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Serum biomarkers for diagnosis and prediction of type 1 diabetes

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Abstract

Type 1 diabetes (T1D) culminates in the autoimmune destruction of the pancreatic β-cells, leading to insufficient production of insulin and development of hyperglycemia. Serum biomarkers including a combination of glucose, glycated molecules, c-peptide, and autoantibodies have been well established for the diagnosis of T1D. However, these molecules often mark a late stage of the disease when ~90% of the pancreatic insulin-producing β-cells have already been lost. With the prevalence of T1D increasing worldwide and because of the physical and psychological burden induced by this disease, there is a great need for prognostic biomarkers to predict T1D development or progression. This would allow us to identify individuals at high risk for early prevention and intervention. Therefore, considerable efforts have been dedicated to the understanding of disease etiology and the discovery of novel biomarkers in the last few decades. The advent of high-throughput and sensitive '-omics' technologies for the study of proteins, nucleic acids, and metabolites have allowed large scale profiling of protein expression and gene changes in T1D patients relative to disease-free controls. In this review, we briefly discuss the classical diagnostic biomarkers of T1D but mainly focus on the novel biomarkers that are identified as markers of β-cell destruction and screened with the use of state-of-the-art '-omics' technologies.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by insulin deficiency as a consequence of autoimmune-mediated destruction of insulin producing pancreatic β-cells within the islets of Langerhans. Although a lifelong administration of exogenous insulin can help to balance glucose homeostasis in T1D patients to a certain degree, currently there are no effective curative therapies available for this disease. At the time of diagnosis, 80% to 90% of β-cell mass has been lost[1] and it is generally accepted that it would be extremely difficult to intervene or reverse the progression of T1D at this late stage.

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[2] Alarmingly, a steady increase in the number of diagnoses of T1D has been observed, with an average annual increase of 2–5% worldwide.[3] The age of symptomatic onset is usually during childhood or adolescent with a peak incidence rate at 12–14 years of age, but the symptoms can also develop at much later ages [4]. Moreover, T1D is often accompanied by sudden and acute complications. Concerns about their future health could lead to development of practical and emotional problems for patients and their families.[5,6] All of these concerns urgently demand more efficient means for early prediction, monitoring of progression, and eventually prevention or reversal of the disease especially at an early stage.

Biomarkers are indicators of normal and abnormal physiological or pathological processes and serve important roles in clinical diagnosis, prognosis, as well as monitoring therapeutic responses. The development of specific blood serum (or plasma) biomarkers are particularly attractive for most diseases because of the nature of blood circulation throughout the whole body, which serves as a carrier of molecules which were changed at localized pathological sites and are relatively easy to access compared to other biological fluids or pathological tissues.[7] However, the development of specific serum biomarkers, especially those reflecting pancreatic β-cell death or stress, has been challenging for T1D because of the fact that T1D is the result of autoimmune attack of β-cel Is, which are only about 0.002% of body mass.[8] Although T1D has been investigated for over a century, the etiology of the disease is still not fully understood. Until now, the biomarkers implemented in clinical practice such as glucose, HbAlc, c-peptide, and autoantibodies (AAb) were mainly diagnostic markers, although AAbs serve as relatively good prognostic markers of the risk of eventual development of the disease. While there has been advances in our understanding of the pathogenesis of T1D[9], we still do not have effective serum markers that can reflect the β-cell function, stress, β-cell mass, or serve as predictors of the progression of disease development. In this review, we will briefly discuss how these traditional biomarkers are used in the clinical diagnosis of T1D followed by the utility of other biomolecules which have resulted in a better understanding of T1D etiology. The contribution of advanced ‘-omics’ technologies leading to the discovery of potential serum biomarkers will also be discussed. Finally, we will discuss the potential biomarkers which can be used to predict disease, including the use of the best currently established AAb-based biomarkers and other novel predictive candidate biomarkers. **Figure 1** shows the major types of potential serum biomarkers for the diagnosis and prediction of T1D.

2. Serum diagnostic markers

Development of T1D involves many factors including genetics, environmental triggering and modifying factors. However, none of these factors can serve as a clear criterion for diagnosis. The current diagnostic biomarkers of T1D still rely on the consequences of hyperglycemia, such as the resulting high glucose or glycated hemoglobin, in combination with other T1D specific biomarkers that discern T1D from other subtypes of diabetes, such as low C-peptide levels or AAbs.

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2.1. Glucose-related biomarkers

Diabetes may be diagnosed with a random plasma glucose 200 mg/dL in patients with classic symptoms of hyperglycemia or hyperglycemic crisis, or two repeated positive results of glucose-related tests in the absence of unequivocal hyperglycemia. In the latter case, the blood-based glucose-related testing includes hemoglobin Ale (HbAlc) (a product of non-enzymatic glycation), the fasting plasma glucose (FPG), and oral glucose tolerance test (OGTT). Each of these tests has its own criteria, sensitivity, and specificity for the diagnosis of diabetes. The American Diabetes Association (ADA) criteria for diagnosis of diabetes is HbAlc 6.5%, or FPG 126 mg/dL, or 2-hour plasma glucose of OGTT 200 mg/dL. HbAlc test provides an indirect measure of the average blood glucose for the past 2 to 3 months, which is a reflection of chronic hyperglycemia, but it is not as sensitive as FPG and OGTT in diabetes diagnosis. FPG and especially OGTT results can be more variable and a close follow-up repeated test is often necessary.

