

Birth and coming of age of islet autoantibodies

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E. Bonifacio *† and

P. Achenbach*§

*Technische Universität Dresden, DFG Center for Regenerative Therapies Dresden, Dresden,

†Paul Langerhans Institute Dresden of the Helmholtz Center Munich at University

Hospital Carl Gustav Carus and Faculty of

Medicine, TU Dresden, Dresden, ‡Institute of Diabetes Research, Helmholtz Zentrum

München, German Research Center for

Environmental Health, Munich-Neuherberg,

and §Technical University of Munich, School of Medicine, Klinikum rechts der

Isar, Forschergruppe Diabetes, Munich,

Germany.

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Correspondence: E. Bonifacio, Center for Regenerative Therapies Dresden, Technische Universität Dresden, Fetscherstrasse 105, 01307 Dresden, Germany.

P. Achenbach, Institute of Diabetes Research, Helmholtz Zentrum München, Heidemannstr.

1, 80939 Munich, Germany.

E-mail: ezio.bonifacio@tu-dresden.de

E-mail: peter.achenbach@helmholtz-muenchen.de

Summary

This review takes the reader through 45 years of islet autoantibody research, from the discovery of islet-cell antibodies in 1974 to today's population-based screening for presymptomatic early-stage type 1 diabetes. The review emphasizes the current practical value of, and factors to be considered in, the measurement of islet autoantibodies.

Keywords: Autoantibodies, ICA, Islet-cell antibodies, Type 1 diabetes

Historical perspectives

Discovery of islet-cell antibodies

The concept of autoimmunity as a pathogenetic mechanism in a subgroup of patients with diabetes was first raised in the 1960s and early 1970s with the observation of insulinitis [1], and the association of juvenile-onset diabetes with certain human leukocyte antigen (HLA) alleles and T cell abnormalities [2–6]. The definitive autoimmune pathogenetic discovery was made in 1974, when two research groups in the United Kingdom reported the identification of islet-cell antibodies (ICA) in patients with so-called 'multiple organ-specific autoimmunity' [7,8].

The first of these publications was by Gian Franco Bottazzo and Deborah Doniach. This research group had discovered thyroid autoimmunity almost 20 years earlier [9], and several other autoantibodies [10–12], and had a treasure chest of samples from patients with various and multiple endocrine autoimmune diseases. Using indirect immunofluorescence, Bottazzo *et al.* detected ICA in these human pancreas samples (Fig. 1a). The manuscript was published in *The Lancet* in November 1974 [7]. The abstract stated: 'Antibodies to pancreatic islet cells were found by immunofluorescence in the sera of 13 patients with multiendocrine deficiencies associated with organ-specific autoimmunity. 10 of these patients were diabetic... The presence of organ-specific pancreatic antibodies supports

