Original Article

Screening for Insulitis in Adult Autoantibody-Positive Organ Donors

Peter In't Veld, Dirk Lievens, Joeri De Grijse, Zhidong Ling, Bart Van der Auwera, Miriam Pipeleers-Marichal, Frans Gorus, and Daniel Pipeleers

Antibodies against islet cell antigens are used as predictive markers of type 1 diabetes, but it is unknown whether they reflect an ongoing autoimmune process in islet tissue. We investigated whether organs from adult donors that are positive for autoantibodies (aAbs) against islet cell antigens exhibit insulitis and/or a reduced β-cell mass. Serum from 1,507 organ donors (age 25–60 years) was analyzed for islet cell antibodies (ICAs), glutamate decarboxylase aAbs (GADAs), insulinoma-associated protein 2 aAbs (IA-2As), and insulin aAbs. Tissue from the 62 aAb$^+$ donors (4.1%) and from matched controls was examined for the presence of insulitis and for the relative area of insulin$^+$ cells. Insulitis was detected in two cases; it was found in 3 and 9% of the islets and consisted of CD3$^+$/CD8$^+$ T-cells and CD68$^+$ macrophages; in one case, it was associated with insulin$^+$ cells that expressed the proliferation marker Ki67. Both subjects belonged to the subgroup of three donors with positivity for ICA, GADA, and IA-2-Ab and for the susceptible *HLA-DQ* genotype. Comparison of relative β-cell area in aAb$^+$ and aAb$^-$ donors did not show a significant difference. Insulitis was found in two of the three cases that presented at least three aAbs but in none of the other 59 antibody$^+$ subjects or 62 matched controls. It was only detected in <10% of the islets, some of which presented signs of β-cell proliferation. No decrease in β-cell mass was detected in cases with insulitis or in the group of antibody$^+$ subjects. *Diabetes* 56:2400–2404, 2007

Type 1 diabetes results from a specific and major loss of insulin-producing β-cells presumably through a T-cell–mediated process (1–5). At clinical onset, patients present circulating autoantibodies (aAbs) against islet cell antigens, which can appear many years before hyperglycemia is established and which are therefore used for prediction of the disease. In first-degree relatives of type 1 diabetic patients, the risk for developing the disease is higher when multiple positivity is present for aAbs against the islet cell cytoplasm (islet cell antibodies [ICA]), insulin (insulin aAbs [IAA]), the 65,000 M_r isoform of glutamate decarboxylase (glutamate decarboxylase aAbs [GADA]), or the insulinoma-associated protein 2 tyrosine phosphatase (insulinoma-associated protein 2 aAbs [IA-2A]) (6–12). Antibody positivity is therefore used for patient recruitment in prevention trials, but it is still unknown whether it corresponds to an insulitis process in the pancreas and, if so, for which combination. There are only few studies available on the histopathology of the pancreas in antibody$^+$ nondiabetic individuals (13,14). In the four reported cases, no leukocytic islet infiltrate or signs of β-cell damage were noticed; three of them were GADA$^+$ patients with polyendocrinopathy, and one was an IA-2A$^+$ organ donor. In the present study we investigated the pancreas in 62 aAb$^+$ organ donors. This larger series allowed us to identify two cases with insulitis, one of whom presenting signs of β-cell proliferation, and to correlate these histopathological findings to the small subgroup of patients with three or four aAbs and a high-risk genotype.

RESEARCH DESIGN AND METHODS

Collection of pancreatic tissue. Pancreas biopsies were obtained from the Beta Cell Bank, which operates for a clinical trial on islet cell transplantation in Belgium (15,16). They were taken as part of a quality control procedure that was approved by the ethics committees of the Belgian Diabetes Registry and participating hospitals. Tissue (~0.5 cm^3) was excised from the body region of cold-preserved (UW flushed) donor organs that were provided by Eurotransplant Foundation (Leiden, The Netherlands). It was fixed in 4% (vol/vol) phosphate-buffered formaldehyde, pH 7.4, or Bouin's fixative; embedded in paraffin; and then histologically analyzed. Between 1989 and 2004, a total of 1,507 biopsies were collected from patients aged 25–60 years for whom serum or plasma was also available for islet cell antibody assays. For none of these donors was diabetes mentioned in the donor information sheets.

