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REVIEW

Immunological biomarkers for the development and progression of type 1 diabetes

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Abstract

Immune biomarkers of type 1 diabetes are many and diverse. Some of these, such as the autoantibodies, are well established but not discriminative enough to deal with the heterogeneity inherent to type 1 diabetes progression. As an alternative, high hopes are placed on T cell assays, which give insight into the cells that actually target the beta cell or play a crucial role in maintaining tolerance. These assays are approaching a level of robustness that may allow for solid conclusions on both disease progression and therapeutic efficacy of immune interventions. In addition, 'omics' approaches to biomarker discovery are rapidly progressing. The potential emergence of novel biomarkers creates a need for the introduction of bioinformatics and 'big data' analysis systems for the integration of the multitude of biomarker data that will be available, to translate these data into clinical tools. It is worth noting that it is unlikely that the same markers will apply to all individuals. Instead, individualised signatures of biomarkers, combining autoantibodies, T cell profiles and other biomarkers, will need to be used to classify at-risk patients into various categories, thus enabling personalised prediction, prevention and treatment approaches. To achieve this goal, the standardisation of assays for biomarker discovery, the integration of analyses and data from biomarker studies and, most importantly, the careful clinical characterisation of individuals providing samples for these studies are critical. Longitudinal sample-collection initiatives, like INNODIA, should lead to novel biomarker discovery, not only providing a better understanding of type 1 diabetes onset and progression, but also yielding biomarkers of therapeutic efficacy of interventions to prevent or arrest type 1 diabetes.

Keywords Autoantibodies · Bioinformatics · Biomarker · Immune · Review · T cell assays · Type 1 diabetes

Abbreviations

FOXP3	Forkhead box P3
GAD	Glutamic acid decarboxylase
IA-2	Islet antigen-2
IDS	Immunology of Diabetes Society
miRNA	MicroRNA
PBMC	Peripheral blood mononuclear cells
Teff	Effector T cells
Treg	Regulatory T cells
ZnT8	Zinc transporter-8

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Introduction

Despite major scientific efforts, the pathophysiology and exact pathways involved in beta cell destruction in type 1 diabetes remain elusive. Several hurdles to our understanding of the disease exist, including the absence of a fully reliable animal model of the disease and the fact that the target organ, the beta cell, is small and well-hidden in the pancreas and, thus, inaccessible for histological assessment in a clinically acceptable manner [1]. What we have learned is that type 1 diabetes is an immune disease, with there being a major role for the immune system in beta cell destruction. In line with this, one of the challenges we are now faced with is to find biomarkers to monitor the behaviour of the immune system in a way that is clinically useful in type 1 diabetes. Although we know that most of the immune activity leading to beta cell destruction takes place in the pancreas and the pancreatic lymph nodes, most of the work on biomarker discovery has focused on the most accessible tissue: the peripheral blood. Hence, the most obvious way forward is to combine biomarkers of immune (dys)function and biomarkers of beta cell (dys)function in type 1 diabetes.

In the present review, we focus on the clinical value of established immune biomarkers in type 1 diabetes, such as HLA genotypes and autoantibodies against beta cell antigens, and also discuss emerging biomarkers, including those discovered using rapidly progressing T cell assays and omics approaches. Whilst touching upon the potential of these novel assays, we caution against having high expectations in the short-term as these assays must be tested for robustness and reproducibility outside the expert discovery laboratories before being ready for 'prime time'. Finally, we outline how the introduction of bioinformatics and 'big data' analysis systems will be crucial for integration of the multitude of biomarker data that will be available in the future. This final step is essential for the translation of these data into clear signals that may aid in a better understanding of type 1 diabetes and, equally as important, into clinical tools for the prediction of disease progression in individuals at risk or living with type 1 diabetes.