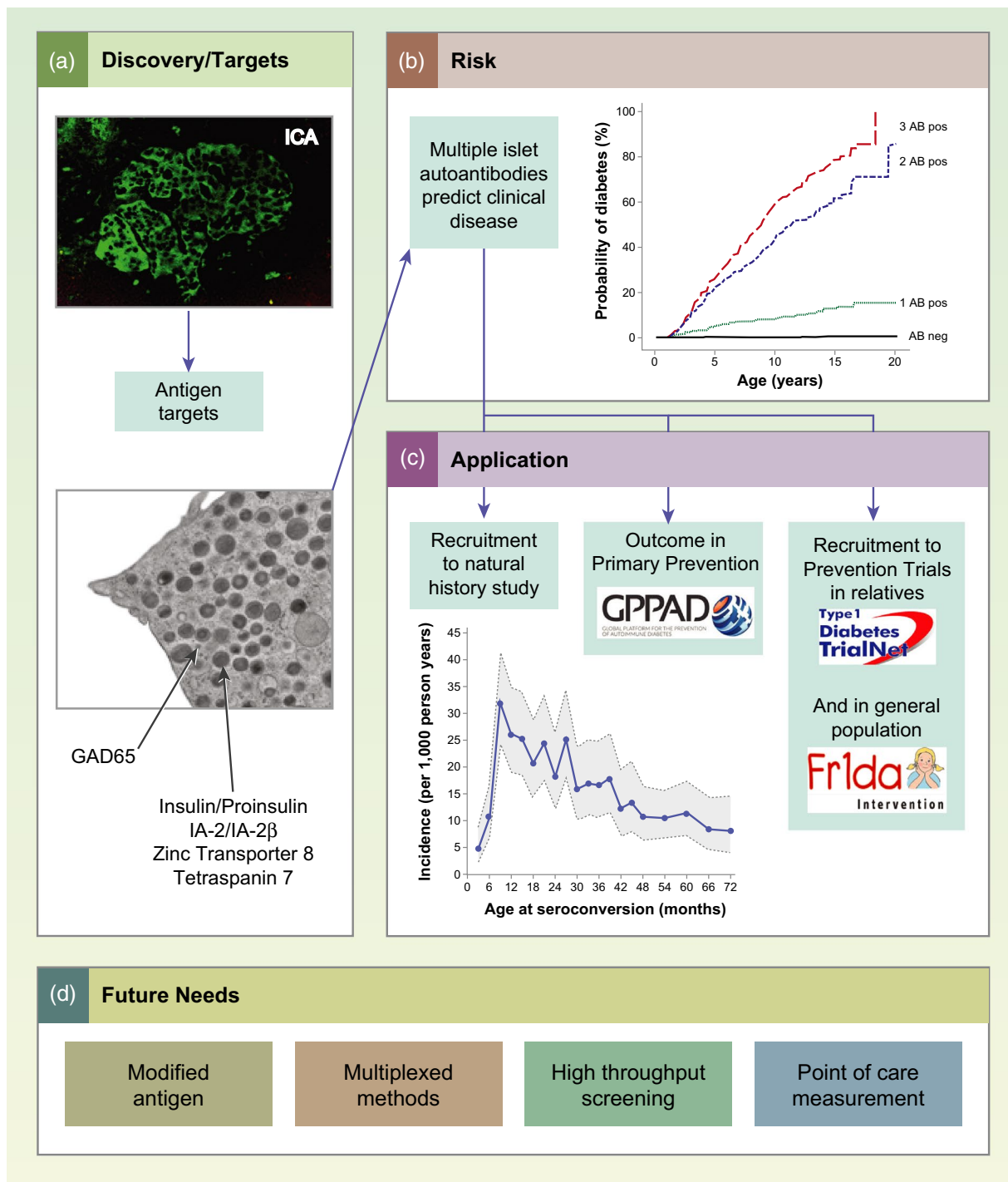


Fig. 1. (a) Discovery of islet-cell antibodies (ICA) and subsequent identification of major β cell autoantigen targets has provided diagnostic markers with which to distinguish autoimmune type 1 diabetes from other non-autoimmune types. (b) Probability of developing clinical type 1 diabetes increases with increasing numbers of different islet autoantibodies, and children with multiple islet autoantibodies will develop clinical diabetes. (c) Currently, islet autoantibody measurements are used to recruit study participants for natural history studies; as outcome markers in primary prevention trials; and for the recruitment of individuals to prevention trials. (d) Future requirements for islet autoantibody diagnostics are stated.

the hypothesis of an autoimmune form of diabetes mellitus put forward to explain the histological ‘insulinitis’ found in selected cases of this disease.’

The second publication was by William J. Irvine’s research group, which had previously reported T cell responses against pancreatic antigens in patients with diabetes [6].

His group also examined a collection of samples from polyendocrine patients, and published their work in *The Lancet* 1 month after the study by Bottazzo *et al.* [8]. Their abstract stated: 'Using an indirect immunofluorescence technique, circulating antibodies to pancreatic islet cells were found in the sera of 5 patients with insulin-dependent diabetes mellitus and coexistent autoimmunity... These findings provide further direct evidence to support the hypothesis of an autoimmune form of diabetes mellitus.'

