 before and ≥5 after cosyntropin administration.

442   Unilateral PA was diagnosed if lateralized index (LI) was ≥2 before and ≥4 after cosyntropin administration. B/L, bilateral.

443   [*], Classification based on post-cosyntropin data. Uni, unilateral; Bi, bilateral; Failed, failure of adrenal vein catheterization.

444   [+], In this patient, AVS data suggested clear lateralization and contralateral suppression prior to cosyntropin stimulation, but bilateral PA after

445   cosyntropin.

446   **Figure 2.** Comparison between CT findings and AVS lateralization.

447   **Figure 3.** Histopathological findings of selected adrenal tissue

448   A and B: Adrenal nodules resected from patients who underwent selective resection of a CT-detected nodule (Cases 2 and 3, Table 2). Both

449   nodules demonstrated no CYP11B2 expression, while CYP17A1 was positive in the nodules. C. Adrenal tissue resected from a patient who had

450   unilaterally unsuccessful AVS. The CT-detected nodule was negative for CYP11B2, while another smaller nodule had CYP11B2 expression.
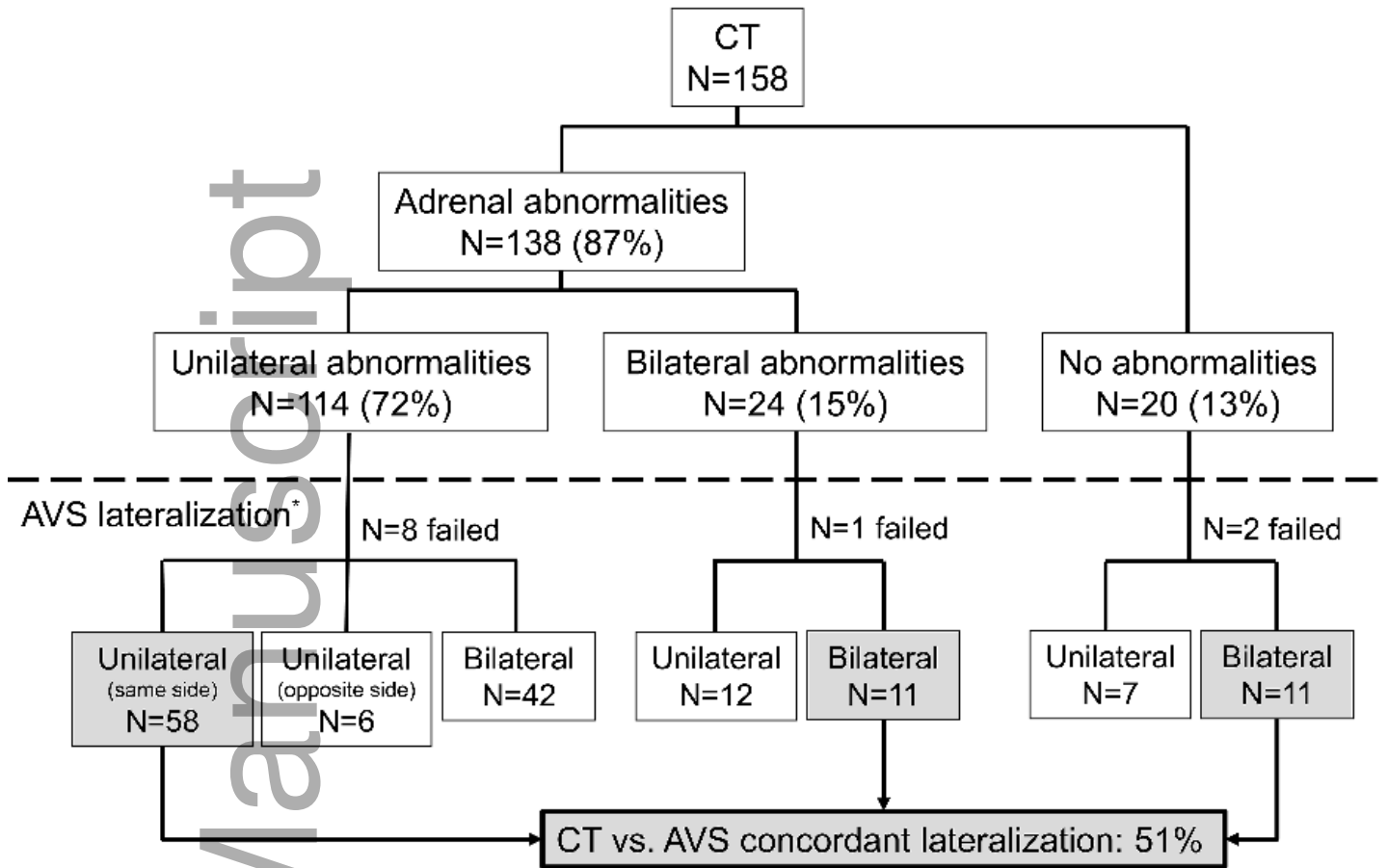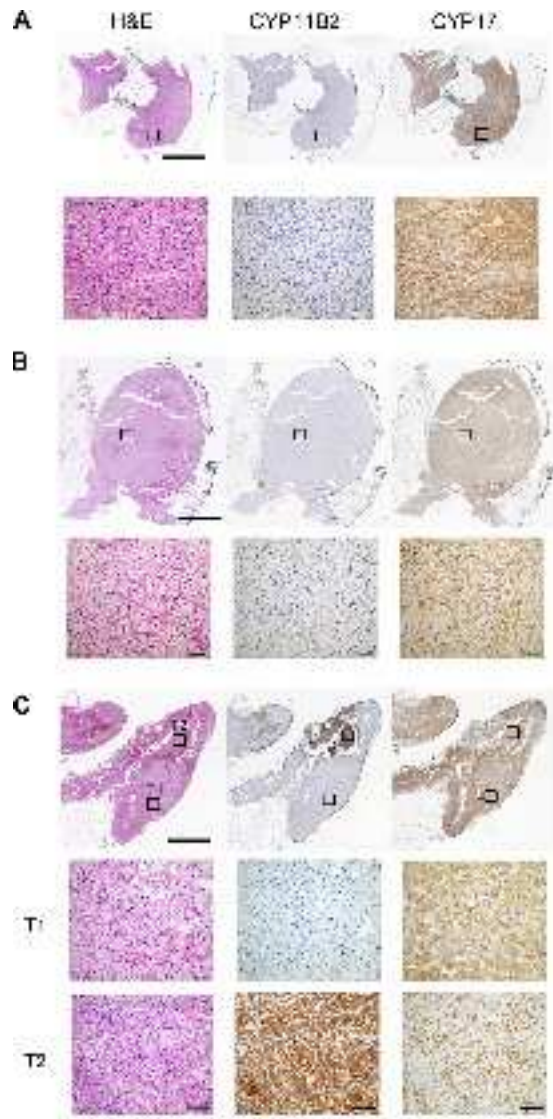
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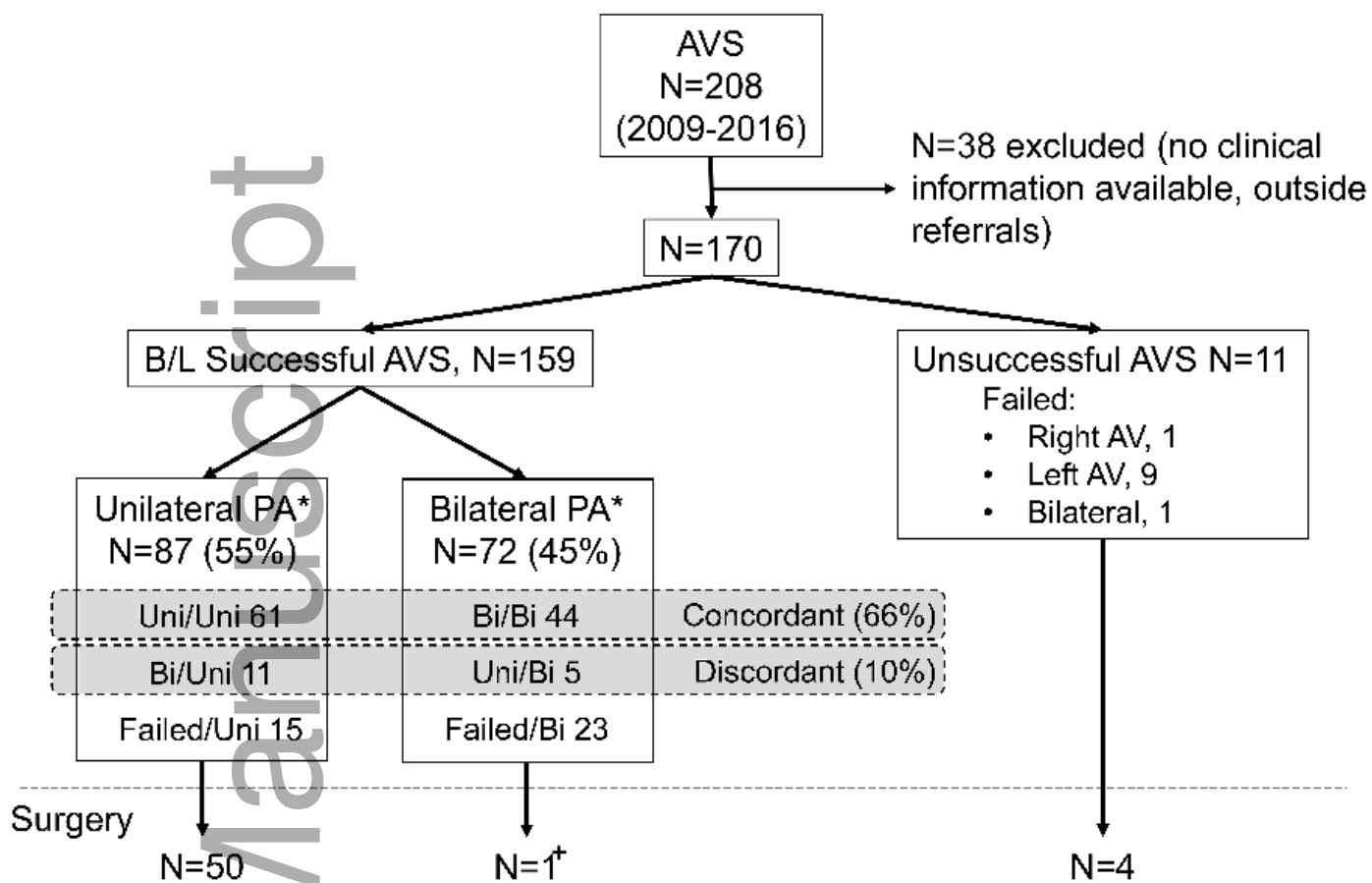
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