Established biomarkers: genes and autoantibodies

Genetic biomarkers

Genetic markers are used to assess predisposition for type 1 diabetes, with studies of large cohorts leading to the identification of over 50 loci contributing to type 1 diabetes risk [2]. However, in analogy with other autoimmune diseases, routine screening for genetic risk for type 1 diabetes is done mainly by HLA typing. In particular, *HLA-DR* (*DR3/4*) and *HLA-DQ* (*DQ8*) genotypes are useful in predicting the risk for developing beta cell autoimmunity. The highest risk *HLA-DR/-DQ* genotypes are present in around 30–40% of individuals with type 1 diabetes and around 2–3% of the background population, thereby increasing the risk by over tenfold relative to the background population. HLA typing alone is, therefore, useful for the enrichment of future cases of type 1 diabetes, but is insufficiently sensitive and specific to be a biomarker for future prevention strategies.

Additional susceptibility loci have much smaller associated risk than *HLA-DR/-DQ*. Nevertheless, they have been shown to improve sensitivity and specificity compared with HLA alone [3]. Indeed, combinations of genetic markers into a genetic risk score do remarkably well to identify infants who are at increased risk for islet autoimmunity, with risk reaching over 10% in the highest risk categories [3]. However, almost 90% of those identified through genetic markers never develop autoimmunity and fewer than half of the cases are identified using genetic-marker combinations. Therefore, additional markers, such as immune correlates or outcomes of the genetic risk, may be helpful.

Autoantibodies

Autoantibodies against beta cell proteins and peptides are now used almost routinely to predict disease and help diagnose type 1 diabetes. Although they do not directly contribute to the pathogenies of the disease, the scientific community has now widely accepted autoantibodies as the hallmark of type 1 diabetes. Their recognition as biomarkers of presymptomatic disease has led to proposals for early type 1 diabetes staging using a range of islet autoantibodies for diagnosis, a concept that is starting to make its way into practice [4].

The autoantibodies currently used as biomarkers of type 1 diabetes in the clinic are mainly those targeting insulin, glutamic acid decarboxylase (GAD), a tyrosine phosphatase-like protein (islet antigen-2 [IA-2]) and zinc transporter-8 (ZnT8). The development of autoantibodies against multiple beta cell antigens is recognised as a critical step in the disease pathogenesis and is associated with a significantly higher type 1 diabetes risk than the presence of just a single autoantibody [5, 6]. Moreover, multiple beta cell autoantibody-positive individuals are at high risk of type 1 diabetes regardless of family history, and children who develop two or more beta cell autoantibody types almost inevitably progress to clinically symptomatic diabetes [7].

The time of progression from presymptomatic to clinical type 1 diabetes varies from weeks to decades in multiple-autoantibody-positive children [7, 8], presenting an opportunity to identify biomarkers that improve prediction. Autoantibody characteristics that allow for stratification of diabetes risk include age at seroconversion, antibody number, titre, affinity, antigen specificity and epitope binding [9, 10]. Using various combinations of these antibody characteristics, multiple-

autoantibody-positive individuals can be stratified by 5 year diabetes risk, ranging from <10% to ~90% [11]. The age of autoantibody development can also be used to stratify individuals with regards to the likelihood of quick progression to clinical diabetes, with more rapid disease progression being observed in children who develop islet autoantibodies early [12]. Of interest, children who develop islet autoantibodies very early in life usually present with insulin autoantibodies first, whilst those who develop autoimmunity later in life present with GAD autoantibodies first [13]. Unfortunately, whilst a useful marker of diabetes progression, age at islet autoantibody development is generally unknown, other than in children who participate in prospective studies from infancy.

Although use of these autoantibodies has reached routine clinical practice, many assays for their measurement exist, with quality varying between them. Exact and reproducible autoantibody measurement is a prerequisite for accurate prediction of type 1 diabetes and diagnostic autoantibody testing. The Islet Autoantibody Standardization Program (IASP), previously known as the Diabetes Antibody Standardization Program (DASP) [14], conducts workshops aimed at the standardisation of islet autoantibody assays and the evaluation of laboratory performance. These are supervised by the Immunology of Diabetes Society (IDS) and organised by the TrialNet Islet Cell Autoantibody Core Laboratory at the University of Florida. Before using an islet autoantibody assay for type 1 diabetes risk assessment and diagnosis, its performance should be ascertained in the IDS-based international workshops.