Analysis of donor blood for aAb and genetic risk markers for type 1 diabetes. Serum samples were prospectively tested for the presence of ICA, IA-2A, and GADA and retrospectively for IAA (17). ICAs were assessed by indirect immunofluorescence, and end-point titers were expressed as Juvenile Diabetes Foundation units (JDFU). IA-2A, GADA, and IAA were measured by liquid-phase radiobinding assay and expressed as the percentage of tracer bound in hemolysis-free sera. Cutoff values for antibody positivity were calculated as the 99th percentile of antibody levels in 790 nondiabetic controls after omission of outlying values (minimally 12 JDFU for ICA, 0.4% for IA-2A, 2.6% for GADA, and 0.6% for IAA). The aAb assays were validated in successive Immunology of Diabetes Workshops and international proficiency testing programs; all positive results were confirmed in a separate subsequent assay (17). Whole blood was haplotyped for DNA polymorphisms at the *HLA-DQA1* and *DQB1* gene loci, and DQ-associated risk was stratified as reported (18).

Screening for insulitis. For detection of insulitis, paraffin sections were immunohistochemically double stained for leukocyte common antigen (LCA) (using mouse anti-LCA from Dako [Glostrup, Denmark]) and the pan-neuroendocrine marker synaptophysin (rabbit anti-synaptophysin; Dako). Binding was detected with biotinylated anti-mouse or anti-rabbit Ig (Amersham, Little

From the Diabetes Research Center, Brussels Free University and Juvenile Diabetes Research Foundation Center for Beta Cell Therapy in Diabetes, Brussels, Belgium.

Address correspondence and reprint requests to Peter In't Veld, PhD, Department of Pathology, Brussels Free University, Laarbeeklaan 103, B-1090 Brussels, Belgium. E-mail: intveld@vub.ac.be.

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aAb, autoantibody; GADA, glutamate decarboxylase autoantibody; IAA, insulin autoantibody; IA-2A, insulinoma-associated protein 2 autoantibody; ICA, islet cell antibody; LCA, leukocyte common antigen.

TABLE 1
Characteristics of aAb$^+$ donors

Case subject	Age (years)	Sex	BMI (kg/m^2)	aAb titers ICA	GADA	IA2-A	IAA	*HLA DQA1*-DQB1** haplotype1/ haplotype2	HLA risk
Positivity for minimally two autoantibodies									
1	59	Male	22	100	1,067.2	2.6	1.7	**0501–0201/0501–0201**	**S**
2	46	Female	21	50	213.5	54.6	<	**0301–0302/0301–0302**	**S**
3	44	Male	24	100	1,928.6	42.6	<	**0301–0302/0501–0201**	**S**
4	50	Female	21	50	567	<	<	02–0202/**0501–0201**	P
5	54	Male	30	25	163.9	<	<	01–0602/**0301–0302**	P
6	49	Male	28	25	3.6	<	<	01–0501/01–0501	N
7	39	Male	26	12	<	1.0	<	0501–0301/0501–0301	P
Positivity for only one of the tested autoantibodies									
$n = 55$	46 (26–60)*	33/22	25 (18–30)*	$n = 19$	$n = 27$	$n = 3$	$n = 6$	NT	
Matched aAb$^-$ controls									
$n = 62$	46 (25–60)*	38/24	24 (18–31)*	$n = 0$	$n = 0$	$n = 0$	$n = 0$	NT	

aAb titers are expressed as JDFU (ICA) or as percentage of tracer binding (GADA, IA2-A, and IAA); <, below cutoff value. *Median (range). HLA risk haplotypes in bold. N, neutral HLA-DQ genotype; NT, not tested; P, protective HLA-DQ genotype; S, susceptible HLA-DQ genotype.

