Biotechnology Advances xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Harmonization of immunoassays for biomarkers in diabetes mellitus

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ARTICLE INFO

Keywords:
Autoantibodies
Biomarker
Classification
C-peptide
Diabetes mellitus
Glycated hemoglobin
Harmonization
Immunoassay
Insulin
Prediction
Standardization

ABSTRACT

Harmonization of biomarkers is important for the comparability of laboratory results as it allows the definition of universal reference values and clinical decision limits. In diabetology, immunoassays are widely used to determine HbA1c, C-peptide, insulin, and autoantibodies to beta cell proteins, which are essential biomarkers for the diagnosis and classification of diabetes mellitus. Furthermore, as large clinical studies have identified HbA1c as a predictor for the development of diabetic complications, HbA1c has evolved as the general treatment target. For decades, the use of non-harmonized assays caused confusion. After the standardization of HbA1c, the worldwide comparability improved and increased the confidence in this laboratory biomarker. Insulin and C-peptide are not only valuable biomarkers to assess beta-cell function, but may also be used to evaluate insulin resistance, a metabolic feature of type 2 diabetes often occurring before its manifestation. Long-lasting efforts led to substantial improvements in the harmonization process of C-peptide assays, but harmonization of insulin assays is still ongoing. Therefore, C-peptide is now sometimes used as a surrogate biomarker for insulin. Furthermore, autoantibodies against beta cell components are important biomarkers for the accurate differentiation of type 1, type 2, and other special types of diabetes. Owing to the heterogeneity of these autoantibodies against beta cell proteins, harmonization is very difficult to achieve. International efforts are in progress to harmonize the current assays, as the presence of autoantibodies against beta cell proteins predicts the development of type 1 diabetes in early life. In conclusion, clinical studies linking diagnosis, classification, prediction, and treatment to laboratory values of the respective biomarkers need to be harmonized to avoid misdiagnosis and incorrect clinical decisions, thus improving patient care and safety.

1. Introduction

1.1. Why do we need harmonization?

Together with clinical examination, laboratory tests provide important mosaic pieces to establish the diagnosis of a disease and are useful for prognosis and treatment monitoring. For interpretation in many cases, fixed reference ranges are used to assess the results of laboratory tests and national and international guidelines use fixed laboratory test values, e.g. cut-off values, for treatment decisions. If

Abbreviations: ADA, American Diabetes Association; CAP, College Of American Pathologists; CE, Capillary Electrophoresis; DASP, Diabetes Antibody Standardization Program; DCCT, Diabetes Control And Complications Trial; DCM, Designated Method Comparison; EASD, European Association For The Study Of Diabetes; ECBS, Expert Committee On Biological Standardization; ECL, Electrochemiluminescence; ELISA, Enzme-Linked Immunosorbant Assay; GAD, Glutamate Decarboxylase; GADA, Autoantibodies To GAD; GHb, Total Glycated Hemoglobin; HbA1c, Glycated Hemoglobin; HOMA, Homeostasis Model Assessment; HPLC, High Pressure Liquid Chromatography; IA-2A, Autoantibodies To IA-2; IAA, insulin autoantibodies; IASP, Islet Autoantibody Standardization Program; ICA, Islet Cell Antibodies; IDF, International Diabetes Federation; IDS, Immunology Of Diabetes Society; IFCC, International Federation Of Clinical Chemistry; IRR, International Reference Reagent; JCTLM, Joint Committee For Traceability In Laboratory Medicine; LADA, Late Onset Autoimmune Diabetes In The Adult; LC-MS, Liquid Chromatography–Tandem Mass Spectrometry; LIPS, Luciferase Immunoprecipitation System; MODY, Maturity Onset Diabetes Of The Young; MS, Mass Spectroscopy; NGSP, National Glycohemoglobin Standardization Program"; NIBSC, National Institute For Biological Standards And Control; NIDDK, National Institute Of Diabetes And Digestive And Kidney Diseases; NMIJ, National Metrology Institute Of Japan; PRM, Primary Reference Material; RBA, Radio-binding Assay; RIA, Radioimmunoassay; RMP, Reference Measurement Procedure; UKPDS, United Kingdom Prospective Diabetes Study; WHO, World Health Organization; ZnT8, Zink Transporter-8; ZnT8A, Autoantibodies To ZnT8

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https://doi.org/10.1016/j.biotechadv.2019.02.015
Received 29 October 2018; Received in revised form 7 February 2019; Accepted 21 February 2019

laboratory results are affected by the analytical procedure, i.e. if they are not harmonized, wrong conclusions may be drawn for the diagnosis or the treatment of a patient, although the doctor is adhering to the guidelines. In these cases, patient safety may be affected (Miller et al., 2014).

Harmonization of laboratory tests indicates that comparable results are obtained independent of when and where the analysis is performed. At best, harmonization can be obtained by standardization of an assay indicating that, among other requirements, a well-defined reference standard together with a reference measurement system is available. The "true" values of biomarkers can be determined only with such laborious systems. To achieve solid harmonization or standardization of laboratory tests, several assumptions and pre-requisites must be considered:

First, the biomarker is well-defined at the molecular level. This means only a single molecular form exists that can be precisely and accurately measured by means of chemical, biochemical, physico-chemical, and other methods. This is especially true for low-molecular-weight biomarkers.

Second, the biomarker is available in pure and stable, i.e. commutable, form.