2.2. Autoantibody-based biomarkers

Of the many types and subtypes of diabetes only 5–10% are classified as T1D. They usually can be differentiated from type 2 diabetes (T2D) and other subtypes based on the presence of specific AAbs. There are five commonly tested AAb markers used in the diagnosis of T1D[10–13] which include ICA (islet-cell cytoplasmic AAb), GADA (glutamic acid decarboxylase (GAD) AAb), IA-2A (insulinoma 2 (IA-2)- associated AAb), IAA (insulin AAb), ZNT8A (zinc transporter 8 AAb). At least 1 autoantibody is present in > 95% of individuals with T1D upon hyperglycemia detection.[14,15] Despite being identified through validated highly confident assays, the commonly tested AAbs have been shown to be insufficient for detection of all T1D cases as there remains a subset of patients who do not have the above listed AAbs at diagnosis, indicating either a possibility of insensitive tests or the potential of different types of autoantigen-mediated β-cell destruction in these T1D patients.

2.3. C-peptide

Insulin is first synthesized as preproinsulin which becomes proinsulin after cleavage of the signal peptide in the endoplasmic reticulum. Proinsulin is packaged into vesicles and is then cleaved into the active insulin hormone and C-peptide and both insulin and C-peptide are released in equimolar amounts from mature granules, together with small amounts of uncleaved proinsulin.[16] Due to hepatic uptake, insulin has a much shorter half-life time (3–5 min) compared to proinsulin and C-peptide. Stimulated serum C-peptide level (as a surrogate for insulin) has been considered a consistent and sensitive measure of β-cell function and can be used to help differentiate autoimmune-diabetes from other diabetes subtypes[17] With the discovery of more and highly prevalent complications, C-peptide test needs to be combined with other factors, such as BMI, for better interpretation of disease progression.[18] C-peptide measurement is also currently the most suitable primary outcome for clinical trials of therapies aimed at preserving or improving endogenous insulin secretion in T1D patients.[19] However, C-peptide measurement may not be as reliable with the increasing obesity epidemic.

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When diagnosing diabetes in the general population, it is most common to detect and evaluate hyperglycemia and blood glucose-related markers. In combination with AAb testing, T1D can be typically differentiated from other forms of diabetes. However, the manifestation of clinical T1D represents the near end-stage of β-cell destruction since only 10–20% of the insulin producing β-cells have been estimated to still be functioning at the time of diagnosis.[20] Therefore, it is imperative to have specific markers that can monitor and predict the disease progression.

3. Predictive biomarkers for T1D development

Prior to clinical T1D, the disease is typically preceded by an asymptomatic period of β-cell destruction that is highly variable in duration, ranging from months to decades.[15,21] It is important to study this silent period of autoimmune destruction of β-cells before the onset of clinical disc to gain understanding of its etiology and discover predictive or prognostic biomarkers 1 development or progression.

3.1. Autoantibodies

Autoantibodies (AAbs) are not only markers for diagnosis and disease classification, but also regarded as the current gold standard for the prediction of T1D development. It is still unclear what the exact role of islet AAbs is in T1D pathogenesis. Many consider it unlikely that islet AAbs are the cause of T1D but rather a reflection of disease progression or a secondary response. They provide proof of immune activation against certain autoantigens in the insulin producing β-cells in AAb individuals.

While the list of known autoantigen recognizing AAbs is still expanding, among all discovered AAbs, ICA, GADA, IAA, IA-2A and ZnT8A remain the most sensitive and specific, and are not difficult to measure thereby warranting their utility in T1D diagnosis and prognosis. The first-degree relatives (FDRs) of patients diagnosed with T1D or those having high-risk human leukocyte antigen (HLA) genotypes, are highly recommended to be screened for these AAbs to estimate the risk of T1D development. Seroconversion has a major impact on the accuracy of predicting T1D. After seroconversion to any two of the five common AAbs, an individual will almost always develop clinical T1D given sufficient time.[15] However the rate of progression depends on many factors, including type and number of AAbs, age, genotype, sex, fitness, etc.

Due to the heterogeneity of the disease, there is not a simple yes/no interpretation for T1D prediction. Autoantibody phenotype and numbers, epitopes, affinity, appearance order, combined with patients' genotypes and ages all contribute to the T1D risks.[22] For example, IAA and GADA are more frequently detected as the first AAb in children but often disappear in 25% and 10% of cases respectively in children at clinical onset. While only a small fraction of the children had IA-2A or ZnT8A as their first AAb and they all persist through diagnosis.[15,23–26] Each of these antibodies also vary in their affinities and epitope specificities, and these variations are connected with different risks for T1D.[27] Therefore, it makes sense to include the AAb phenotype and epitopes when defining the T1D risks. However, individuals positive for a single islet AAb are far less likely to develop T1D than individuals who are positive for multiple islet AAbs. Progression to T1D in children with a

single AAb is about 10% at 10 years, and the incidence of developing clinical T1D after more than 2 AAbs are present is 11% each year and >70% in the ensuing 10 years.[15,22,28] In general, the number of islet AAbs expressed by an individual is a more important predictor of T1D than any specific combination of islet AAbs.[29]