Recently, novel autoantibodies have been described, including those targeting neo-antigens generated in beta cells under conditions of stress (e.g. immune stress, metabolic stress, etc). These include antibodies against modified beta cell-derived peptides or proteins generated through stress-induced post-translational modifications, like citrullination [15]. Although these novel autoantibodies may help us to understand the pathogenesis of type 1 diabetes, it is as yet unclear how they will contribute to better detection of autoimmunity or prediction of disease progression.

In summary, whilst autoantibody screening can be used to establish an increased risk of type 1 diabetes, since the time from seroconversion to diagnosis can vary from weeks to decades, additional biomarkers that may be used before or after seroconversion to predict rapid vs slow progression would be valuable.

T cell biomarkers: our progress so far

The rationale for T cell biomarkers

Whilst several components of both the innate and adaptive immune systems are implicated in the beta cell destruction that leads to type 1 diabetes, current evidence suggests that T cells are the main mediators [16, 17]. Currently, the natural history of type 1 diabetes is routinely monitored using measurements of glucose metabolism (insulin or C-peptide) to assess residual beta cell function, but the change in these biomarkers lags behind the destructive process. Whilst measurements of islet-specific autoantibodies provide a useful biomarker of future disease, autoantibodies do not play a direct pathogenic role and have shown limited use in monitoring disease progression in immunotherapy trials. Assays that measure the frequency and/or functional capacity of T cells, which are associated with beta cell destruction, are therefore uniquely placed to gain key insights into the pathogenesis and progression of type 1 diabetes. Such T cell biomarkers will increase our understanding at each stage of disease, from the initial loss of tolerance to progression to clinical disease. They will also provide insight into the rate of beta cell loss following diagnosis. T cell biomarkers are also becoming a vital component of immunotherapy trials in type 1 diabetes, identifying logical targets for intervention, providing novel insights into why (or in whom) treatments succeed or fail and providing potential for participant stratification [18].

Measuring T cells in type 1 diabetes

T cell biomarkers in type 1 diabetes can be broadly divided into two categories: antigen specific and antigen non-specific.

Antigen-specific assays Antigen-specific assays aim to enumerate and phenotype T cells with reactivity towards islet antigens. CD4 T cells are commonly detected by measuring proliferation, cytokine production, or upregulation of markers associated with cellular activation following incubation of peripheral blood mononuclear cells (PBMC) with recombinant islet antigens or peptides. Most assays use ‘classical’ antigens, like peptides originating from preproinsulin, but as with novel autoantibodies, T cell reactivity against neo-epitopes has also been described (e.g. against hybrid peptides present in beta cells) [19]. CD4 and CD8 T cells can also be detected using soluble, multimeric MHC molecules loaded with islet peptide (p-MHC) which, when combined with multiparameter flow cytometry, enables the enumeration and phenotypic characterisation of these cells. In many cases, whilst these technologies demonstrate that beta cell-specific T cells are readily detectable in peripheral blood of individuals with type 1 diabetes, similar cells are also detected using these methods in individuals without diabetes who lack evidence of pathological autoimmunity [16, 20]. However, careful phenotyping has revealed important differences in the differentiation and polarisation of these cells depending on the clinical state of participants. For example, compared with individuals without diabetes, islet cell-specific CD4 T cells in type 1 diabetes are more proliferative and less reliant on co-stimulation, which is suggestive of previous in vivo activation [21]. In type 1