Third, the biomarker is well-defined on the molecular level, but the molecular weight is much higher, e.g. peptides or proteins. In this case, the biomarker may be unstable under physiological conditions and may not be commutable.

Fourth, the biomarker, e.g. a protein, is well-defined but post-translationally modified; thus, the biomarker occurs in different molecular entities that are ill-defined and/or the modification, but not the protein, is the clinically relevant biomarker.

Fifth, the biomarker is not well-defined i.e. it exists in several (iso)-forms or its structure is unknown on the molecular level. Consequently, no primary reference material is available. In such cases, standardization is not possible.

From these considerations, schematically summarized in Fig. 1, it is obvious that standardization can only be achieved if an appropriate reference material is available. Otherwise, the assay needs to be harmonized by defining a "reference material", which can be communicated for calibration of an assay to a network of reference laboratories and manufacturers. In spite of clear differences between harmonization and standardization, both are used interchangeably in the literature.

A general model for a "comprehensive, coherent measurement system" based on establishing a reference material and developing a

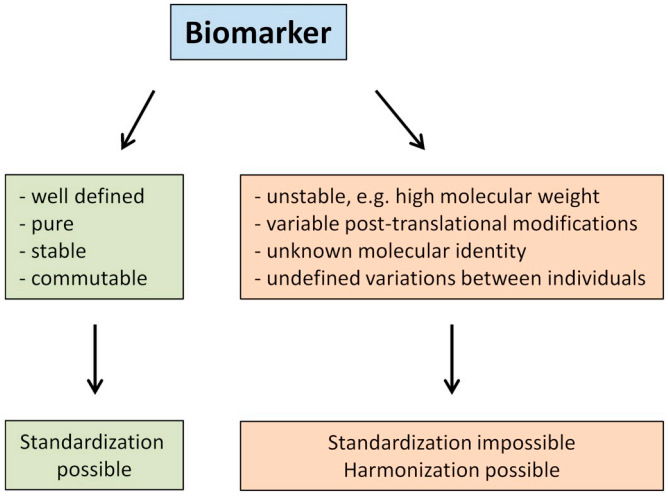

Fig. 1. Assumptions and pre-requisites for harmonization and standardization of biomarkers. If the analyte is well defined on the molecular level, available in stable, commutable and pure form, the harmonization and standardization is possible; if these pre-requisites are not fulfilled standardization is not possible. In this case harmonization should be sought.

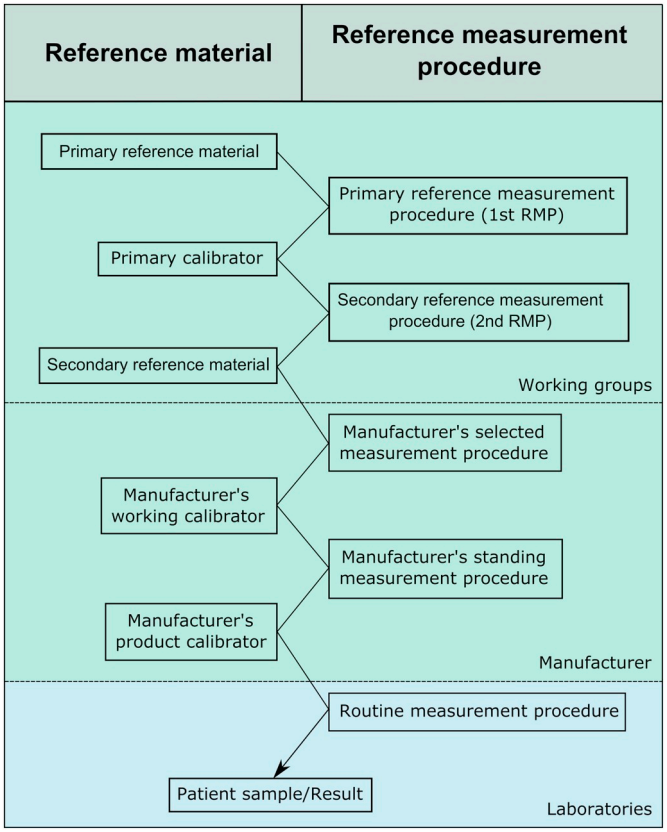

Fig. 2. The traceability chain of laboratory biomarkers: from primary reference material to patients' results.
The hierarchical structure of a comprehensive measurement system illustrating the interrelationship between the reference material (left) and the reference measurement procedure (right) is shown. Working groups establish primary reference materials (PRM) and respective reference measurement procedures (RMP) which are used by manufacturers for calibration of their assays. Via this traceability chain, the laboratory results of patients are traceable to the primary reference material based on ISO 17511 (ISO, 2003).

reference measurement procedure (RMP) was first proposed in 1979 (Tietz, 1979). As schematically outlined in Fig. 2, such a reference system consists of defined reference material on one side and definitive methods on the other. In essence, Tietz described a hierarchical structure of the reference measurement system including a general procedure on how the system can be implemented via secondary methods and secondary reference material in peripheral clinical laboratories. This system is the basis for the harmonization/standardization of laboratory tests in clinical chemistry and it has been refined and described in ISO 17511 (ISO, 2003). This document specifies how to assure the metrological traceability of values assigned to calibrators and control materials by establishing or verifying trueness of measurement. Furthermore, the use of commutability calibrators and control material for internal and external quality assessment is described. Therefore, the patients' routine laboratory results can be traced to the secondary reference material provided by the manufacturer. In addition to the analytical procedures, harmonization/standardization systems also include pre- and post-analytical steps concerning the sample material, sample stability, reporting, reference ranges and interpretation, respectively.

