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Anti-ZnT8 autoantibodies: a new marker to be screened in patients with anti-adrenal antibodies

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Short title: ZnT8A in anti-adrenal antibodies positive patients

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1 **Introduction**

2 Patients with autoimmune Addison's disease (AAD) have higher susceptibility to develop
3 other autoimmune diseases. Thus, along with the classical markers of AAD (anti-adrenal
4 cortex (ACA) or anti-21-hydroxylase (21-OH) autoantibodies) these patients often display
5 other non-adrenal autoantibodies with a prevalence that could reach 88% [1–3]. Among these
6 non-adrenal autoantibodies, anti-insulin (IAA), anti-insulinoma-associated protein 2 (IA-2A)
7 and anti-glutamic acid decarboxylase (GADA) autoantibodies detected in type 1 diabetes
8 (T1D), have been found in 10% of AAD patients [2,4]. The more recent described
9 autoantibodies in T1D, anti-Zinc transporter 8 protein antibodies (ZnT8A), are present in both
10 juvenile-onset and adult-onset T1D. They are considered to be important biomarkers for
11 predicting the clinical onset of this autoimmune disease especially as they are found in 18 to
12 30 percent of T1D patients without any other autoantibodies [5–8]. Among the well-
13 established T1D associated autoantibodies, ZnT8A and AAD association is the least studied
14 [2,4,9]. Moreover, the ZnT8 protein was found in rat adrenal glands [10], thus ZnT8A could
15 also be a biomarker of autoimmunity pathogenesis against adrenal tissue.

16 As T1D is frequently associated with AAD and since ZnT8A are rarely studied in AAD
17 patients, the aim of the present study is to evaluate the prevalence of ZnT8A in patients with
18 positive ACA in order to define the usefulness of ZnT8A for prediabetes screening in AAD.

19

20 **Material and Methods**

21 **Patients**

22 The presence of ZnT8A was investigated in 36 patients positive for ACA from 2 different
23 French hospitals (12 were from Toulouse University Hospital and 24 from Hospices Civils of
24 Lyon). Twenty-six women and 10 men were included of which 19 were children (<18 years).

25 The sera of these patients have been obtained between 2001 and 2019 and kept frozen at -
26 20°C in the immunological laboratories of the 2 hospitals.

27 Clinical diagnosis was obtained for all patients. Autoimmune Addison's disease was
28 diagnosed according to SFE/SFEDP adrenal insufficiency French consensus criteria [11].
29 Autoimmune Addison's disease was defined by the presence of ACA associated with a panel
30 of non-specific (except melanoderma) clinical signs and symptoms of hypoadrenalism, with
31 low plasma cortisol and increased ACTH levels at 8am and/or an insufficient increase of
32 plasma cortisol after synacthen administration [11–13]. **Type 1 diabetes was determined by**
33 **the original clinician according to the latest American Diabetes Association diagnostic criteria**
34 **recommendations [14] i.e, the presence of multiple autoantibodies with or without**
35 **hyperglycemia (stage 2 and 3).**

36 Observational studies conducted in France using data obtained from a retrospective study
37 without any additional therapeutic or monitoring procedure do not require informed consent.
38 This was the case herein as autoantibodies were analyzed as part of diagnostic investigation.
39 Patient records and information was anonymized and de-identified prior to analysis. The study
40 was approved by the institutional review board and the ethics committee of the Hospices
41 Civils de Lyon and the ethic committee of Southwest and overseas II.

42 **Anti-adrenal cortex autoantibodies detection**

43 The presence of ACA has been analyzed using indirect immunofluorescence technique on
44 primate adrenal tissue (INOVA Lite Adrenal primate IFA, Werfen, Le Pré-Saint-Gervais,
45 France) according to the manufacturer's recommendations (**Figure 1**).