Chalfont, U.K.) and, respectively, streptavidin horseradish peroxidase or alkaline phosphatase complex (Dako), using diaminobenzidine or new fuchsin as substrate. Sections were also double stained for insulin and glucagon (guinea pig anti-insulin and rabbit anti-glucagon were a gift of Dr. Van Schravendijk [Brussels Free University, Brussels, Belgium]). For each case, an average of 180 ± 16 (means ± SE) islets were screened. Insulitis was arbitrarily defined as an infiltrate of ≥15 LCA$^+$ cells within the islet (central insulitis) or directly surrounding the islet (peri-insulitis). This number was set after determining mean and range of the number of LCA$^+$ cells per islet in 62 islet aAb$^-$ controls: 0.35 ± 0.04 (range 0–7) LCA$^+$ cells per islet (1,550 islets investigated); the "insulitis" level was set at twice the maximum number encountered in these controls. Although arbitrary and therefore susceptible to discussion, this definition provides a more quantitative basis than that used in other studies for comparing the occurrence of insulitis. Only one study on human insulitis has defined insulitis on the basis of the number of leukocytes per islet (Gianani et al, [14] examined 14 normal controls to determine the number of leukocytes in the 10 islets with the largest mononuclear infiltrate).

Characterization of leukocytic infiltrates. Leukocytic infiltrates were immunophenotyped on paraffin sections using immunofluorescent double and triple staining with the following antibodies: rabbit anti-CD3 and anti-LCA (Dako), mouse anti-CD4 and anti-CD8 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), and mouse anti-CD20 and anti-CD68 (Dako). Binding was visualized with anti-rabbit fluorescein isothiocyanate or anti-mouse Cy3 (Jackson Immunoresearch, Soham, U.K.) and examined in an Axioskop M fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with an Orca AG camera (Hamamatsu, Japan) and Smartcapture imaging software (Digital Scientific, Cambridge, U.K.). For negative controls, primary antibodies were omitted; positive controls were conducted on paraffin-embedded human tonsils.

Quantification of relative β-cell area and β-cell proliferation. Relative insulin$^+$ cell area was measured according to Rahier et al. (19) on coded slides using a 266-point counting grid in 10 randomly chosen microscope fields at a final magnification of ×140. The number of points hitting insulin immunoreactive cells (N_i) and pancreatic parenchyma (N_p) were counted in 10 randomly chosen microscope fields per case. Relative β-cell area was expressed as a percentage and calculated as $(N_i/N_p) \times 100$. All morphometric analyses were carried out blinded on coded slides. Two-color immunohistochemistry using antibodies for Ki67 (Dako) and insulin was used to determine the percentage of proliferating β-cells. Reproducibility of the point-counting technique was evaluated with the formula of Weibel (19); the calculated relative error in each section was ~11% for a mean relative β-cell area of 1.2%.

RESULTS

Screening of organ donors for risk markers of type 1 diabetes. Testing for the four IAAs resulted in 62 positive cases (aAb$^+$) of 1,507 donors in the age-group of 25–60 years (4.1%). Most cases were positive for a single aAb ($n = 55$), with only four double, two triple, and one quadruple positive case(s) (Table 1). The three donors with ≥3 aAbs displayed a susceptible HLA-DQ genotype, whereas the four cases with only two aAbs exhibited a neutral or protective HLA-DQ genotype (Table 1). We observed lower aAb titers in the single aAb cases than in the multiple aAb cases (results not shown), probably as a result of their high fraction of "statistical positives " (1% cutoff). A control group of 62 aAb$^-$ donors was selected from the total donor group by matching for age, sex, and BMI.

Screening of organ donors for insulitis and histopathology of positive cases. Of the 62 antibody$^+$ and 62 antibody$^-$ donors, only two cases presented islets with insulitis as defined under RESEARCH DESIGN AND METHODS; they belonged to the small subgroup ($n = 3$) with positivity for ≥3 aAbs (Table 1). Case subject 1 (M-59y) died 10 h after hospitalization for a subdural hematoma (plasma glycemia 6.4 mmol/l at admission), and case subject 2 (F-46y) died 43 h after hospitalization for subarachnoidal hemorrhage (plasma glycemia 8.2 mmol/l at admission).