2. Immunoassay

Immunoassays are biochemical test systems for determining the concentrations of proteins or small molecules by using antibodies. Immunoassays are based on the ability of antibodies to recognize their

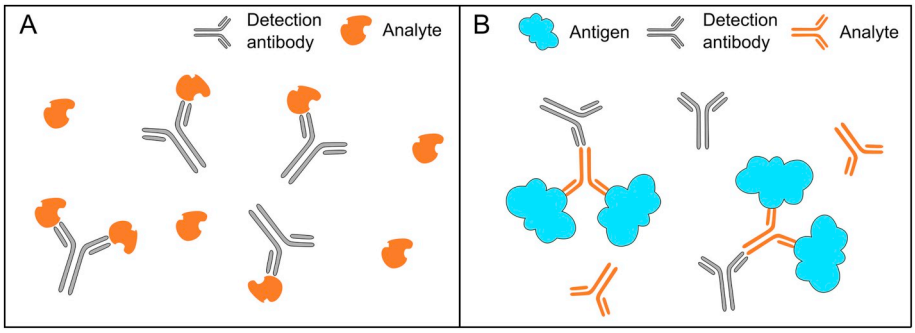

Fig. 3. Principles of immunoassays. (A) Biologically generated antibodies may be used as specific recognition elements for the determination of biomarkers (orange). (B) Conversely, the in vivo generated human antibodies (orange) recognizing specific endogenous human proteins (blue) may be the biomarker of clinical interest. For detection an anti-human IgG antibody is used (detection antibody).

target by binding to specific epitopes in complex biological solutions. However, in particular cases it is clinically important to detect the presence of antibodies to an endogenous antigen and quantify the concentration of this specific antibody. Here, the antibody is the biomarker rather than the antigen to be determined. The two principles are schematically shown in Fig. 3.

For quantification of biomarkers in clinical chemistry, two general formats are used: the competitive and the non-competitive immunoassay. While the competitive immunoassay is based on competition between the biomarker and a fixed amount of labeled antigen binding to a limited amount of the antibody, the non-competitive immunoassay uses an excess of labeled specific antibody recognizing the biomarker. Numerous formats of immunoassays have been developed. The assay may be varied by a) the animal source of the antibody b) the antibody makeup, be it mono- or polyclonal c) the detection system, including the labeling of the antibody, d) the method by which bound and unbound antigen are separated or detected without separation, and e) the assay format, be it homo- or heterogeneous etc.

However, harmonization of immunoassays is a common problem for all formats, because antibodies are heterogeneous by nature. Furthermore, their affinity for the antigen of interest may vary over time, even from batch to batch. While some of the problems can be avoided by using monoclonal antibodies, establishment of an RMP with commutable reagents remains a challenge. The specific problems of harmonization across immunoassays of specific biomarkers widely used in the diagnosis, classification, and monitoring of diabetes will be discussed below.

3. Diagnosis of diabetes mellitus

Diabetes mellitus is increasing worldwide. Since 1980, the prevalence rose from 108 to ~ 422 million of the worldwide population (NCD-RisC, 2016). It is estimated that about 90% of all individuals with diabetes mellitus have type 2 diabetes (Zheng et al., 2018). Leading causes of morbidity and mortality in these patients are cardiovascular events, kidney complications, blindness, stroke, and lower limb amputations. The pathogenesis of these vascular complications may lie in the prediabetic phase before type 2 diabetic patients manifestation i.e. during the insulin resistant state (Artunc et al., 2016).

By definition, diabetes is a metabolic disorder characterized by chronic hyperglycemia. Apart from clinical aspects, laboratory test results are a major backbone for firm establishment of the diagnosis. Detailed cut-off values have been defined and are reported in national and international guidelines (ADA, WHO and most European countries), supporting the diabetes diagnosis if one or more of the following cut-off values are exceeded (American Diabetes Association, 2018):

- Fasting plasma glucose ≥126 mg/dL (≥7.0 mmol/L) or
- HbA1c ≥ 6.5% (≥48 mmol/mol) or
- 2 h-plasma glucose during oGTT ≥ 200 mg/dL (≥11.1 mmol/L) or
- Random plasma glucose ≥200 mg/dL (≥11.1 mmol/L)[#]

[#] in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis.

Clinical practice and evaluation of the pathogenesis revealed that diabetes mellitus is a very heterogeneous disease. Therefore, diabetes is classified, although the diagnostic criteria from above are valid for all classes except minor differences in cut-off values for gestational diabetes mellitus. According to the American Diabetes Association and most national and international diabetes organizations, diabetes can be classified into the following general categories (American Diabetes Association, 2018):

- Type 1 diabetes (due to autoimmune beta cell destruction, usually leading to absolute insulin deficiency)
- Type 2 diabetes (due to a progressive loss of beta cell insulin secretion usually with a background of insulin resistance)
- Gestational diabetes mellitus
- Specific types of diabetes due to other causes

In most cases the patient's clinic, together with the laboratory values of HbA1c and plasma glucose, may support the diagnosis; however, additional biomarkers are needed to confirm or exclude the diagnosis of type 1, type 2, or other specific types of diabetes if the diagnosis is uncertain. According to an evaluation of the survival analysis using the data base of UK Bio-Bank, C-peptide is an important and reliable biomarker for distinguishing type 1, type 2, and possibly other specific types of diabetes (Jones and Hattersley, 2013). Along those same lines, the determination of autoantibodies may be useful for the differentiation of type 1 and type 2 diabetes, as well as the proper diagnosis of specific diabetes forms such as MODY (Maturity Onset Diabetes of the Young).