46 **As the main target of ACA has been described to be 21-OH [15,16], 22 out of 24 patients**
47 **from Hospices Civils of Lyon were also analyzed for anti-21-OH autoantibodies in the initial**
48 **diagnostic investigation** using an enzyme-linked immunosorbent assay (ELISA) technique
49 (21-OH-AB ELISA, RSR, Cardiff; United Kingdom) or a radio-immunological assay (RIA)

50 (21-OH-AB, RSR, Cardiff; United Kingdom); the cut-off for positivity was chosen according
51 to the manufacturer's recommendations at 0.4 U/mL or 1.1 U/mL respectively. The 2
52 techniques have been shown to give comparable sensitivity with 87% of concordance on 30
53 patients in Lyon immunological laboratory (personal data not shown).

54 **Anti-ZnT8 autoantibodies detection**

55 ZnT8A were analyzed using 2 ELISA techniques (ZnT8/96D Elisa, RSR, Cardiff, United
56 Kingdom or Medizym®, Medipan, Dahlewitz, Germany). The cut-off for positivity was
57 chosen according to the manufacturer's recommendations at ≥ 15 IU/ml in both assays. These
58 two ELISA tests have been compared in Toulouse immunological laboratory and showed the
59 same performance on 32 patients with 100% of concordance between the 2 assays (personal
60 data not shown).

61 **Anti-IA2 and anti-GAD autoantibodies detection assays**

62 In 22 patients, IA-2A and GADA were also analyzed at the time of diagnosis or
63 retrospectively when testing for ZnT8A. IA-2A were detected using 2 ELISA kits (ELISA
64 RSR IA-2 Ab, Cardiff, United Kingdom and Medizym®, Medipan, Dahlewitz, Germany).
65 The cut-off for positivity was chosen according to the manufacturer's recommendations at
66 ≥ 7.5 U/mL or ≥ 10 U/mL, respectively. For GADA detection, 2 ELISA kits were used (ELISA
67 RSR GADAb, RSR, Cardiff, United Kingdom and Medizym® anti GAD, Medipan,
68 Dahlewitz, Germany) with the same cut-off of positivity at ≥ 5 U/mL.

69 The 2 techniques for both IA2A and GADA antibodies have been shown to give comparable
70 sensitivity and specificity according to the results of Diabetes Antibody Standardization
71 Program [17]. When IA-2A and GADA were explored at time of diagnosis a
72 radioimmunoassay could have been employed (cut off positivity at ≥ 30 cpm) (1 patient).

73

74 **Statistical analysis**

75 All data are reported as the means \pm sem. Sex of patients involved in this study are
76 indicated in the tables but are not included in statistical analysis of the data. Comparisons
77 between groups were performed using Wilcoxon rank sum test or two-sided Fisher's Exact
78 test as appropriate. A p-value <0.05 was considered significant. All data were analyzed using
79 the R software (v3.6.3).

80

81 **Results**

82 **Clinical data and anti-adrenal cortex autoantibodies**

83 The cohort included a total of 36 patients (19 children and 17 adults) with an average age
84 of 21.6 years (mean \pm sem: 21.6 ± 2.3 years)

85 Thirty-one of 36 patients displaying ACA were diagnosed to suffer from AAD. Most of
86 them were newly diagnosed; indeed the mean time between AAD diagnosis and ZnT8A
87 detection was 1.7 years (median of 2 months). Among the 36 positive ACA patients, 22
88 patients were analyzed for anti-21-OH autoantibodies and were all positive for these
89 autoantibodies (16 using RIA, mean \pm sem: $959,6 \pm 534,7$ and 6 using ELISA, mean \pm sem :
90 $71,7 \pm 15,7$ U/mL).