Despite the clear utility of the AAbs combined with genetic susceptibility in T1D prediction, they have several limitations.[30–32] First, AAb screening is only recommended for FDRs of T1D patients in the settings of clinical research studies[33] and more than 85% of people who develop T1D do not have family history of disease. Second, only a subset of the AAb-positive subjects will progress to clinical diabetes, all in different time frames, and the presence and levels of AAbs after seroconversion do not track disease progression. Therefore, it is critical to have additional biomarkers that can predict the stages of progression and identify the best timing for intervention and therapy. Finally, AAbs are also not useful as biomarkers for therapeutic outcomes. Consequently, in the last few decades, considerable efforts have been dedicated utilizing new technologies for the discovery of novel serum biomarkers that can serve as predictors of disease progression, β-cell function and mass, and functional monitoring of therapeutic responses.

3.2. Potential novel biomarkers

The development of high-throughput '-omics' technologies has provided some excellent platforms for the discovery of novel biomarker candidates by allowing a systems-wide coverage of molecular changes during disease progression. Often these studies are conducted using cultured primary cells,[34–36] immortalized cells,[37] mouse islets,[38] or with human pancreas tissues[39] including small quantity islets isolated by laser microdissection,[40,41] single islet sections,[42] or sorted cell sources.[43,44]. While many of these studies may not directly involve serum, they are useful in identifying potential serum biomarker candidates while also elucidating dysregulated pathways underlying the disease. These methods can aid in identifying novel biomarker candidates potentially useful for indicating the level of β-cell destruction, dysfunction, and mass as well as the ongoing immunological response in serum. In this part, we mainly discuss three types of novel biomarkers including proteins, nucleic acids, and metabolites. Figure 2 illustrates the types of markers and the main omics technologies or assay platforms applied in the discovery and validation of these types of serum biomarkers in T1D.

3.2.1. Protein biomarkers

3.2.1.1. Serum protein biomarkers—Proteins are the direct executors for all aberrant genetic changes and the identification of specific serum proteins as the source of biomarkers for specific diseases perhaps is the most accepted concept in the biomarker field. Proteomics analyses of protein/peptide expression and post-translational modifications (PTMs) for T1D has the potential to make significant contributions to aid in disease prediction and prognosis. Comprehensive analysis of the serum proteins is generally challenging because of the extreme complexity and high dynamic range of the serum proteome. This difficulty is especially true considering the extreme low abundance of potential biomarkers from disease-specific tissues or cells such as pancreatic β-cells. Proteomics analysis of serum is also complicated by the heterogeneity of the disease, individual variations, different disease

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stages, and the often subtle yet significant abundance changes. Therefore, well-designed strategies that incorporate sensitive proteomics technologies and experimental designs with statistical considerations are critical for addressing each of the aforementioned barriers. Perhaps due to the extremely low mass of pancreatic β cells in total body mass and the long progressive nature of β-cell loss, the identification of serum protein biomarkers specific to T1D is still in its early stage. Herein, we will present a brief discussion of the current proteomics workflow for biomarker development and a summary of candidate protein biomarkers reported for T1D from recent literature.

A general proteomics workflow used for the discovery of novel serum biomarkers usually consists of two phases: a discovery phase using bottom-up (peptide centric) proteomics to search for biomarker candidates, and a verification phase of selected candidates using targeted proteomics analysis and/or immunoassays. The extremely high dynamic range of serum proteome often requires the removal or depletion of major high-abundance proteins using immobilized antibodies in order to enhance the detection of low-abundance candidate biomarkers.[45,46] While such strategies make the low-abundance proteins relatively accessible, they could inadvertently remove proteins of possible interest through binding (specific or nonspecific) to targeted high-abundance proteins. A second strategy to deal with the high dynamic range issue is extensive serum protein/peptide fractionation, usually with chromatography methods that are orthogonal to the final dimension of low-pH reverse phase liquid chromatography (RPLC) before the MS analysis.[47] Both of these strategies have been employed or combined to profile serum proteome to a depth of several hundreds to thousands of proteins[48–53] from people without and with prediabetes/diabetes. However, the extensive fractionation decreases the analytical throughput of sample analysis and thus limits the number of samples to be analyzed. The throughput can be partially recovered by multiplexing with unique sample specific mass tags such as iTRAQ[54] (isobaric tag for relative and absolute quantification) or TMT[55] (tandem mass tags) and this approach has been implemented in longitudinal proteomics profiling of human plasma.[52,53] Typically, potential protein biomarkers that differ between cases and controls with statistical significance are then followed by verification with targeted proteomics and/or immunoassay in an independent cohort of samples. Table 1 lists the biomarker candidates that were reported from at least two independent research papers. Unfortunately, most of these proteins are highly abundant in plasma and lack specificity to pancreatic β cells and the overlap between the different studies is limited. Moreover, some of them showed opposite changes in terms of up/down expression regulation in different studies. The observed inconsistency and current lack of highly promising protein biomarkers could be a result of the limitations of the shotgun proteomics approach in identifying low abundance proteins specific to pancreatic islet cells and the heterogeneity of disease and individuals masking the “true biomarkers”. More in-depth profiling with advanced technologies along with improved experiment designs (e.g., longitudinal samples) will be necessary to discover more specific protein biomarkers that show statistically significant changes during disease progression.[56]

3.2.1.2. PTMs—The mechanisms underlying the breach of immune tolerance to β-cell antigens is still poorly understood. A prominent role for inflammation in cross-talk between the β-cell and immune system has been demonstrated in the pathogenesis of T1D.