The sequence of pathophysiological events occurring during the development of type 1 and type 2 diabetes is associated with the occurrence of autoantibodies and changes in C-peptide and insulin plasma concentrations (see Fig. 4).

While a standardization procedure for glucose measurements with primary reference material has been achieved (Andreis et al., 2014; Hannestad and Lundblad, 1997; ISO, 2003), the harmonization of other important biomarkers used in clinical diabetes (e.g. HbA1c, insulin, C-peptide, and autoantibodies to beta cell proteins) is only partly solved. Here, we review the way forward for harmonizing the immunoassays of these biomarkers and reporting on the current status of harmonization and standardization of these biomarkers.

4. Glycated hemoglobin (HbA1c)

As outlined above, hemoglobin A1c (HbA1c) is one of the most important laboratory biomarkers for the diagnosis of diabetes and for monitoring metabolic control in patients with diabetes to reduce complications. However, it took decades until the molecular nature of HbA1c was defined and the standardization could be initiated. Rahbar and coworkers described a minor, fast hemoglobin fraction that was increased in patients with diabetes while they were screening for

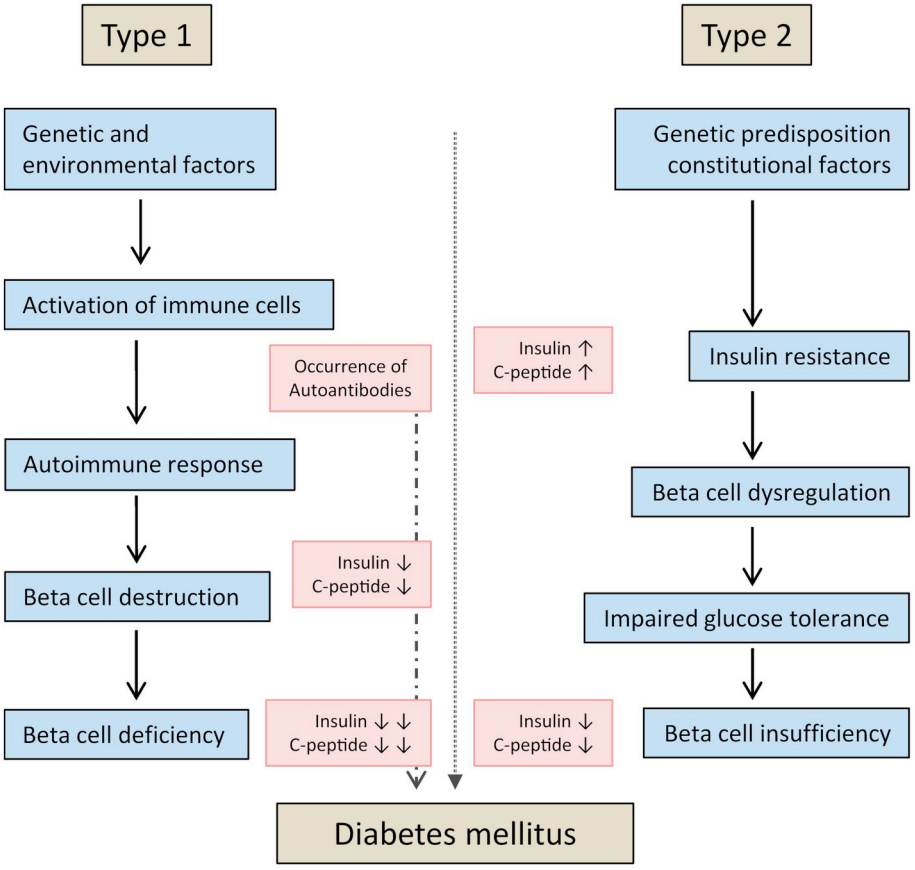

Fig. 4. Pathological events leading to type 1 and type 2 diabetes mellitus.
The sequence of events leading to type 1 and type 2 diabetes mellitus is schematically shown together with relevant biomarkers. Development of type 2 diabetes is caused by constitutional factors and genetic predisposition. These factors favor insulin resistance which is accompanied by elevated insulin and C-peptide concentrations as counter regulatory mechanism in the early stage of disease progression. Consequently, this may lead to a dysregulation of the pancreatic beta cells characterized by an impaired glucose tolerance. Further exacerbation finally ends in the manifestation of type 2 diabetes due to beta cell insufficiency accompanied by inadequately reduced insulin and C-peptide secretion and plasma concentrations. In contrast, the development of type 1 diabetes mellitus is assumed to be associated with genetic and environmental factors. The initial step in the progress of type 1 diabetes is not fully understood. It is presumed that activation of immune cells leads to an autoimmune response accompanied by the formation of high-affinity autoantibodies against antigens of the pancreatic beta cell. Subsequent beta cell destruction leads to insulin and C-peptide deficiency and finally to the manifestation of type 1 diabetes mellitus.

hemoglobinopathies (Rahbar et al., 1969). Although the molecular nature of this minor hemoglobin fraction was unknown, this finding drew the attention of many diabetes centers because no other biomarker for assessing glycemia was known, except for acute blood glucose concentration. Using a modified chromatographic method, Trivelli et al. reported that 100 patients with diabetes showed an approximate two-fold increase in fast hemoglobin components (glycohemoglobin) over values found in 20 normal subjects (Trivelli et al., 1971). From their chromatographic method it appeared that the “fast” hemoglobin fraction (HbA1) is more than one fraction indicating heterogeneity.