In case subject 1, 5 of the 58 examined islets (9%) showed peri- or central insulitis. Four of these islets contained both insulin$^+$ and glucagon$^+$ cells, whereas one was insulin negative and mainly composed of glucagon$^+$ cells. No other insulin$^-$/glucagon$^+$ islets were detected in this donor. In case subject 2, 27 of 917 islets (3%) presented insulitis (Fig. 1*A* and *B*), all islets containing insulin$^+$ and glucagon$^+$ cells. Another 3% of the islets were insulin negative and mainly composed of glucagon$^+$ cells; these islets did not present signs of insulitis (Fig. 1*C*).

In both cases, the infiltrating cells predominantly corresponded to CD3$^+$CD8$^+$ T-cells (Fig. 1*G–I*) and CD68$^+$ macrophages (Fig. 1*F*), with a few CD20$^+$ B-cells and CD3$^+$CD4$^+$ T-cells detected (Table 2).

β-Cell surface area and proliferation in donors with high-risk markers for type 1 diabetes. When the average β-cell surface area in antibody$^+$ donors with ≥2 aAbs was compared with that in donors with a single aAb or that in antibody$^-$ controls, no significant difference was noted (Table 3). Individual values in case subjects with multiple aAbs fell within the range of the control group.

The average percentage of insulin$^+$ cells that were also positive for the proliferation marker Ki67 was very low (<1‰) in the three groups. The range in aAb negatives was 0–7‰. Only one aAb$^+$ case presented a value outside this range (49‰). This case was characterized by insulitis and positivity for the four aAbs (case subject 1); the Ki67$^+$