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Inflammation triggers β-cell oxidative and endoplasmic reticulum (ER) stress, and may lead to alternative splicing and misfolding of β-cell proteins as well as post-translational modifications (PTMs).[57,58] Being different from the native proteins, the modified format of autoantigens or neoantigens (i.e., antigens expressed under specific conditions, rather than ubiquitously) maybe recognized as foreign and result in a breakdown of tolerance. More importantly, the newly discovered antigens can potentially be used as therapeutic agents to suppress autoimmunity in those at risk for the development of T1D, as well as in those with established disease who received islet replacement or regeneration therapy. Additionally, antibodies against these neoantigens could be supplementary to the current T1D AAb panel for diagnosis and prediction, since 2–5% of patients who are diagnosed in the clinic with T1D are found to be negative for common AAbs.[59] Despite the great potential of neoantigens in helping predict and prevent T1D, investigation of neoantigens is still in its infancy.

Increased recognition of post-translationally modified autoantigens by autoreactive T and B cells has been observed in other human autoimmune diseases, including rheumatoid arthritis and celiac disease. The similar PTMs observed in T1D include citrullination and deamidation of GAD65,[60] citrullination of ER chaperone 78 kDa glucose-regulated protein (GRP78),[61] deamidation of proinsulin peptide[62], ROS modified collagen type II (CM),[63] oxidative insulin,[64] phosphorylation of peripherin,[65] and formation of vicinal disulfide bond between adjacent cysteine residues of insulin A-chain.[66] PTMs of autoantigens enhance the immune response and recognition by T cells by either increasing the binding affinity to major histocompatibility complex class II (MHC II) molecules[63] or by modifying amino acids at T-cell contact positions.[60,66] These modified antigens activate antigen recognition by T cells isolated from T1D donors. More impressively, T-cell clones have been shown to respond specifically to these modified antigens which have been detected in the blood of mouse and human donors with prediabetes. Other types of modifications not previously mentioned that could play a role in immunogenicity, as observed in T1D or other autoimmune diseases, include phosphorylation[67], lipidation[68], glycation[69], etc. Another type of recently discovered novel antigen is hybrid insulin peptides (HIPs) which result from covalent crosslinking of proinsulin fragments with other peptides present in β-cell secretory granules.[70] HIPs are detectable in mouse pancreas and CD4 T cells isolated from T1D donors mount a memory recognition response to these HIPs. The potential utility of specific PTM signatures of autoantigens in serum for predicting T1D is still in exploratory stage.

3.2.1.3. Cytokines—Another type of important protein biomarkers are cytokines and chemokines since they play significant roles in the stimulation, regulation, and intercellular signaling of immune cells, mediating insulitis and β-cell destruction.[71] Many cytokines are upregulated in prediabetes[72] and their roles as surrogate serum markers of disease have been investigated. A screening of 65 cytokines in a nested case-control study of 67 children revealed that 15 cytokines, chemokines, and growth factors were elevated in AAb positive subjects and two specific cytokines, IL-10 and IL-21, can even differentiate enterovirus infected groups.[73] Serum CXCL1 was demonstrated to discriminate T1D from T2D.[74] An impressive study screened 13 cytokines from two independent sample sets across thousands

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of subjects. Interestingly, they discovered four cytokines (IL8, IL-IRa, MCP-1, and MIP-Iβ) were all downregulated in the serum of T1D patients. They suspected those cytokines may play protective roles against T1D but additional studies would be required to understand their mechanisms.[75] All these studies used well established immunoassays but did not show much overlap to other studied cytokines or their trend between different studies. Cytokines are powerful mediators of inflammation and microbial elimination. However, a potential problem for the use of cytokines as disease serum biomarkers is the lack of organ specificity to T1D since serum cytokines can change in response to injury and damage in all types of tissues and in many inflammation or immune-related diseases.[76,77]

3.2.1.4. Proinsulin/C-peptide ratio—An elevation in the ratio of proinsulin to C-peptide (measurement of insulin) is indicative of β-cell dysfunction and is primarily thought of as a reflection of alterations in insulin protein folding and processing that originate in the endoplasmic reticulum.[78,79] Data has shown that the elevated ratio of serum proinsulin to C-peptide, as an indication of β-cell ER dysfunction, precedes T1D onset especially in younger children. [80] Future studies measuring proinsulin to C-peptide ratios, combined with other biomarkers, are needed to demonstrate their utility in predicting the onset of T1D in the pre- symptomatic phase.[80,81]

3.2.1.5. Other novel AAbs and autoantigens—There have been a number of reports of novel or rarer types of autoantigens and associated AAbs in T1D, including tetraspanin-7 (a membrane glycoprotein),[82], islet cell surface antigen(ICSA),[83] islet of Langerhans regenerating protein-la (REGIA),[84] pancreatic duodenal homeobox 1 (PDX1),[85] chemokine (C-C motif) ligand 3 (CCL3)[86] protein disulfide isomerase (PDl),[87] golgin-160,[87] voltage-gated potassium channel (Kvl.3),[87] Rab GDP dissociation inhibitor beta (GDIβ),[59] amylase alpha-2A,[88] L-type voltage-gated calcium channel s(VGCCs),[89,90] aminoacyl-tRNA synthetase,[91] protein family members of commonly tested autoantigens GAD67[92] and IA–2β,[93] splicing variants of the commonly tested autoantigens[94–96], " as well as to interesting protein/peptide fusion products. [70,97] More targel are likely to exist as has been demonstrated using risk assessment analyses based on total proteome measurements[98] and bioinformatics expression analyses[99].