4.1. Clinical utility

After these promising results, a multicenter, randomized, clinical study was designed to determine whether an intensive treatment regimen directed at maintaining blood glucose concentrations as close to normal as possible would affect the appearance or progression of early vascular complications in type 1 diabetes patients. The results of this long-term prospective study, “Diabetes Control and Complications Trial” (DCCT), demonstrated that the risk for development and progression of diabetic retinopathy is closely related to the degree of glycemic control as assessed by hemoglobin A1c (HbA1c) (Nathan et al., 1993). In addition, the study provided clear evidence for a relation of HbA1c values to mean blood glucose values. In this trial, glycated hemoglobin was analyzed by ion exchange chromatography using “Bio-Rex 70” as the separation column, yielding a better separation of the HbA1 fraction into several subfractions termed HbA1a,b,c. As the HbA1c fraction was associated with glycemia in patients, this fraction was used to assess glycemia in further studies. The results obtained for patients with type 1 diabetes in the DCCT were largely supported by the outcome of the United Kingdom Prospective Diabetes Study (UKPDS) performed in patients with type 2 diabetes (UKPDS Group, 1998). Both studies demonstrated that HbA1c is a reliable biomarker of glycemic control and supported the use of HbA1c values as treatment goals. However, the widespread use of the HbA1c assay was not possible, as the assay was not harmonized.

4.2. Definition of the biomarker

While the clinical studies were running HbA1, HbA1c and total glycated hemoglobin (GHb) were introduced into clinical practice, although the molecular nature of these hemoglobin fractions was unclear. Step by step, the molecular identity of glycated hemoglobin was revealed (Bunn et al., 1975; Holmquist and Schroeder, 1966; Koenig et al., 1977). The formation of glycated hemoglobin was discovered in 1976 (Bunn et al., 1976). After addition of glucose to hemoglobin, a labile Schiff base is formed (aldimine), which is stabilized by Amadori rearrangement to a stable ketoamine (Fig. 5). Kinetic analysis of the reaction indicated that the first reversible step was faster than the second irreversible step (Bunn, 1981). The glycation of hemoglobin attracted even more attention when it was discovered that other serum proteins and particularly tissues prone to develop diabetic complications (e.g. coronary artery, aorta, lung, glomerulus, peripheral nerves) showed increased glycation in patients with diabetes (Vogt et al., 1982).

4.3. Harmonization and implementation

The obvious wide-spread usefulness of HbA1c in clinical practice caused a boost of new HbA1c assays (> 30) developed by different companies using different test principles including cation exchange chromatography, affinity chromatography (which determines total glycated hemoglobin), enzymatic assays, and immunoassays (Lenters-Westra et al., 2013; Weykamp, 2013). Not surprisingly, these non-harmonized, non-standardized assays yielded variable results causing confusion in the diabetes community. To address this problem the

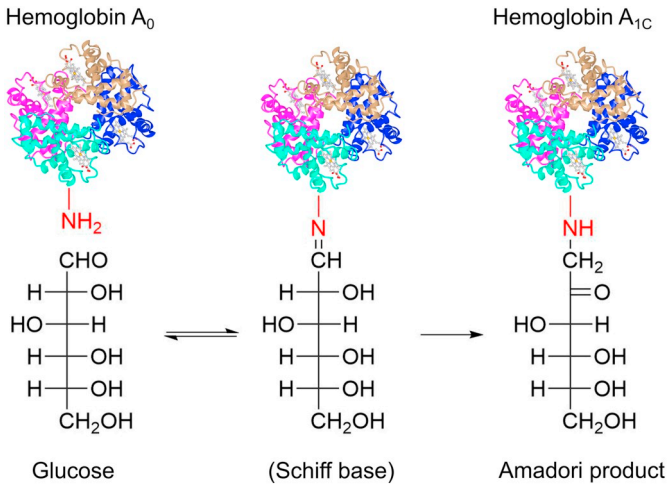

Fig. 5. Schematic illustration of the formation of glycated hemoglobin (HbA1c).
In a first reversible step, glucose is attached to the N-terminal valine of the hemoglobin ß-chain forming a labile Schiff Base, which is rearranged to a stabile ketoamine bond yielding HbA1c. The concentration of HbA1c is proportional to both the patient's erythrocyte life span and the mean glucose concentration during that life span of approximately 120 days.

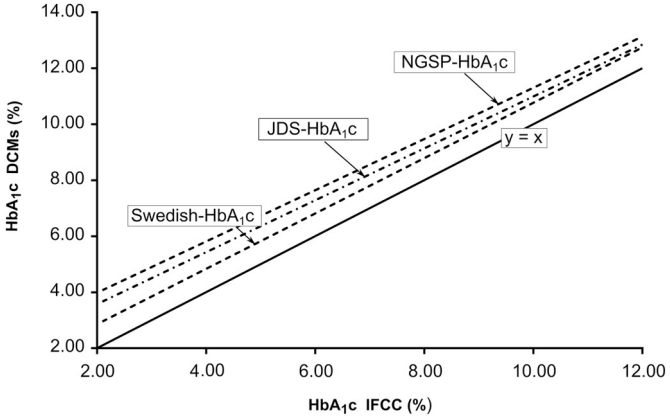

Fig. 6. Comparison of HbA1c values obtained by different national harmonization programs.
In addition to the U.S., Japan and Sweden set up programs for harmonization of HbA1c comparison of distributed blood samples showed that HbA1c values were lower in Japan and much more so in Sweden, using the highly efficient separation column Mono-S, than US values (Miedema, 2004). DCM, designated comparison method. Reprinted by permission from Springer Customer Service Centre GmbH: Springer, Diabetologia, Towards worldwide standardization of HbA1c determination, Miedema K., c 2004.