diabetes, these cells typically secrete higher levels of proinflammatory cytokines (including IFN-γ, granulocyte-macrophage colony-stimulating factor [GM-CSF] or IL-17) [22, 23], although controversy exists regarding the precise role that each of these cytokines play in islet destruction. In contrast, T cells secreting the immunosuppressive cytokine IL-10 are characteristic of those without disease, those who develop type 1 diabetes at a later age or those who show a beneficial clinical response following antigen-specific immunotherapy [24, 25]. These findings suggest that the balance of responses, rather than the presence of islet-specific T cells per se, is key in determining the rate of beta cell destruction. Similarly, although islet-specific CD8 T cells can be detected in both individuals with and without type 1 diabetes, studies have suggested that they are increased in frequency and have a more antigen-experienced phenotype and enhanced effector function in individuals with type 1 diabetes, and these features inversely correlate with a positive outcome following immunotherapy [26, 27]. Recently, the field is moving towards use of single-cell omics platforms to gain deeper insight into the functional phenotype of islet-specific T cells. This approach has already yielded novel findings, which suggest that the proinflammatory signature of islet-specific CD4 T cells is established early in life, pre-dating autoantibody production, and may be targetable by immune intervention [28].

Non-antigen-specific assays Disease-relevant biomarkers can also be derived by measuring the frequencies of specific T cell subsets and studying their functional characteristics or transcriptional profiles. T cell frequencies are routinely measured using multiparameter flow cytometry, using either PBMC or whole blood samples. This approach delivers rich and robust datasets from limited biological material. Such studies have identified biomarkers associated with progression to clinical type 1 diabetes and subsequent beta cell destruction. For example, an increased frequency of follicular helper cells has been observed before and at the time of type 1 diabetes diagnosis and may inversely correlate with C-peptide levels [29, 30]. Similarly, biomarkers of clinical efficacy (as indicated by a slower rate of C-peptide decline) have been identified in participants following immunotherapy, including increased levels of anergic or exhausted CD8 T cells following treatment with teplizumab (anti-CD3) [31], an increased frequency of central memory CD4 T cells following abatacept therapy [32] and increased levels of forkhead box P3 (FOXP3) in subsets of memory regulatory T cells (Treg) following peptide immunotherapy [25]. Additional testing of these biomarkers in other settings will be required to establish if they are general biomarkers of beta cell decline or treatment specific.

The functional potential of T cell populations can also be tested through a range of in vitro assays, to reveal key biomarkers. For example, although there is no difference in the frequency of CD4 FOXP3$^+$ Treg, the ability of these cells to control autologous effector T cells (Teff) is significantly reduced in individuals with type 1 diabetes, both before and after clinical diagnosis. Moreover, this functional deficiency appears to be influenced by type 1 diabetes susceptibility loci, suggesting that it may play a causative role in disease pathogenesis [33].

Detailed investigations have highlighted both Teff resistance to suppression and intrinsic Treg dysfunction (in at least a subset of individuals). This has led to the discovery of tractable biomarkers, such as reduced Treg stability and altered transcriptional signature and altered cytokine signalling (decreased IL-2 signalling in Treg and increased IL-6 signalling in Teff) [34–38], but has also revealed therapeutic opportunities (e.g. low dose IL-2 administration or anti-IL-6R therapy).

Scaling for widespread use

Despite their importance, developing standardised T cell biomarkers for routine use in type 1 diabetes remains a challenge. The inability to biopsy the site of tissue damage and the low frequency and affinity of islet-specific T cells in peripheral blood (typically 10–100-fold lower than pathogen-specific T cells) remain major hindrances. Emerging single-cell technologies will allow for deeper insights to be gained using lower sample quantity requirements, but these may have limited widespread use due to their high cost. Standardisation of immune phenotyping, as has been so successfully performed in the immune-oncology field, is a pressing need that will allow for easier comparisons of studies and faster confirmation of biomarkers using independent validation cohorts. Standardisation of sample preparation and preservation will also be required especially when assessing biomarkers of functional activity. A coordinated, collaborative approach, including large international consortia, will be necessary for progress in this area, to maximise the potential of T cell biomarkers in type 1 diabetes.