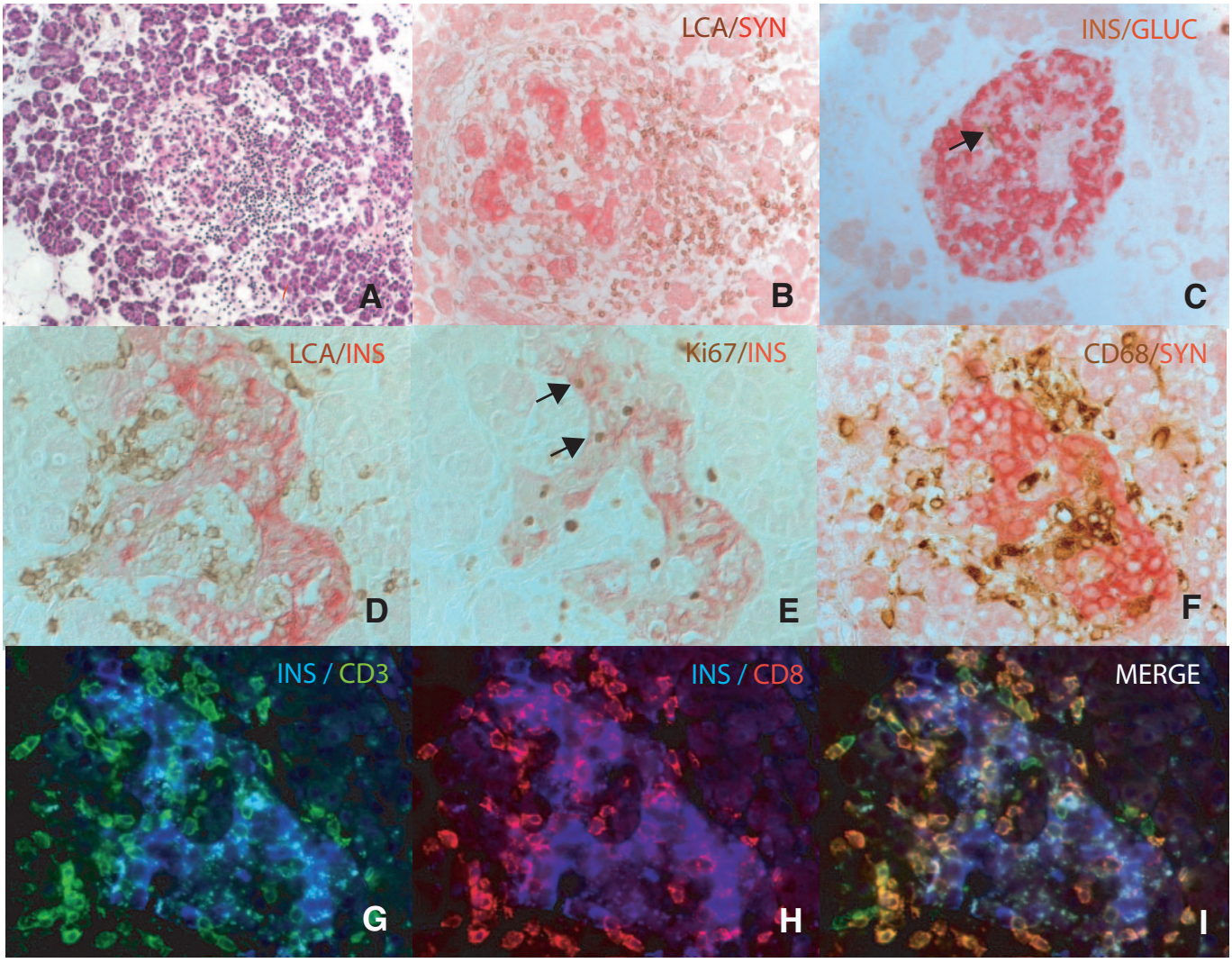

FIG. 1. Histology of the islets of Langerhans in adult organ donors with multiple (≥3) aAbs against islet cell antigens. Islets from case subject 2 show infiltration by mononuclear cells (*A*: hematoxylin-eosin staining; *B*: immunostaining of a consecutive section for LCA and the pan-neuroendocrine marker synaptophysin [SYN]). Double staining for glucagon (GLUC) and insulin (INS) shows that some islets predominantly exist of glucagon$^+$ cells with only a few insulin$^+$ cells (*C*, arrow). In case subject 1, double staining of consecutive sections for LCA and insulin (*D*) or Ki67 and insulin (*E*) shows the presence of Ki67$^+$ cells in insulitic islets, with some cells coexpressing both insulin and Ki67 (arrows). The leukocytic infiltrate consists of both CD68$^+$ macrophages (*F*) and lymphocytes. *G–I*: Immunofluorescent triple staining of an infiltrated islet for insulin, CD3, and CD8 shows that the infiltrate predominantly consists of CD3$^+$CD8$^+$ T-cells.

cells were only noticed in islets with a leukocytic infiltrate (Fig. 1*D* and *E*; Table 3).

DISCUSSION

In the present study, we have screened for insulitis in pancreatic tissue from 62 adult organ donors carrying aAb risk markers for type 1 diabetes. Insulitis was detected in two of three cases with at least three aAbs, be it in <10% of the islets. It was not found in any of the 59 case subjects with only one or two aAbs or in any of the 62 antibody$^-$ control subjects. Both case subjects also presented a susceptible HLA-DQ genotype. There were no signs of a reduced β-cell mass in the two insulitis case subjects nor in the group of aAb$^+$ donors. These data demonstrate that insulitis is a rare phenomenon in aAb$^+$ nondiabetic adults.

Insulitis is also a rare finding at clinical onset of type 1 diabetes after 30 years of age, which contrasts with its detection in all onset patients younger than 7 years of age (4). Although type 1 diabetes in adults is, by definition, characterized by the presence of aAbs, a combination of

TABLE 2
Immunophenotype of the infiltrating cells in islets with insulitis

Case subject	No. of islets with insulitis examined	No. of CD68$^+$ cells per islet	No. of LCA$^+$ cells per islet	No. of CD20$^+$ cells per islet	CD3$^+$		
					No. of cells per islet	CD3$^+$ CD4$^+$ (%)	CD3$^+$ CD8$^+$ (%)
1	5	24.4 (4–53)	63.0 (16–101)	4.0 (0–13)	53.6 (20–84)	3.8 ± 1.2	82.2 ± 2.8
2	11	42.3 (19–78)	58.0 (26–137)	7.4 (0–34)	60.5 (18–169)	3.1 ± 1.3	70.9 ± 2.8

Data are means ± SE (range).