91 For 5 out of 36 patients, Addison's disease diagnosis was not retained; there were 4 children
92 (10, 12, 14 and 15-years-old) and 1 young adult (26 years-old). All 5 patients had other
93 autoimmune disorders: 3 T1D (with one of them associated with ZnT8A and anti-TPO
94 autoantibodies, and one of them associated with only ZnT8A) and 2 hypoparathyroidisms
95 (with one suffering of an autoimmune polyendocrine syndrome (APS) type 1). Three of these
96 5 patients displayed anti-21-OH autoantibodies with significant titers above the cut-off of
97 positivity (31 and >100 U/mL using ELISA and 17 U/mL using RIA). Data on anti-21-OH
98 autoantibodies titers for the remained 2 patients were not available.

99 Concerning T1D status, 27.8% (10 patients out of 36) and 22.6% (7 patients out of 31) within
100 positive ACA patients and AAD cohort suffered from T1D respectively (**Table 1, Figure 2**) .

101

102 **Prevalence of ZnT8A**

103 Seven (4 children and 3 adults) of 36 patients (19.4%) had ZnT8A (**Table 1**). ZnT8A was
104 observed in 5 patients out of 31 with AAD (16.1%) and in 2 patients out of 5 with no AAD
105 (40.0%) (**Figure 2**) but no statistically significant difference was noticed in prevalence of
106 ZnT8A in AAD versus no AAD patients (p-value = 0.244).

107 Among the 7 positive ZnT8 patients, 71.4% (3 children, 2 adults) suffered from T1D at the
108 time of ZnT8A detection (**Table 1, Figure 2**). The titers of ZnT8A, when positive, were
109 higher for the T1D patients but there was no statistical difference when compared with
110 patients with no T1D (mean \pm sem : 348.84 U/mL \pm 170.8, n=5 versus 33.5 U/mL \pm 2.5, n=2,
111 p-value = 0.381 with Wilcoxon rank sum test).

112 The frequency of T1D was significantly different between patients with positive ZnT8A
113 versus patients with negative ZnT8A (71.4% vs 17.2%, p-value = 0.010) (**Table 1**).
114 Characteristics regarding ZnT8A frequency, ZnT8A titer and T1D association were the same
115 in both children and adult population (**Table 2**).

116 Concerning the frequency of autoimmune diseases associated with positive ACA other
117 than T1D alone, the differences were not statistically significant (42.9% vs 44.8%) (**Table 1**).

118

119 ***Anti-IA2 and anti-GAD autoantibodies***

120 Twenty two AAD patients were tested for all the T1D associated autoantibodies. Ten patients
121 were positive for one or more autoantibodies and 5 (22.7%), 9 (40.9%) and 2 (9.1%) of them
122 had ZnT8A, GADA and IA-2A respectively.

123 Two patients positive for GADA and IA-2A were also positive for ZnT8A and they suffered
124 from a T1D. Five patients (3 with T1D, one presenting an APS type 1 and one with anti-TPO
125 and anti-TG antibodies) present only GADA. One positive ZnT8A patient (165.1 U/mL) had
126 positive GADA (3667 cpm) but with no IA-2A and had a T1D and anti-TPO. One positive
127 ZnT8A patient (97.5 U/mL) was weakly positive for GADA (5.8 U/mL) but with no IA-2A
128 (1.4 U/mL) and had a T1D. One positive ZnT8A (31.0 U/mL) patient had neither GADA (1.0
129 U/mL) nor IA-2A (1.4 U/mL) and did not suffer from a T1D despite having an AAD evolving
130 for 13 years at the time of the sample collection integrating into an APS type 1. Clinicians
131 continue to monitor his glycaemia due to his young age and ZnT8A status.
132 Twelve patients were negative for all of ZnT8A, GADA and IA-2A; among them only one
133 patient suffered from a T1D.