Thus, the discovery and validation of ICA were reported by two independent research groups within the space of a month. These were discovered in polyendocrine patients rather than in typical patients with type 1 diabetes. Moreover, the classification of diabetes into types 1 and 2, which had been introduced decades earlier, had not yet taken root, and terms such as 'juvenile,' 'adult-onset,' 'insulin-dependent' and 'non-insulin-dependent' were used to distinguish age- and therapy-related forms of the disease. In 1975, Lendrum *et al.* reported ICA in the sera of 51 of 105 children with recent-onset diabetes [13], revealing an autoimmune pathogenetic component in a large proportion of childhood cases of diabetes.

Prediabetes

Perhaps the most important discoveries were those that led to the notion of a 'prediabetic' stage of the disease. Lendrum *et al.* examined ICA in the diabetic twin cohort of David Pyke and in 1976, reported that the antibodies could be present years before the onset of diabetes [14]. Also in 1976, Irvine *et al.* reported that the antibodies could precede diabetes onset by several years [15]. In 1981, Gorsuch *et al.* measured ICA in the first-degree relatives of patients with insulin-dependent diabetes and discovered that patients who developed insulin-dependent diabetes had ICA up to 30 months before the onset of diabetes [16]. These early findings eventually led to the notion that type 1 diabetes is a chronic autoimmune disease, as described by George Eisenbarth in 1986 [17].

Standardization

The number of methods for detecting ICA and reports of ICA has increased rapidly since the pivotal studies described above. These reports discussed complement-fixing antibodies [18], bovine-pancreas-positive ICA [19], the two-color fluorescence detection method [20], the protein-A detection method [21] and islet-cell-surface antibodies [22], among many others. What started as a clear concept soon became complex and confused. A workshop to standardize ICA measurements was convened, and after the sobering realization of how variable these measurements could be [23], an exemplary standardization program that introduced common standards [24] and

international units [25,26] was established, and was subsequently used for antigen-specific islet autoantibody measurements [27–29]. Importantly, the program gave credibility to the antibodies as markers of prediabetes [30–32] and many poorly performing detection methods became obsolete.

Islet-cell antibodies are heterogeneous and target multiple antigens

The ICA immunofluorescence test had become standard, but the identification of their target antigens became an urgent undertaking (Fig. 1a). MacCuish *et al.* had demonstrated T cell responses to insulin fragments in patients with and without insulin treatment in 1975 [33]. In 1983, Palmer *et al.* showed that children who developed type 1 diabetes had insulin autoantibodies (IAA) before they were treated with insulin [34]. This was an important breakthrough in the field. It also signaled the presence of multiple autoantibodies, because insulin is only expressed in pancreatic islet β cells, whereas all islet cells stained for ICA [7,8].

In 1982, Baekkeskov *et al.* reported autoantibodies against a 64-kDa islet protein [35,36], and in 1990 Christie *et al.* described autoantibodies against 40-kDa and 37-kDa fragments of islet proteins [37]. The 64-kDa target of autoantibodies was later identified as glutamate decarboxylase (GAD65) [38], a known antigenic target of autoantibodies in the neurological disorder stiff-person syndrome [39]. The 40-kDa and 37-kDa fragments were identified as ICA512 (now also known as IA-2) [40] and the related protein phogrin (also known as IA-2 β) [41], respectively, both of which were identified separately as the targets of autoantibodies in type 1 diabetes [42,43]. GAD65-directed autoantibodies (GADA) were shown to be part of the ICA reaction, with a β cell-specific staining pattern [44]. The IA-2-directed autoantibodies (IA-2A) were shown to be part of the pan-islet-cell staining of ICA [45]. Other proteins, such as ICA69, were claimed to be targets of ICA [46], but were not confirmed by other groups or in standardization workshops [47]. The lipid antigens GM2-1 and sulfatides were also reported to be targeted by ICA [48,49], but no methods have been developed for robust assessment of their validity. In contrast, the β cell zinc transporter 8 (ZnT8) protein has been confirmed to be a target of autoantibodies (ZnT8A) in more than 50% of patients with type 1 diabetes [50,51], and tetraspanin 7 was identified as the 38-kDa target of autoantibodies against glima [52,53]. These autoantibodies were present in more than 30% of patients with type 1 diabetes [54].