"National Glycohemoglobin Standardization Program" (NGSP) was established in 1996 (http://www.ngsp.org/). The purpose of this program was to relate HbA1c results to those of the DCCT and UKPDS, thus linking clinical outcomes to a laboratory test. Other countries set up programs accordingly to promote harmonization e.g. in Japan and Sweden (Mono-S) (Panteghini et al., 2007). However, the data showed that HbA1c values were lower in Japan and much more so in Sweden, using the highly efficient separation column Mono-S, than the US values (Fig. 6). This situation made clear that a worldwide standardization of HbA1c was necessary (Miedema, 2004).

To achieve a uniform international standardization, the International Federation of Clinical Chemistry (IFCC) established a working group in 1995. Based on the definition of HbA1c as hemoglobin that is irreversibly glycated at one or both N-terminal valines of the beta chains, candidate reference methods based on peptide

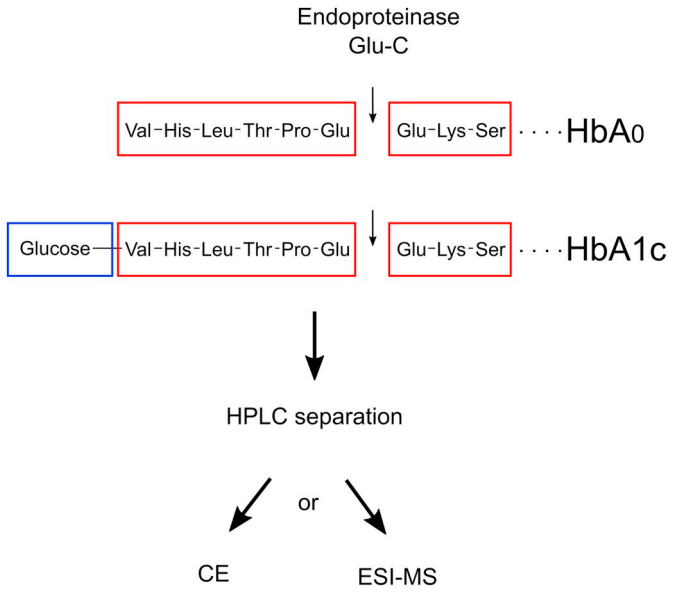

Fig. 7. Reference method for HbA1c.
Hemoglobin from lysed human erythrocytes is digested with endoproteinase Glu-C, which specifically splits the peptide bond between two adjacent glutamates liberating N-terminal hexapeptide, regardless of glycation state. The peptides were separated by HPLC and quantified either by electrospray ionization mass spectrometry (ESI-MS) or by capillary electrophoresis (CE) using UV detection at 214 nm. Both methods yielded identical results (Jeppsson et al., 2002).

mapping were suggested (Kobold et al., 1997). On the basis of this suggestion, a reference standard procedure could be established for HbA1c (Jeppsson et al., 2002). The principle is schematically outlined in Fig. 7. In a first step hemoglobin obtained from lysed erythrocytes is specifically cleaved by the endoproteinase Glu-C, liberating the first 6 amino acids of the beta-chain, no matter if glycated or non-glycated. In a second step the proteolytic peptides are separated by HPLC and quantified either by electrospray ionization mass spectrometry or by capillary electrophoresis. HbA1c is determined as the ratio of HbA1c and total hemoglobin. For calibration defined mixtures of highly purified HbA1c and HbAo were used (Finke et al., 1998). Evaluation of the suggested reference method by an international network of reference laboratories showed excellent precision with intra- and inter-laboratory coefficients of variation below 2.0 and 2.3%, respectively (Jeppsson et al., 2002). These reference methods were approved by the IFCC in July 2001. With the IFCC-RMP, which is based on the concept of metrological traceability, HbA1c values are assigned to whole blood panels that serve as calibrators for manufacturers. Thus, the complete quality chain from IFCC-RMP to the patient is created. To implement this reference procedure, an IFCC laboratory network has been established embedding the IFCC-RMP in a global network of reference laboratories in Europe, Asia, and the US (Hoelzel et al., 2004).

Comparison of blood samples determined either by IFCC or NGSP procedures correlated excellently, but IFCC results are consistently lower (approximately 2% HbA1c) than NGSP based results. Both results can be interconverted by the formula indicated in the next paragraph. While IFCC results are accuracy-based, NGSP results are directly related to clinical outcomes, where the master equation relates accuracy-based HbA1c values to clinical outcomes.

The ADA, EASD, IDF, and IFCC approved statements for HbA1c standardization are essentially as follows (Weykamp et al., 2008):

1. HbA1c test results should be standardized worldwide, including the reference system and results reporting.
2. The IFCC-RMP for HbA1c represents the only valid anchor to implement standardization of the measurement.