134

135 **Discussion**

136 This study confirmed that ZnT8A are present in patients suffering from AAD in both
137 children and adults with an overall prevalence of 19% of the patients tested herein. The
138 presence of ZnT8A in AAD has been demonstrated in other cohorts [4,18] although the
139 prevalence was lower with 8% in both studies. It is important to note that the sensitivity of
140 autoantibodies in general varies among reports and this may be mainly explained by
141 differences in the populations studied in terms of age and duration of the disease. An
142 important part of the variation may also be related to the choice of the analytical test for
143 ZnT8A since several ELISA techniques exist; however, this is not the case since all the
144 ELISA techniques have been shown to give comparable performances according to the results
145 of Diabetes Antibody Standardization Program [17] and personal data.

146 The frequency of T1D in our AAD cohort reached 22.6% which is higher than reported in
147 previous studies (ranging from 1.2% to 13% in more recent studies [2,4,9,19,20]). An

148 interesting issue arising from the present study, which could explain the higher frequency of
149 T1D, is the difference in the population studied particularly the importance of the children
150 population making up more than 50% of our cohort. To the best of our knowledge, the only
151 large AAD cohort reported included few children and did not differentiate them in data
152 interpretation [4]. The mean age of 22 years is lower in comparison with other studies with a
153 mean age between 30 and 50 years. The evolution of diagnostic criteria according to the
154 *American Diabetes Association* classification could also contribute to the higher proportion of
155 T1D observed in our study. Indeed the recent inclusion of latent autoimmune diabetes in
156 adults in T1D [14] and the inclusion of diabetes autoantibodies positivity in diagnostic criteria
157 has modified diabetes classified as T1D and can explain the higher number of T1D patients
158 in comparison to the literature.

159 The prevalence of ZnT8A has been found to be correlated with the age of diabetes onset in
160 the literature. Anti-ZnT8 autoantibodies seemed to be better markers at diabetes onset
161 between 6 to 17 years of age in comparison with patients of less than 5 years-old and adults
162 [21,22]. This has also been found in other cohorts of patients showing a high prevalence of
163 ZnT8A positive patients between 8-16 years and 10-19 years [23–25]. We, thus, investigated
164 the age of ZnT8A positive patients but we did not find any statistical difference in comparison
165 with the negative cohort.

166 Neither age nor sex seemed to influence the frequency of T1D association or ZnT8A
167 positivity in positive ACA patients. This is of particular interest if we take into account that
168 highest prevalence of ZnT8A are mainly reported in T1D children [26]. However, our cohort
169 is too small to reach conclusions with a statistical power.

170 In ZnT8A-positive patients, we observed a titer about 10 times higher in T1D patients
171 versus no T1D patients. Indeed it was already reported that, at onset of T1D, a high proportion

172 of children who present autoantibodies related to other autoimmune diseases have a very high
173 ZnT8A titer [21].

174 In our cohort, most patients with positive ZnT8A had IA-2A and/or GADA and suffered
175 from a T1D. Two patients had ZnT8A with no other autoantibodies or with a low titer of
176 GADA. One of these 2 patients already had T1D, but the remaining patient had to be
177 followed.

178 Our data also indicate that positive ZnT8A are associated with coexisting T1D in more
179 than 70% of our patients and that by consequence 30% of the population with positive ZnT8A
180 did not suffer from T1D at the time of the study.

181 The zinc transporter 8 localized on the membrane of insulin containing secretory granules
182 plays an essential role in biosynthesis, storage and release of insulin [27–29]. A previous
183 report also showed an expression of this protein in rat adrenal endocrine cells [30]. Therefore,
184 ZnT8A could be a marker of autoimmunity associated to the adrenal gland destruction in
185 AAD, with no development of T1D; a similar event was reported with GADA in some type 1
186 APS [2,31,32]. However, since humoral autoreactivity to ZnT8 has been demonstrated to be
187 an independent marker of disease susceptibility in pre-diabetic patients and associated with
188 faster development of T1D, one third of patients with no T1D have to be followed due to the
189 potential risk of developing T1D [33].