3.2.2. Nucleic acid biomarkers

The presence of low-concentration small circulating DNA fragments in serum documented.[100] The DNA fragments are thought to be the result of apoptosis, necrosis, or through active and as-yet-unknown secretion pathways.[101] These nucleic acids might carry a variety of genetic and epigenetic alternations related to disease development and progression. Recently, circulating unmethylated insulin DNA has aroused great interest as a biomarker target for early detection of β-cell death in T1D.[102,103] This is based on the fact that certain cytosine-guanine (CpG) sites in the insulin gene are specifically unmethylated in pancreatic β-cells and methylated in most other tissues. During progression of T1D, fragments of the characteristic unmethylated insulin DNA due to β-cell death are leaked into the bloodstream and become detectable. Therefore, measurement of the amount of unmethylated insulin DNA in the peripheral circulation may reflect the amount of β-cell death.[104] In addition, the new methylation-specific real-time PCR and sequencing

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techniques have proven to be sensitive, specific, and cost-effective assays[104–107] for identifying and quantifying unmethylated insulin DNA in blood.

Herold et al. led the screening of serum unmethylated insulin DNA as T1D biomarkers in several different cohorts and demonstrated the ratio of unmethylated/methylated INS DNA was consistently and significantly higher in patients with recent onset T1D compared to healthy groups, patients with long standing T1D, and the at-risk population with at least one AAb and normal OGTT.[103,104,106,108] The receiver operating characteristic (ROC) analysis revealed the ratio of unmethylated/methylated insulin DNA allows for discrimination between T1D and healthy groups with a sensitivity of 38% and a specificity of 95%.[106] Another recent work by Fisher *et al.* they reported the absolute levels of both unmethylated and methylated insulin DNA were higher in new onset T1D patients compared to control individuals and emphasized methylated insulin DNA may also be informative of the underlying disease process.[107] Investigation of unmethylated circulating DNA as a T1D biomarker is not restricted to insulin. Amylin is a glucose regulating hormone highly expressed and co-secreted with insulin from β-cells and interestingly, its unmethylated DNA is correlated with unmethylated insulin DNA as well in recent onset T1D and healthy subjects.[109] However, the utility of circulating DNA markers in T1D diagnosis and prognosis still needs to be further validated.

Another type of important nucleic acid biomarkers is called the transcriptomic fingerprint. Transcriptomic profiles in T1D have also been studied in samples of whole-blood,[110,111] peripheral blood mononuclear cells (PBMCs),[112] and other blood derived immunocyte subsets.[113] The suitability of the sample types used for transcriptional signatures has yet to be rigorously evaluated and the cellular composition of analyzed samples should be considered in the interpretation of the blood-based transcriptional data. A genome-wide transcriptomics analysis of whole blood RNA samples revealed the type 1 interferon (IFN) related transcriptomics signatures were already detectable in children at increased risk of T1D before the T1D-associated autoantibodies were detected.[110] This upregulation of IFN-inducible genes were found to be transient in isolated PBMCs from a different longitudinal cohort of children with a genetic predisposition to T1D.[112] Mehdi et al., combined the gene expression data from these studies and corrected it for time of seroconversion. This sophisticated statistical analysis identified differentially expressed genes that contribute to T cell-, DC-, and B cell-related immune response.

MicroRNAs (miRNAs) are a class of small (21–23 nucleotides) non-coding RNAs that are generally considered as regulators of gene expression through targeting of mRNAs or aiding mRNA stability. In recent years, new roles for miRNAs have been shown both in the regulation of β-cell function and the pathogenesis of T1D. A recent review reported that more than 200 dysregulated miRNAs have been identified in different tissues, cells, and serum/plasma in human and murine samples of T1D.[114] Among the miRNAs that were dysregulated in serum from human samples, several of them have the potentials of being used as circulating biomarkers of T1D. Increased serum levels of miR-375 have been considered to be linked with β-cell injury and significant increase of circulating miR-375 levels has been measured in subjects with T1D[115] and T2D.[116] However, Latrielle et al. showed that β-cell derived miR-375 only contributes to about 1% of the total serum

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miR-375 and questioned its capability of providing insights into β-cell loss.[117] Comparable or even decreased levels of miR-375 were also reported in disease cases.[118,119] Osipova *et al.* investigated circulating levels of three specific miRNAs using qPCR techniques and revealed that miR-21 and miR-210 were significantly increased in serum of subjects with T1D.[120] Similar to the discovery of proteomics biomarkers, global microarray and sequencing analysis of the serum miRNA for discovery and qPCR for validation were employed to screen miRNA biomarkers in T1D and healthy control subjects.[121] Among these analyzed miRNAs, miR-21,[115,120,121] miR-24,[115,121,122] miR-148a,[115,121] miR-181a-5p,[121,123] miR-210–5p,[120,121] are shown to be more consistently upregulated in serum from T1D cases in at least two studies.