TABLE 3
Insulitis, β-cell surface area, and β-cell proliferation in aAb$^+$ organ donors

No. of aAbs	Case subject	No. of islets with insulitis/total islets examined	Relative β-cell surface area [% (range)]	Ki67$^+$ INS$^+$ cells/INS$^+$ cells ‰ (range)
4	1	5/58	1.95	49*/0†
3	2	27/917	1.59	0
	3	0/99	1.46	0
2	4	0/116	1.50	0
	5	0/137	0.82	0
	6	0/141	0.79	0
	7	0/331	1.28	0
1	$n = 55$	0/10,223	1.13 (0.50–2.80)	0.4 (0–5)
0	$n = 62$	0/10,334	1.21 (0.51–2.61)	0.4 (0–7)

*Ki67$^+$ ins$^+$ cell number in islets with insulitis. †Ki67$^+$ ins$^+$ cell number in islets with no signs of insulitis.

three or more of such aAbs in association with a high-risk genotype is infrequent, especially in latent autoimmune diabetes in adults (20). The absence of insulitis in most adult aAb$^+$ donors does therefore not exclude progression to the disease.

The present observations should, however, be interpreted with caution in terms of their possible significance for the development of type 1 diabetes. The seven donors with more than one antibody risk marker were older than 38 years, an age that is not typical for the development of classical type 1 diabetes. Moreover, four of them presented with a protective HLA-DQ genotype that may have modulated an autoimmune response. It is nevertheless conceivable that the presence of insulitis in two subjects with four risk markers illustrates a stage in the disease process that might eventually lead toward a sufficient β-cell loss such that diabetes develops. However, no decrease in β-cell mass became apparent after measuring relative β-cell surface area. There were also no substantial numbers of pseudo-atrophic islets (1) as a remnant of prior destructions and a sign of self-limiting insulitis at an earlier age. It is conceivable that both subjects exhibit a low-intensity autoimmune process affecting only a small percentage of the islets. The occurrence of Ki67$^+$ β-cells in some of the infiltrated islets raises the possibility that β-cell proliferation can compensate for any losses. Such a subclinical autoimmune process may at a later stage result in slowly progressive type 1 diabetes or latent autoimmune diabetes in adults (21,22). The histopathology of late-onset type 1 diabetes has not been well studied. One case has been described: A 65-year-old female with positivity for two aAbs (GADA and IA-2A) and a HLA-DQ/DR risk profile was initially diagnosed with type 2 diabetes and then shown to present several islets with predominantly CD4$^+$ T-cell infiltrates, without signs of β-cell destruction (23). In the presently described cases, infiltrating leukocytes mainly corresponded to CD8$^+$ T-cells and CD68$^+$ macrophages, as was also the case in type 1 diabetic patients with insulitis (2,24–26).

Our data do not strengthen or weaken the significance of the detected circulating markers as predictors for type 1 diabetes. They indicate an association between triple antibody positivity with a high-risk genotype and an insulitis process in the pancreas. In elder individuals, this insulitis process appears limited to <10% of the islets and may thus not lead to type 1 diabetes or may lead only to a mild form. We cannot exclude that cases with a low percentage of infiltrated islets were missed as a result of our sampling in one region (body) and of the relatively small number of analyzed islet sections (averaging 180 islets per organ); more extensive sampling was precluded by the islet isolation procedure for which these organs were harvested. For the same reason, we may have missed differences in β-cell mass if these would have occurred in other regions.

Despite the limitations imposed by the small tissue specimen, the nature and extent of our study provide information with respect to the use of organs from adult aAb$^+$ donors for transplantation. Absence of histopathological changes in all 59 donors with one or two aAbs questions exclusion of these organs, while the detection of insulitis in triple antibody$^+$ donors can be seen as an exclusion criterion.

In conclusion, we have screened 62 nondiabetic aAb$^+$ organ donors older than 25 years for the presence of insulitis. Insulitis was found in two of the three case subjects who presented at least three antibodies but in none of the other 59 antibody$^+$ subjects or 62 matched controls. Presence of one or more antibodies was not related to a decrease in β-cell mass. These observations can be used to include or exclude organs from aAb$^+$ donors for transplantation in diabetic recipients. They also need consideration when recruiting adult aAb$^+$ subjects for prevention trials.

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