190 Other autoimmune disorders classically associated with AAD were present in our cohort.
191 Autoimmune thyroid disorders were present in 25% of the cases, in lower proportion than
192 already reported [2,9,19], certainly due to the age of our patients. Parathyroid disorders or
193 APS were present in 11% of the cases. The frequency of associated autoimmune disorders is
194 higher, although not significantly, in AAD patients with ZnT8A than in patients with no
195 ZnT8A. However, this trend was no longer observed when T1D patients without other
196 associated autoimmune disorders were excluded suggesting that T1D represents the main

197 associated autoimmune disease in ZnT8A-positive AAD patients, as reported in a previous
198 report [4].

199 More than ninety percent of patients with AAD develop anti-21-OH autoantibodies [34].
200 In our study, 3 patients with positive anti-21-OH autoantibodies showed no AAD. Those
201 patients were young and should be followed as their titers of anti-21-OH autoantibodies are
202 high and can thus be predictive for the development of AAD [35,36].

203 In light of these data and as the onsite implementation of ZnT8A monitoring in the clinical
204 practice for T1D, it should also be included in the screening panel of AAD population. This
205 finding is of importance for clinicians as the presence of ZnT8A can identify subjects at high
206 risk to develop T1D. Further investigation of the role of ZnT8A in AAD with no T1D has also
207 to be performed.

208

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219

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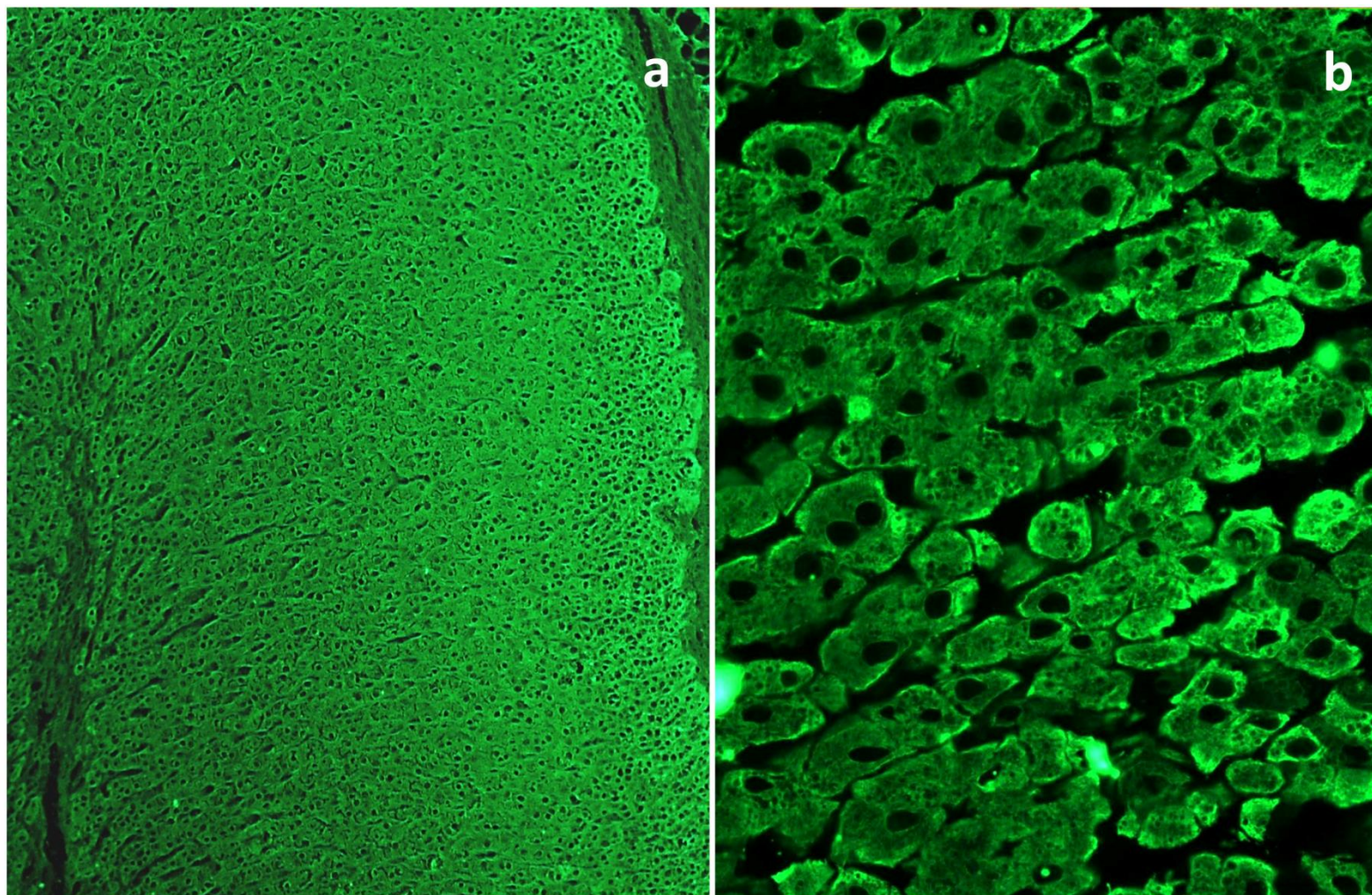
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342 **Figure 1:** Anti-adrenal cortex autoantibodies positive sample detected by indirect
343 immunofluorescence on primate adrenal tissue sections (INOVA Lite Adrenal primate IFA,
344 Werfen, Le Pré-Saint-Gervais, France) with FITC anti-Human IgG. IgG stain with a diffuse
345 pattern on cytoplasm of the cells of the three layers of the adrenal cortex at a) x20
346 magnification b) x40 magnification.