Emerging biomarkers: omics

Various omics technologies have been used to map the changes that occur during the course of type 1 diabetes development. Analysis of longitudinal samples from individuals with increased risk for type 1 diabetes and samples from their carefully matched controls have revealed that changes associated with the development of type 1 diabetes can be detected very early on in disease progression. Longitudinal samples, collected from birth to the appearance of autoantibodies and clinical type 1 diabetes diagnosis, have enabled the discovery of innate and adaptive immunity-related transcriptome signatures before seroconversion. [28, 39, 40] Also, recent studies have reported serum proteomes as indicators of disease progression in individuals with type 1 diabetes, during various stages of the disease, from early infancy to seroconversion and

diagnosis [41–43]. In such studies, special attention should be given to the selection of adequate reference samples as age has a profound effect on the proteome, especially during early childhood [44–46]. The same is true for many of the emerging omics analyses.

Of special interest are the T and B cell repertoires, which can now be measured at the single-cell level. These could provide the opportunity to track emerging populations of cells during an individual's life. For example, it may be possible to link new clones of CD8 memory T cells with specific infections or autoantibody patterns that link the environment (e.g. infections, pollution) and autoimmunity. It may also be possible to identify T or B cell receptors that are autoantigen- or disease-specific in the peripheral blood. Furthermore, the ability to obtain T or B cell receptor profiles and gene expression profiles from the same cell makes it possible to determine the phenotype of individual clones. Thus far, these studies are in their infancy and relatively few samples have been analysed, but it is an exciting opportunity for the identification of type 1 diabetes biomarkers, especially if applied to sequential samples from multiple individuals.

MicroRNAs (miRNA; small non-coding RNAs) act as another set of potential immune biomarkers. Studies analysing the miRNA expression profiles in serum or plasma of people with type 1 diabetes have revealed altered miRNA signatures, some reflecting beta cell dysfunction, whilst others correlate with immune activation [47]. However, due to a lack of strictly standardised sample processing procedures and circulating miRNA analysis guidelines, only few studies are reproducible. Levels of miRNAs are very sensitive to pre-analytical procedures associated with sample collection and processing. Before being able to judge their value as a biomarker, more work using rigorously standardised analytical approaches and demonstrating the tissue origin of the different miRNAs described will be needed.

Metabolomic and lipidomic signatures are likely to reflect changes in immune and metabolic status, which will be informative with respect to the pathogenesis of type 1 diabetes. They will also likely yield a new class of immune markers. Metagenomic studies are also emerging [48] and the opportunity to link metabolomic profiles to microbiome analysis may provide insight into how the environment and immunity may shape each other. Finally, it is imperative that we trace changes back to the genetics of type 1 diabetes since many of the changes observed are strongly influenced by genomic variation, as was recently shown for DNA methylation [49].

The range of omics approaches currently available, along with emerging novel ones, provide exciting opportunities for revealing new insights into the pathogenesis of type 1 diabetes. Further development of advanced methods for data analysis and integration are urgently needed to enable interpretation of large and varied datasets. Longitudinal measurements, as well as computational nonlinear analysis methods, are likely to provide promising biomarker candidates that complement the existing ones. Targeted detection of selected analytes may, in turn, lead to the identification of useful markers, such as those for participant stratification and follow-up of individuals to assess their response to treatment.

The need for longitudinal and integrated biomarker studies

Most progress in immune-biomarker discovery is owing to the establishment of longitudinal cohorts of individuals willing to donate tissue, mainly blood, for many years. These cohorts has been derived from various sources, ranging from studies of genetically at-risk individuals from birth (e.g. The Environmental Determinants of Diabetes in the Young [TEDDY] Study, BABYDIAB, and the Type 1 Diabetes Prediction and Prevention [DIPP] Study) to studies of newly diagnosed individuals with type 1 diabetes (e.g. the Belgian Diabetes Registry [BDR]).