Prediction of clinical disease

The notion that ICA and other islet autoantibodies precede the onset of type 1 diabetes allows the prediction of future

disease. As early as 1977, Irvine's group showed that the presence of ICA identified adult patients treated with oral hypoglycemic agents who would later require insulin treatment [55]. Numerous studies, including a prominent study in triplets [56], had identified occasional cases of ICA-positive individuals who later developed diabetes, but it was not until 1988 that an analysis of the Barts–Windsor Family Study showed that it was indeed possible to estimate the risk in ICA-positive relatives of patients with type 1 diabetes [57]. This was followed by the establishment of risk estimates using the standardized international units for ICA [30]. The higher the titre of ICA, the higher the risk that an ICA-positive relative would develop type 1 diabetes. The risk reached 100% within 10 years in relatives who had ICA titres of > 80 Juvenile Diabetes Foundation (JDF) units/ml. These studies provided the foundation for later prevention trials in ICA-positive first-degree relatives [58].

The inclusion of IAA, GADA and IA-2A further improved our ability to stratify the risk of type 1 diabetes. The first reported use of autoantibody combinations to improve diabetes prediction was in twins in 1992, when a combination of ICA, IAA, GADA and antibodies against the 37-kDa and 40-kDa fragments was used [59]. This was followed in 1994 by a study in relatives of patients with type 1 diabetes, which found that 8% of relatives with ICA only and 88% of those with ICA plus IAA, GADA or antibodies to the 37-kDa or 40-kDa fragments developed diabetes [60]. It is noteworthy that not all the antibodies are useful in every situation. For example, the prediction of insulin requirement in adult-onset diabetes is made by testing for ICA [61], GADA [62] and IA-2A [63], but IAA are rare in patients in this age group [64].

Antibody combinations were subsequently used to select at-risk relatives for clinical trials [58,65], and it is now well established that the diabetes risk associated with the presence of multiple islet autoantibodies (two or more of IAA, GADA, IA-2A and ZnT8A) is markedly greater than the risk in people with a single autoantibody [66–69]. A landmark study, that involved combined analysis of more than 13 000 individuals from three birth cohorts, demonstrated that almost all children with genetic susceptibility to type 1 diabetes who developed multiple islet autoantibodies progressed to diabetes (Fig. 1b) [70]. This has paved the way for population-based screening [71].

Natural history of islet autoantibodies

Prospective birth-cohort studies have made invaluable contributions to our knowledge of the appearance and progression of islet autoantibodies in childhood, including the German BABYDIAB Study [72], the Finnish DIPP Project [73], the DAISY from Colorado [74] and the TEDDY Study [75], which have now been running for up to three

decades. These studies have shown that in genetically predisposed children, autoantibody seroconversion occurs relatively frequently between the ages of 6 months and 3 years, with the incidence of autoantibodies peaking at an age of 1 year (Fig. 1c) [76–78]. The typical natural history of type 1 diabetes in children is the appearance of the first high-affinity autoantibody [79], which is usually IAA in the youngest children, followed by the appearance of other islet autoantibodies [80], usually within 3 years [81], and eventually the development of diabetes. Two islet-autoimmunity endotypes are distinguished [82]. One is characterized by the first appearance of IAA in children carrying HLA-DR4, and occurs in the first years of life. The second is characterized by the first appearance of GADA in children carrying HLA-DR3, and is the endotype most frequently observed in children who seroconvert after age 2 years. Based on the different associations of the two endotypes and environmental factors, it has been suggested that the endotypes have different etiologies [82]. However, age is an important confounder and it is possible that these differences are merely age-related. In contrast to IAA and GADA, IA-2A usually occurs together with autoantibodies against other β cell antigens and is therefore a very specific and highly predictive immune marker for progression to clinical type 1 diabetes [83,84], particularly if its reactivity spreads to epitopes on the homologous protein IA-2 β [84–86]. ZnT8A also usually appears later in the development of the disease [87].