348

349 **Table 1:** Comparison between the ZnT8A-positive and ZnT8A-negative group among
 350 positive anti adrenal cortex autoantibodies patients (mean± sem or effective (percentage of
 351 patients).

	ZnT8A-positive group	ZnT8A-negative group	P-value
Patients' characteristics			
n	7	29	NA
Sex (women/men)	5/2	21/8	1.000
Age (years) at ZnT8A dosage	19.1 ± 3.6	22.2 ± 2.7	0.968
ZnT8A titer (U/mL)	258.8 ± 131.4	3.8 ± 0.6	NA
Auto-immune diseases			
Addison disease diagnosis at time of ZnT8A dosage	5 (71.4%)	26 (89.7%)	0.244
Total T1D	5 (71.4%)	5 (17.2%)	0.010*
Total autoimmune disorders associated or not with ACA:	6 (85.7%)	18 (62.1%)	0.384
<i>T1D alone (with or without AAD)</i>	<i>3 (42.9%)</i>	<i>5 (17.2%)</i>	0.168
<i>Other autoimmune disorders:</i>	<i>3 (42.9%)</i>	<i>13 (44.8%)</i>	1.000
- Anti-TPO and/or anti-Tg with or without hypothyroidism	2 (whose 2 associated T1D)	6	
- Graves' disease	0	1	
- Parathyroid disorders or APECED syndrome (APS type 1)	1	3	
- APS type 2 with no T1D	0	1	
- Goodpasture syndrome	0	1	
- Autoimmune ovaritis	0	1	
Without other autoimmune disorders associated with AAD	1 (14.3%)	11 (37.9%)	0.384

352 *Significant at 0.05 threshold

353 AAD : autoimmune Addison's disease ; APECED: auto-immune polyendocrinopathy candidiasis
 354 ectodermal dystrophy; APS : autoimmune polyendocrine syndrome ; NA: not applicable T1D: type
 355 1 diabetes; TPO : thyroperoxidase ; Tg : thyroglobulin ; ZnT8A : zinc transporter type 8
 356 autoantibodies.

357 **Table 2:** Comparison of children and adult populations, with positive ZnT8A status (mean±
 358 sem or effective (percentage of patients)).