In Europe, several interesting projects are ongoing, gathering blood and other accessible samples, such as urine and stool samples, enabling biomarker discovery. One of these projects, the Global Platform for the Prevention of Autoimmune Diabetes (GPPAD; www.gppad.org/de, accessed 19 August 2018), collects biomaterial in neonates and early childhood in the general population, thus focusing on biomarkers predicting autoimmune disease development in young children, and is aimed at intervening using a prevention trial. Another project, INNODIA (Translational approaches to disease modifying therapy of type 1 diabetes: an innovative approach towards understanding and arresting type 1 diabetes; www.innodia.eu, accessed 19 August 2018), is focusing specifically on novel biomarker discovery in type 1 diabetes. INNODIA was conceived with the idea of bringing academic researchers, from basic to clinical research, together with industry and to involve the most important stakeholders: the people with type 1 diabetes and their families, from the beginning. Throughout Europe, INNODIA is collecting, in a standardised manner, blood samples and data from newly diagnosed individuals with type 1 diabetes and their first-degree relatives, to learn more about the pathogenesis of type 1 diabetes and to find new biomarkers of type 1 diabetes. What is innovative is that these samples are collected in a standardised manner, using tools that allow assays coming from the basic research in INNODIA to be applied without delay. A suite of standardised assays, ranging from genetic screening, autoantibody determination and fresh T cell analyses, to a suite of novel biomarkers, including miRNAs and several omics assays, including proteomics and metabolomics, is run on these samples. All clinical data are centralised, together with data coming from basic research laboratories (e.g. sequencing and omics data), in a secure INNODIA database, thus establishing a systems biology

platform allowing integrated analysis of all data and subsequent modelling of type 1 diabetes in silico.

Conclusions

Biomarkers of the destruction of beta cells by the immune system in type 1 diabetes are needed to guide our understanding of the disease and help us to evaluate the effects of immune interventions aimed at preventing type 1 diabetes or arresting disease progression. Autoantibodies against beta cell antigens (insulin, GAD, IA-2 and ZnT8) have come to age and are now used as robust biomarkers that aid clinicians in the diagnosis of people with type 1 diabetes, in particular when presenting with diabetes in adulthood, where the differential diagnosis between type 1 and type 2 diabetes is sometimes difficult. Moreover, a combination of autoantibodies (potentially combined with genetic markers in the future), allows for relative risk assessment of progression to type 1 diabetes, both in family members of people with type 1 diabetes and in the general population.

However, more robust and subtle immune biomarkers are needed to better understand disease progression in individuals with type 1 diabetes, as heterogeneity in this process clearly exists. Here, high hopes are placed on T cell analyses that, through the standardisation of assays, are now approaching a level of robustness that allows for solid conclusions to be made with regards to disease progression. These T cell assays may also open avenues for biomarkers of therapeutic efficacy of immune interventions in type 1 diabetes, obviating the need for longitudinal, expensive clinical trials.

Novel biomarkers are emerging, including those from proteomic, metabolomic and transcriptomic (including miRNA and single-cell T cell) profiling. Advanced computational methods are required to analyse and integrate large datasets and guide interpretation of data. Analysis of longitudinal samples, first for discovery and then, importantly, for validation of selected sets of candidate biomarkers will be crucial in future research.

Finally, it is important to acknowledge that type 1 diabetes is not a unidirectional disease where the immune system attacks the passive and innocent beta cell. Indeed, clear indications are there to accept an active role of the beta cell in its own destruction. Biomarkers of beta cell stress and beta cell (dys)function are, therefore, as important as immune biomarkers in helping to deepen our understanding of the pathogenesis of type 1 diabetes. Moreover, these may also contribute to better stratification of individuals for trials of type 1 diabetes and help in the prediction of progression of disease. Early detection of the disease process is important to enable the timely introduction of prevention or treatment strategies. The identification of subsets of individuals with different underlying pathogeneses and differences in speed of disease progression will facilitate personalised prediction, prevention and treatment of type 1 diabetes.

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