Practical perspectives

Antibody titre, affinity and specificity

There are differences in the target autoantigens and epitopes, the titres, affinities and subclasses of islet autoantibodies. These characteristics are associated with the subject's age and HLA genotype, and in some cases can help to distinguish diabetes-associated islet autoantibodies from non-disease-associated autoantibody signals [88].

The intensity and maturity of the antibody response are reflected in the antibody titre, affinity, immunoglobulin (Ig)G subclass, and target epitopes on single or multiple islet autoantigens. Islet autoantibodies with high titres usually involve multiple IgG subclasses and are directed against multiple epitopes on the target antigen. Similar to ICA [30], high titres of IAA [64,84] or IA-2A [84] are associated with faster progression to clinical type 1 diabetes. Moreover, IAA or IA-2A responses that include IgG2, IgG3 and/or IgG4 as well as IgG1 are associated with an increased risk, even if the antibody titres are not high. By combining these antibody characteristics, the 5-year diabetes risk in islet-autoantibody-positive relatives can be stratified from less than 10% to more than 90% [84].

The affinity (binding strength) of the autoantibody to the target antigen is closely related to the intensity of the antibody response. Accordingly, high-affinity islet autoantibodies are associated with progression to clinical type 1 diabetes, even if the antibody titre is relatively low, whereas low-affinity antibodies are unrelated to the development of diabetes, even if the capacity and titre of the antibody are high [79,89–92]. Consistent with their high disease specificity, IA-2A are characterized by high affinity [92]. In contrast, both IAA and GADA can range in affinity by more than 1000-fold [79,89–91]. The highest affinities are $> 10^{11}$ l/mol. For IAA and GADA the low- and high-affinity autoantibodies appear to bind to different epitopes [79,90,91]. For example, high-affinity IAA require the preservation of amino acids 8–13 in the insulin A chain to bind to human insulin, and also bind proinsulin. In contrast, the majority of low-affinity IAA are dependent on the COOH-terminal residues of the insulin B chain and usually do not bind proinsulin [79]. Low-affinity antibodies are seen more frequently in individuals who do not have a strong genetic susceptibility to type 1 diabetes and in children who remain positive for only IAA or GADA [79,80,93]. The affinities and epitope specificities of IAA and GADA can be used to stratify the progression to type 1 diabetes [79,90,94,95], and those for GADA can predict insulin therapy in individuals with adult-onset diabetes [96,97]. The spread of IA-2A reactivity against epitopes on the homologous IA-2 β protein is associated with the rapid development of diabetes [84–86].

Therefore, it is useful to identify and/or exclude low-affinity signals in risk screening for clinical trials, particularly in individuals with only IAA or GADA, who may be at an early stage of the disease process and may progress to producing multiple islet autoantibodies [79,80,90]. The identification of markers associated with the risk of progression from single to multiple islet autoantibodies has been investigated in studies within the TrialNet Consortium [68,98–103]. Genetic risk may also be used to select single islet autoantibody-positive children who are most likely to progress to producing multiple islet autoantibodies [68,102,103]. A low GAD autoantibody titre is associated with a low risk of progression to multiple islet autoantibodies [98].

Why do multiple antibodies or multiple tests work?