MiRNAs have also been analyzed in blood derived exosome samples since exosomes are found to be the transporter of miRNAs in cell-to-cell communication *in vitro*.[124] Lakhter et al. found that serum exosomal miR-21–5p was increased in new onset T1D patients compared with healthy individuals while total serum miR-21–5p were reduced, suggesting that serum exosome miR-21–5p maybe a promising marker of T1D development.[125] Garcia-Contreras et al. discovered 7 differently regulated exosomal miRNAs in 12 pairs of case-control subjects using microarray techniques and only 3 of them had significant changes in a different cohort by qRT-PCR.[126] The authors suggested that this could be related to the differences of sensitivity and efficiency of the techniques and different disease status in the study population.

3.2.3. Metabolomics biomarkers

Glucose is a simple and useful metabolic biomarker used for the diagnosis of diabetes, but it is not specific to T1D. Research focusing on serum metabolomics biomarkers (comprising metabolites, lipids, and small molecules) is still in its infancy but is becoming more studied. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry are the two techniques used to profile serum metabolic fingerprint of T1D.[127,128]. A few amino acids and lipid metabolites were found to be associated with T1D. Methionine is one typical amino acid that is involved in DNA methylation and could be relevant to the timing of appearance of autoantibodies.[127] Lipidomics studies of young and at-risk patients that progressed to clinical disease revealed that some classes of lipids show dysregulation in the blood.[129] Sphingolipids play important roles in inflammation and insulin signaling and have been analyzed for biomarker utility.[130] Torre et al. demonstrated that phospholipids were decreased in cord blood of T1D patients at the time of birth, long before diagnosis.[131] The lipids most decreased were phosphatidylcholines and phosphatidylethanolamines. A particularly interesting aspect of the study was the strong correlation of known cases of first trimester gestational infection and the corresponding decrease in measured phospholipids at birth, an observation made from several studies.[131–133] It has been proposed that low levels of phospholipids at birth may represent a higher risk factor for developing T1D due to the important role phospholipids play in immune system development and modulation. With further advances in discovery and validation efforts, metabolomics biomarkers has pot environmental factors contributing to the disease and in environmental factors contributing to the disease and in predicting the progression of the disease.

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4. Future directions

While the diagnosis, or even prognosis, of T1D based on genotyping and the presence of multiple AAbs has been well established, it is still extremely challenging to develop specific biomarkers for predicting T1D development or progression. Considerable progress has been made in the application of various '-omics' technologies toward the identification and validation of different kinds of biomarkers. Some of the biomarkers such as the methylation patterns of circulating DNA[103,105] have shown promising aspects in the detection of β-cell death and predicting of T1D development. Nevertheless, there are still many hurdles on the way to find novel biomarkers that will be specific to pancreatic β-cells in terms of β-cell function, stress, or death. It is important that the biomarkers are capable of detecting T1D development at very early stage, predicting the disease progression, stratifying high-risk population for intervention, and monitoring the efficacy of novel therapies.

While the most significant advances to date have occurred in the area of nucleic acid-based predictive biomarkers,[103,105,107] the direction of developing of novel β-cell specific proteins or PTM biomarkers along with immune-responsive markers for predicting T1D are still very attractive because proteins/PTMs (e.g. C-peptide) are more likely to provide an assessment of β-cell function or dysfunction. However, proteomics based discovery of β-cell specific markers has been challenged by the complexity of the serum/plasma sample, the extreme low-abundance of the candidates, and the sensitivity of the existing technologies. However, we anticipate that the currently used advanced proteomics technologies[134] should be sufficiently sensitive to detect and validate β-cell specific biomarkers in serum with well-designed sample cohorts. Moreover, the quality of the sample sources utilized for biomarker development is another critical point for success. The sample sets need to provide sufficient statistical power for candidate discovery and validation in order to establish the specificity and sensitivity. In order to develop a biomarker capable of accurately predicting progression, longitudinal samples from clinical trials such as T1D TrialNet for both high-risk subjects that progressed to T1D and those that did not progress to T1D will be important for such development.

Finally, besides serum/plasma, circulatory exosomes or extracellular vesicles may also serve as an interesting source for biomarkers in T1D.[135–137] Several studies have shown that β-cells, under conditions of insulitis, release exosomes to antigen presenting cells (APCs) which can lead to their activation. These exosomes contain several well documented auto-antigens including GAD65, IA-2, and proinsulin.[34] Conditions within exosomes may also cause differential modifications of the contents[138] and impaired immune phenotyping.[139] It is also possible that there isn't a single ideal predictive biomarker. Instead multiple biomarkers may constitute a multi-marker panel of proteins, PTMs, or nucleic acids from serum and/or serum exosomes that will provide a more accurate prediction or measurement of the disease progression.