3. HbA1c results are to be reported worldwide in IFCC units (mmol/mol) and derived NGSP units (%), using the IFCC-NGSP master equation:

$$\text{HbA1c [mmol/mol]} = (\text{HbA1c [\%]} - 2.15) \times 10.929$$

$$\text{HbA1c [\%]} = \text{HbA1c [mmol/mol]} \times 0.0915 + 2.15$$

These statements, acknowledged worldwide, transformed the previously chaotic situation of HbA1c measurements to a more ordered state (Gillery, 2013; Little et al., 2011).

Before standardization, most widely used methods for determination of HbA1c including immunoassays were originally harmonized to NGSP/DCCT and later calibrated to match the IFCC reference method. Important to note that the harmonization and standardization is independent from the different assay principles and methods of the manufacturers; all use secondary reference material to calibrate the assay. Thus the results should be comparable. Since the standardization of all HbA1c assay principles have been extensively reviewed, e.g. (Weykamp, 2013), we will focus on immunoassays of HbA1c in the following section.

4.4. Immunoassay for HbA1c

Immunoassays for HbA1c were developed using the glycated N-terminal hexapeptide as the hapten and monospecific antibodies have been generated that specifically recognize the N-terminal glycation of the hemoglobin beta chain, but not the non-glycated terminus or other modifications (e.g. carbamylation) of the N-terminus. Most commercially available immunoassays for HbA1c use antibodies raised against glycated N-terminal peptides of different sizes. Thus, depending on the manufacturer the antibodies used in the immunoassay recognizes the first four or more amino acids of the glycated N-terminus of the hemoglobin ß-chain. Several formats of immunoassays have been developed: a) the antibody reacts with HbA1c yielding soluble immune complexes. Polyhaptens bind excess antibodies and the resulting agglutinated complexes are measured using immunoturbidimetry at 340 nm (Roche, Basel, Switzerland), b) using the enzyme immune assay principle with the sample coated to a titer plate (DAKO, Glostrup, Denmark) and c) a synthetic polymer containing multiple hapten copies causes agglutination of latex particles coated with an HbA1c mouse monoclonal antibody. As HbA1c in the patients' samples compete with the limited number of antibody-latex binding sites, agglutination is reduced, and light scattering is decreased. This immunoassay format is used in the point of care device DCA (Siemens Healthineers, Erlangen, Germany). Various other immunoassays are offered by different manufacturers. All assays are calibrated according to the IFCC reference method.

4.5. Does the harmonization of HbA1c improve assay performance and patient care/safety?

To assess the effect of harmonization on the variability of glycated hemoglobin values, the College of American Pathologists (CAP) started proficiency surveys in the mid-1980s. For example in 1993, a CAP survey revealed that 50% of the participating laboratories reported their values as HbA1c, 21% as HbA1 and 29% as total glycated hemoglobin (GHb) (Little et al., 2011). In 1999, 80% of the results were reported as HbA1c, followed by 100% from 2010 to the present, indicating that all laboratories are determining the same biomarker. In parallel, the accuracy and precision improved substantially. While in 1993 reported mean values of the circulated HbA1c sample ranged from 3.6–6.5% HbA1c (target value 4.8% HbA1c) with a bias up to 1.8% HbA1c, these values improved to 5.6–6.4% (target value 5.9% HbA1c) with a bias up to 0.5% HbA1c in 2010. Numerous national laboratory organizations also reported an improvement of the accuracy and precision of HbA1c over time, particularly after the National Glycated Hemoglobin Standardization Program (NGSP) has been established. A recent large proficiency testing/external quality assessment in Europe (EurA1c) including 2166 laboratories across 17 countries and 24 manufacturers showed that the majority of the participating countries and manufacturers met the strong IFCC criterion (European HbA1c Trial, 2018). Between laboratory variabilities were the main contributors to the overall variability, indicating that more educational work needs to be done.

The impressive improvements of the comparability of HbA1c values obtained by the standardization efforts make laboratory values traceable to the clinical studies. Therefore, the suggested fixed ranges for diabetes diagnosis and the HbA1c target values for metabolic control are valid. Strict standardization enables the definition of universal reference values and clinical decision limits (Weykamp, 2013). HbA1c is also an accepted biomarker for assessing the effectiveness of newly developed antidiabetic drugs.

5. Insulin and C-peptide

Insulin is an endocrine hormone synthesized by the beta cells of the islets of Langerhans in the pancreas. It is an anabolic hormone composed of 51 amino acids that is stored in vesicles in the beta cells and is released by elevated glucose concentrations in the blood. It is generated from its precursor proinsulin by limited proteolysis yielding C-peptide and the anabolic insulin consisting of an A- and B-chain (Fig. 8 (Rubenstein et al., 1969)). Subsequently, both substrates are released in equimolar concentrations into circulation. Following secretion, large amounts of insulin are cleared by the liver, whereas C-peptide passes the liver almost unaltered. As a consequence, insulin concentrations in the peripheral blood are significantly decreased, in contrast to C-peptide concentrations showing constant blood concentrations. Insulin is the only hormone capable of reducing blood glucose levels and is therefore essential to maintaining glucose homeostasis in various conditions. In contrast to the defined biological functions of insulin, the physiological role of the C-peptide remains elusive. In clinical practice, C-peptide measurement is mainly used as a surrogate marker of insulin release. C-peptide levels are more suitable for assessing insulin secretion and insulin resistance because C-peptide has the following advantages compared to insulin: a) it has a longer plasma half-life in vivo, b) it is less affected by hemolysis, and c) the standardization effort is already well advanced (see below).