Multiple antibodies and multiple tests have the mathematical advantage of increasing the a priori probability of a true result in the samples selected for the second measurement, as may be expected from Bayes' theorem [104]. This can be illustrated theoretically in the example shown in Fig. 2. The example assumes that 0.3% of

preschool children are true positives and will develop type 1 diabetes. In a population of 100 000, this corresponds to 300 children. A single islet autoantibody measurement (e.g. IAA) with a sensitivity of 70% will identify 1000 children when its threshold is set to the 99th percentile of the population. These children will have a 21% risk (positive predictive value) of developing type 1 diabetes. A second test with similar characteristics will identify a similar number of children with a similar risk. However, there will be a marked enrichment of future cases of type 1 diabetes in the children who have both autoantibodies: there would be 155 children with both IAA and GADA, 147 of whom would develop diabetes (95% risk; 49% sensitivity); and of 1690 with only IAA or GADA, 126 would develop diabetes (7.5% risk; 42% sensitivity). Adding more antibodies, such as IA-2A, would identify multiple islet autoantibodies in another 70% (88) of children with a single IAA or GADA who will develop diabetes, thereby increasing the sensitivity (78%) with only a slight reduction in the risk. Adding another antibody (e.g. ZnT8A) will provide a limited improvement in test performance. In reality, IAA and GADA, etc. are not completely independent and there are age relationships, so it is not quite this simple. Nevertheless, for many children who develop the disease, the high risk associated with multiple antibodies has more to do with Bayes' theorem and perhaps less to do with a multiple-hit disease process that progresses from single to multiple antibodies. A similar principle applies when a second test is performed (e.g. an electrochemiluminescence assay [105,106], luciferase immunoprecipitation system assay [107,108] or IAA affinity assay [109,110]) using samples that were previously identified as positive on a radiobinding assay. A very similar outcome would also be expected if the order of the tests were reversed. The thresholds for the different antibodies can also be adjusted to obtain the best possible combination of sensitivity and risk [111].

Modelling islet autoantibody profiles

Prospective studies have shown that the natural progression to type 1 diabetes is not uniform in children and adolescents. Based on the individuals' different genetic backgrounds and environments, islet autoimmunity may develop at different ages, show different longitudinal autoantibody profiles and progress to clinical diabetes at various rates. Today, we are unable to predict the individual's progression exactly or to link etiological factors to the dynamics of islet autoantibody patterns over time. However, recent studies have started to develop mathematical algorithms to model complex longitudinal autoantibody profiles and stratify progression rates [112,113]. Children who develop multiple islet autoantibodies can be clustered