359

	Children <18 years (n=19)		Adults ≥ 18 years (n=17)		P-value [†]
	ZnT8A- positive group	ZnT8A- negative group	ZnT8A- positive group	ZnT8A- negative group	
Patients' characteristics					
n	4 (21.1%)	15 (78.9%)	3 (17.6%)	14 (82.4%)	1.000
Sex (women/men)	3/1	11/4	2/1	10/4	1.000
Age (years) at ZnT8A dosage	12.0 ± 2.1	11.9 ± 0.9	28.7 ± 1.5	33.3 ± 3.8	NA
ZnT8A titer (U/mL)	181.7 ± 110.7	5.3 ± 1.0	361.5 ± 295.2	2.2 ± 0.5	0.629
Auto-immune diseases					
Addison disease diagnosis at time of ZnT8A dosage	2 (50.0%)	13 (86.7%)	3 (100.0%)	13 (92.9%)	0.429
Total T1D	3 (75.0%)	3 (20.0%)	2 (66.7%)	2 (14.3%)	1.000
Total autoimmune disorders associated or not with AAD:	4 (100.0%)	10 (66.7%)	2 (66.7%)	8 (57.1%)	0.429
<i>T1D alone with or without AAD</i>	2	3	1	2	
<i>Other autoimmune disorders</i>	2 (whose 1 T1D + hypothyroidism)	7	1 (whose 1 T1D + hypothyroidism)	6	
Without other autoimmune disorders associated with AAD	0 (0.0%)	5 (33.3%)	1 (33.3%)	6 (42.9%)	0.429

360 [†] P-values concerning comparison between ZnT8A-positive group in children versus ZnT8A-

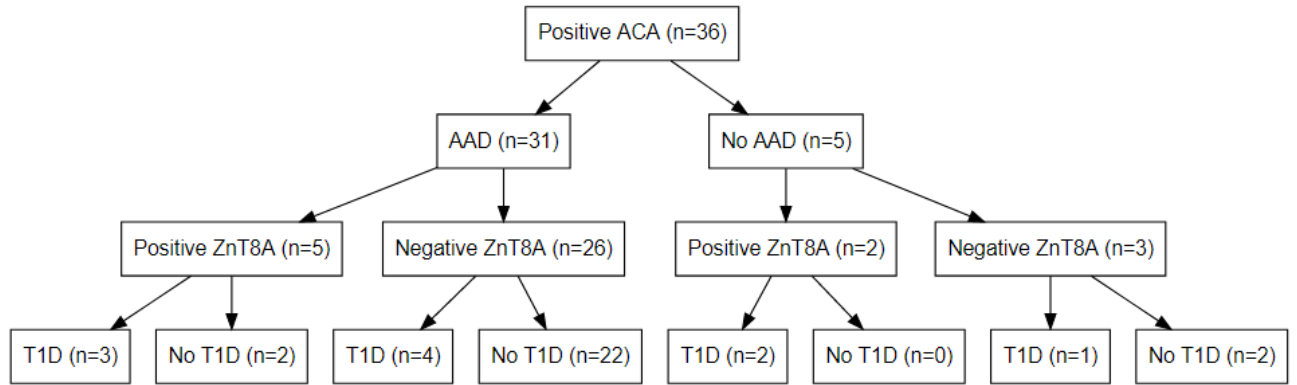
361 positive group in adults.

362 AAD : autoimmune Addison's disease ; NA: not applicable T1D: type 1 diabetes ; ZnT8A :

363 zinc transporter type 8 autoantibodies.

364 **Figure 2:** Flowchart according to the presence of autoimmune Addison's disease, ZnT8A and
365 T1D.

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368 ACA, anti adrenal cortex antibodies ; AAD, autoimmune Addison's disease; ZnT8A, zinc

369 transporter type 8 autoantibodies; T1D, type 1 diabetes.