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Abbreviations:

T1D	type 1 diabetes
T2D	type 2 diabetes
AAb	autoantibody
HbA1c	hemoglobin A1c
FPG	fasting plasma glucose
OGTT	oral glucose tolerance test
ICA	islet-cell cytoplasmic autoantibody
GAD	glutamic acid decarboxylase
GADA	GAD autoantibody
IA-2A	insulinoma 2- associated autoantibody
IAA	insulin autoantibody
ZnT8A	zinc transporter 8 autoantibody
PTM	post-translational modifications

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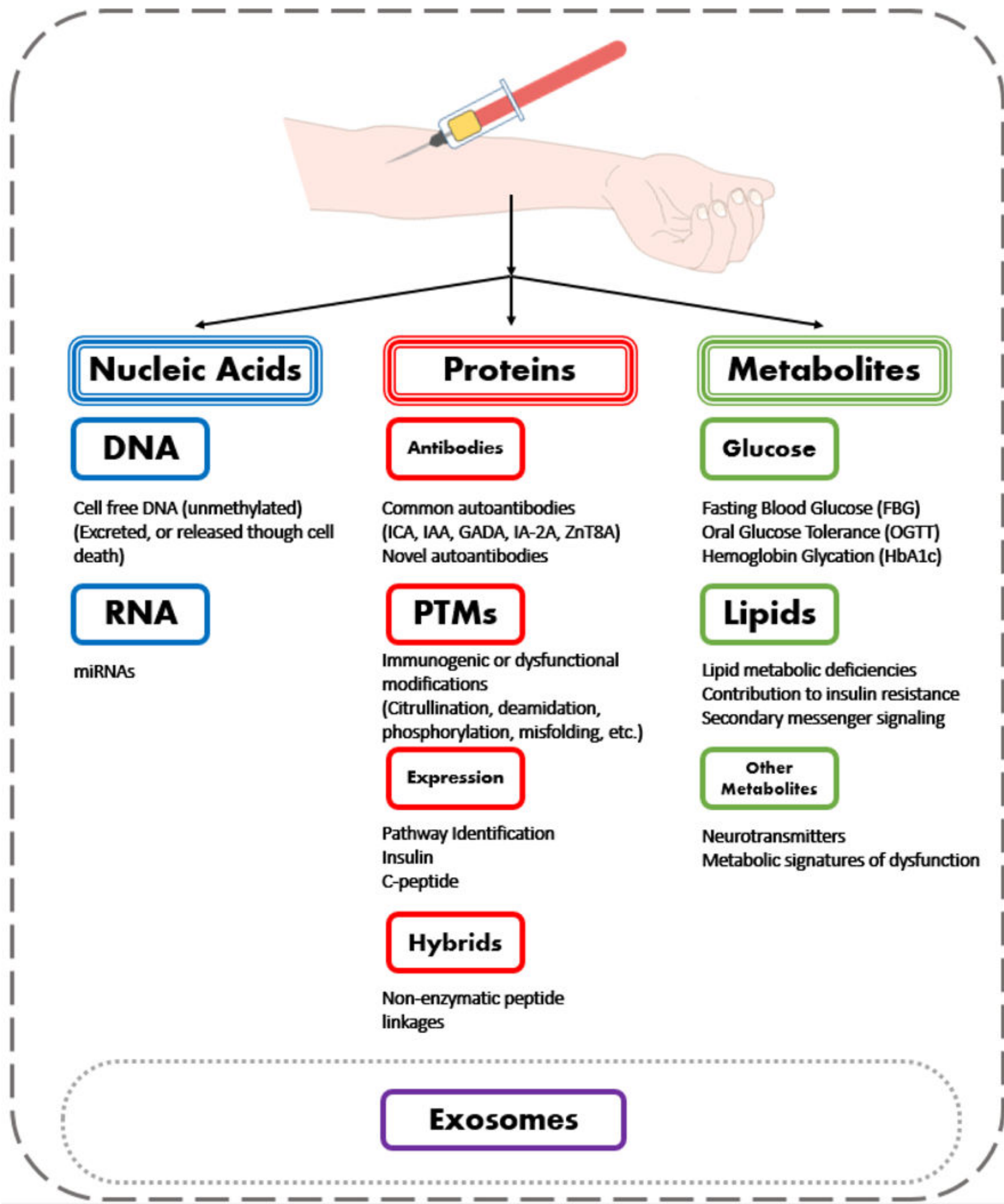

Figure 1. The many types of currently used and potential biomarkers from serum for the diagnosis and prediction of T1D.

Types of validated clinical diagnostic biomarkers and potential new types of biomarkers are illustrated.