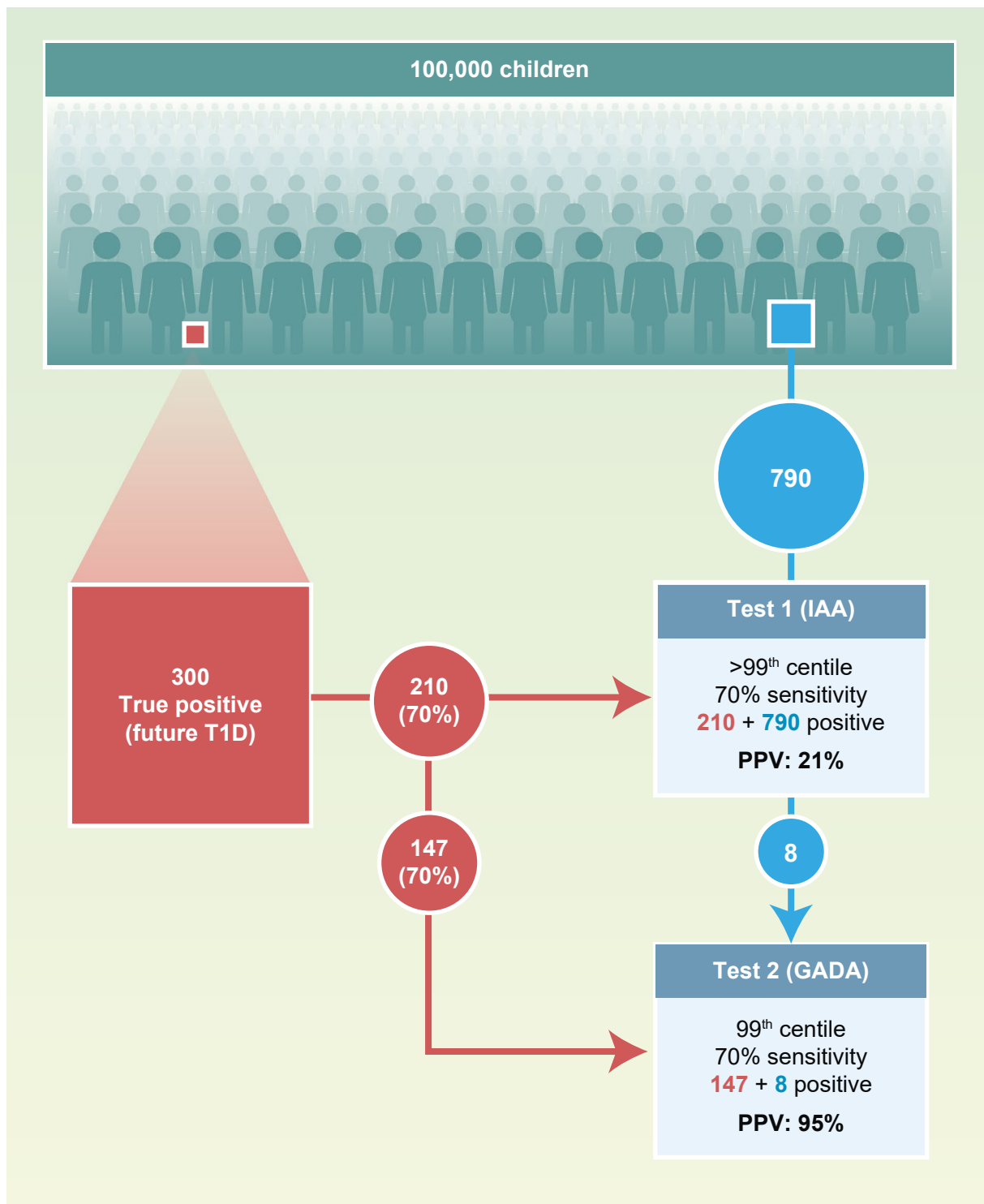


Fig. 2. Illustration of how the application of Bayes' theorem to islet autoantibodies can confer high positive predictive values in the diagnosis of future type 1 diabetes using multiple islet autoantibodies. A population of 100 000 unselected children, including 300 (0.3%) who will develop type 1 diabetes, is tested for insulin autoantibodies (IAA) with a test that has a threshold selected at the upper 99th percentile (1% positive) and 70% sensitivity. Under these assumptions, 210 of the true future cases of type 1 diabetes will be identified (filled red box) and 790 of the 99 700 who will not develop type 1 diabetes will be positive (filled blue box). This provides a positive predictive value [(PPV) or risk] of 21%. A second test with a similar threshold and sensitivity [e.g. for glutamic acid decarboxylase antibodies (GADA)], applied to the same 100 000 children, will yield a similar number of positives and predictive value. However, among the IAA-positive children, there will be a marked enrichment of true positives who are also GADA-positive, yielding a PPV of 95%.

according to their longitudinal profiles, and it has been shown that the likelihood of progressing from seroconversion to clinical diabetes within 5 years ranges in these clusters from below 10% to above 80%. Those children who seroconverted in the first years of life and expressed stable IAA and IA-2A responses had the highest risk of diabetes. Interestingly, this risk was unaffected by the child's GADA status [113]. A cluster analysis also revealed that losing IAA reactivity was associated with delayed progression to type 1 diabetes in children who were positive for multiple islet autoantibodies [112]. Mathematical approaches applied to data from prospective cohorts have strong potential utility as novel tools for the stratification of islet-autoantibody-positive individuals and offer new opportunities to clarify the disease mechanisms.

Islet autoantibodies used to select for trials and as study outcomes

Clinical trials to investigate the treatment of type 1 diabetes commonly use the participant's autoantibody positivity as an inclusion criterion (Fig. 1c) [58,65,114]. In trials that recruit individuals with clinical diabetes, islet autoantibodies are used to distinguish type 1 diabetes from other types. In addition to the recruitment of trial participants, islet autoantibodies have been used as outcome markers in several studies (Fig. 1c). These include natural history studies such as TEDDY [115] and primary prevention studies such as BABYDIET [116], TRIGR [117] and POInT [118]. The availability of high-quality, high-throughput and harmonized autoantibody tests [119] mean that stable longitudinal measurements of the outcomes are possible. As discussed in above, the definition of outcome can be improved by including confirmation with a second laboratory test or the use of multiple assays.

The age at screening is also important. For first-degree relatives of patients with type 1 diabetes, the risk of developing islet autoantibodies decreases exponentially with age, with a half-life of 3–4 years [120]. This has practical implications. First, if we consider that the peak incidence of islet autoantibody seroconversion occurs in the first 3 years of life, screening is likely to be most effective in preschool years. Secondly, relatives who remain negative through to their teenage years will have an eightfold lower risk of developing islet autoantibodies than they had when they were born.

Extension to the population at large

The development of multiple islet autoantibodies has long been recognized as a critical step in the pathogenesis and diagnosis of type 1 diabetes [59,60], culminating in the finding that almost all children who develop multiple islet

autoantibodies will develop clinical symptomatic diabetes, regardless of whether they have an a priori family history of the disease [70]. This has led to a new staging strategy for type 1 diabetes, in which the presence of multiple islet autoantibodies is now used as a criterion for the diagnosis of presymptomatic early-stage type 1 diabetes [121]. An early diagnosis of type 1 diabetes can prevent the severe metabolic decompensation that is frequently observed at the onset of clinical diabetes [122–124]. Screening for islet autoantibodies can be performed with capillary blood samples or dried blood spots [125–128]. The Fr1da Study started in 2015 as a model project investigating public health screening for early-stage type 1 diabetes (confirmed with positivity for multiple islet autoantibodies) in Bavaria, Germany [71]. It assesses: (1) whether the early diagnosis of type 1 diabetes in the context of regular medical check-ups in childhood is feasible and efficient; (2) whether ketoacidosis and the hospitalization of children can be prevented by screening; and (3) whether psychological distress can be reduced with the early diagnosis of diabetes, education and care. Similar studies have already commenced in Lower Saxony, Germany, with additional screening for low-density lipoprotein–hypercholesterolemia (Fr1dolin Study) [129] and in Colorado, with additional screening for celiac disease (ASK Study) [130].

The future

We envisage two areas of activity in the next few years (Fig. 1d). From a practical perspective, a technology is required that facilitates the widespread use of islet autoantibody testing for the diagnosis of presymptomatic type 1 diabetes in the public health context [131]. From the research perspective, activities to identify modified protein targets, both to generate better assays and to identify pathogenetic disease mechanisms, are highly likely.

Technological advances should drive down costs and allow simple high-throughput screening, which will favor its widespread application. Cost is a clear factor, because population-based screening requires that tens or hundreds of thousands of children be tested, more than 99% of whom will be negative. This will require a sensitive first-line test that covers the majority of the major islet autoantibodies (IAA, GADA, IA-2A, ZnT8A), with follow-up tests for those who are positive to confirm and stratify their risk [71,127,132,133]. Point-of-care testing may be one approach to achieving this. This technology should be coupled to careful application of Bayes' modeling, including additional risk factors such as genetics [134], family history and age. This sort of modeling will be possible once much larger numbers of children have been tested and followed, emphasizing the need to introduce broad testing programs in many regions and countries.

Modified islet antigens have been reported in the literature [135,136], but we conclude that a number of these are unlikely to be validated because of weaknesses in the assays. Increased antibody binding to a modified form of the tetraspanin 7 protein was observed in some patients [137], but it is difficult to determine whether this is a favored *in-vivo* target or an artificial *in-vitro* modification. Smart systems that reliably identify antibodies that bind to proteins from unperturbed and perturbed islets should be possible, and will probably reveal a range of variations in the autoantibody–autoantigen targets that we know today.

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