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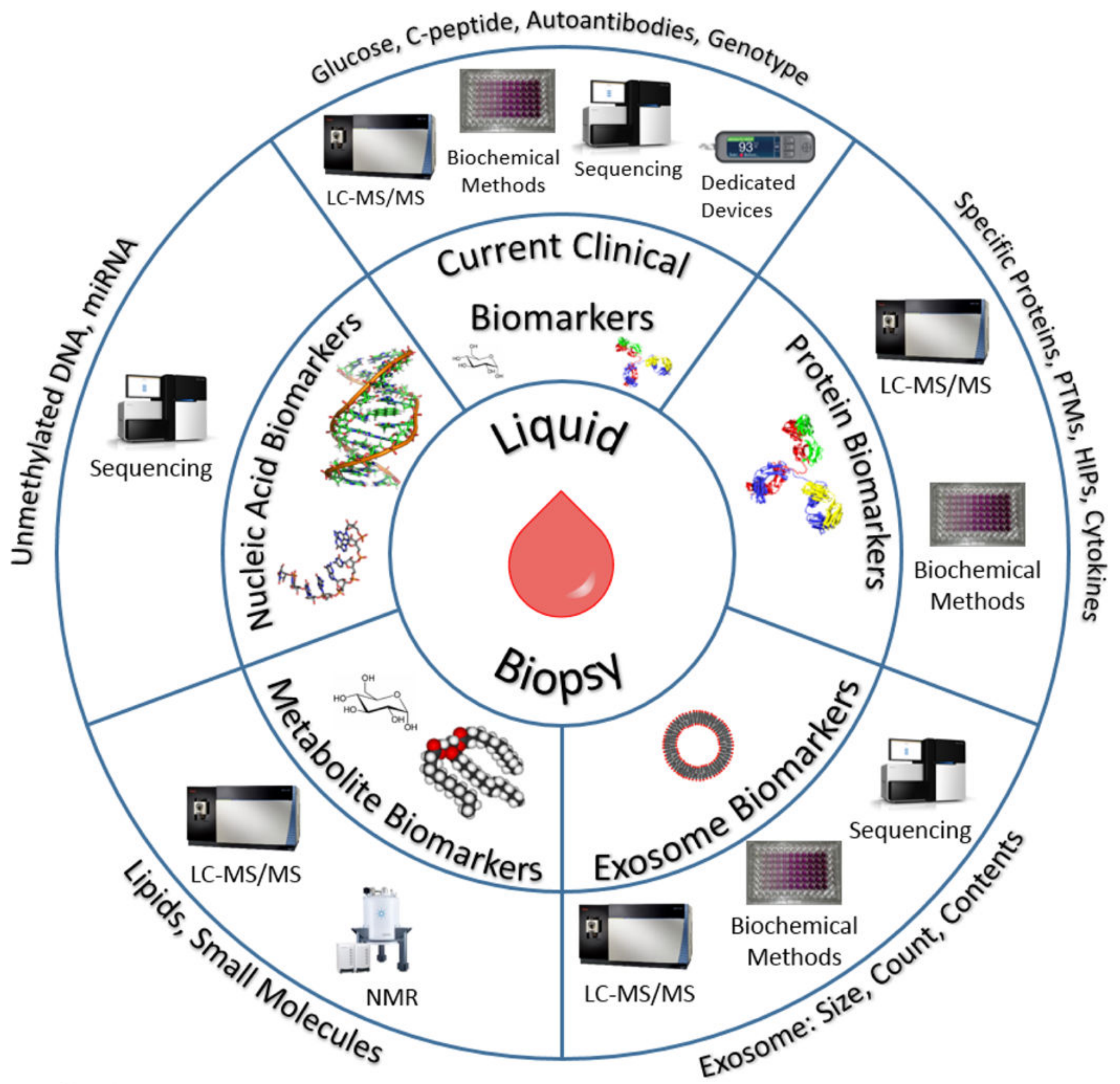

Figure 2.
Different types of potential serum biomarkers for T1D and the associated omics technologies and assay platforms for their discovery and measurements.

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Table 1.

Protein candidate biomarkers discovered by proteomics.

Gene Symbol	Protein name	Regulation	Function
ADIPOQ	Adiponectin	Zhi et al,[48] up * Moudler et al,[52] down *	Glucose homeostasis
APOA4	Apolipoprotein A-IV	Zhi et al,[48] up von Tonerne, et al,[49] down Moudler et al,[52] down	Leukocyte adhesion
APOC4	Apolipoprotein C-IV	von Tonerne, et al,[49] down Moudler et al,[52] down	Viral infection
AZGP1	a-2-glycoprotein 1 (zinc)	Metz et al,[50] up Zhang et al,[51] up	Lipid mobilization activity
BTD	Biotinidase	von Tonerne, et al,[49] up Zhang et al,[51] up	Biotinidase activity
C3	Complement C3b	Zhi et al,[48] down von Tonerne, et al,[49] down Zhang et al,[51] down	Innate immunity, complement activation
C4A	Complement C4-A	Zhi et al,[48] down von Tonerne, et al,[49] down Zhang et al,[51] down	Innate immunity, complement activation
CLU	Clusterin	von Tonerne, et al,[49] down Metz et al,[50] down Zhang et al,[51] down	Cytoprotective capability
KNG1	Kininogen 1 isoform 1	Zhi et al,[48] up von Tonerne, et al,[49] down Zhang et al,[51] up	Innate immunity, DC activation
LUM	Lumican	Metz et al,[50] up Zhang et al,[51] up	Extracellular matrix structural constituent
SERPINA6	Corticosteroid-binding protein	Metz et al,[50] up Zhang et al,[51] up	Correlate to insulin deficiency or response
SERPINF2	Alpha-2-antiplasmin	von Tonerne, et al,[49] down Zhang et al,[51] up	Serine protease inhibitor
TTR	transthyretin	von Tonerne, et al,[49] down Zhang et al,[51] down	Involve in β-cell stimulus- secretion coupling

* Up/down inc licates that the protein is significantly up/down regulated in serum of patients with iabetes diabetes/prec relative to the healthy controls.