5.1. Clinical utility

The development and manifestation of diabetes mellitus is associated with impairment (type 2) or loss (type 1) of beta cell function. As outlined above, insulin and C-peptide are directly connected and related to beta cell function. They are therefore suitable for evaluating the secretion rate and for monitoring the function of beta cells. In detail, insulin measurements allow determination of the insulin secretion rate or the insulin sensitivity and resistance of individuals. Type 2 diabetes is a heterogeneous disease with different causes. Manifestation of the disease can occur due to decreased beta cell function, accompanied by reduced insulin secretion, or due to increased insulin resistance, or both. Beta cell function and insulin resistance can be assessed during an oral glucose tolerance test measuring insulin and glucose concentrations (Stumvoll et al., 2000). Insulin resistance is a key driver and an independent risk factor for the development of the metabolic syndrome and type 2 diabetes (Lillioja et al., 1993; Rewers et al., 2004; Weyer et al., 2001). Thus, quantification of insulin supports the risk assessment of individuals susceptible to the development of type 2 diabetes. For this purpose, specified calculations are conducted to quantify insulin resistance, insulin sensitivity, and beta-cell function. For example, the Homeostasis model assessment (HOMA) score is applied to determine insulin resistance using the concentrations of insulin and glucose (Matthews et al., 1985). The determination of C-peptide

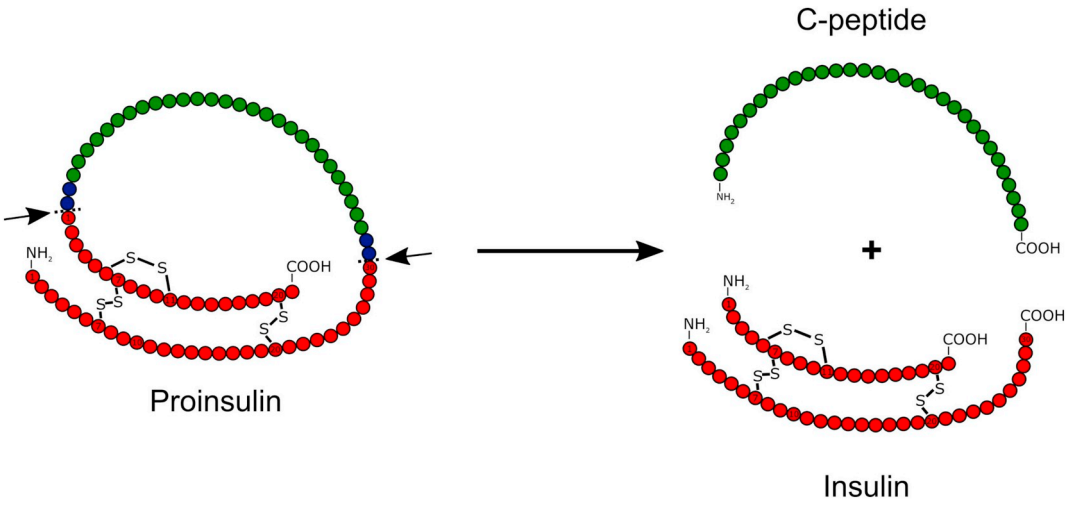

Fig. 8. Schematic illustration of insulin synthesis by proinsulin cleavage.
After formation of proinsulin in the endoplasmic reticulum, proinsulin is packed in granules and split into insulin and C-peptide by limited proteolysis (arrows). Upon stimulation (e.g. by glucose), both peptides are rapidly secreted into circulation at an equimolar ratio. As insulin is rapidly degraded by the liver, insulin concentrations in the peripheral blood are markedly reduced compared to C-peptide concentrations.

concentration is also important for the differentiation between type 1 and type 2 diabetes and the classification of diabetes subtypes (Jones and Hattersley, 2013; Oram et al., 2014). The evaluation of hypoglycemia in nondiabetic individuals is another important indication for the determination of insulin and C-peptide. An insulinoma is an uncommon disease characterized by hyperinsulinemia due to enhanced insulin secretion by neoplastic cells, mainly localized in the pancreas. The diagnosis can be made when a symptomatic patient has significantly reduced blood glucose concentrations while at the same time the insulin or C-peptide concentrations are inappropriately high (Cryer et al., 2009).

In summary, insulin and C-peptide are useful laboratory biomarkers for clinical and research approaches to diagnose, classify, and monitor diabetes mellitus. It is therefore essential to use assays that are harmonized, thereby ensuring the accurate, comparable, and reliable determination of insulin and C-peptide.

5.2. Definition of the biomarker

As shown in Fig. 8, insulin and C-peptide are well defined on the molecular level. The first insulin immunoassay, a radioimmunoassay (RIA), was described by Yalow and Berson (Yalow and Berson, 1959) and used polyclonal guinea-pig anti-bovine insulin antibodies. However, it could not discriminate between insulin, proinsulin, or different proinsulin variants. This was a particular problem for patients with type 2 diabetes or enhanced insulin resistance, who show increased proinsulin levels. For these patients, there was no possibility of correctly determining insulin secretion using the insulin assays available at this time (Temple et al., 1992). With the development of monoclonal anti-insulin antibodies, the sensitivity and specificity of insulin immunoassays improved and automated assays with higher reliability and reproducibility could be developed (Schroer et al., 1983). Cross-reactivity of insulin with proinsulin and its variants was reduced, making it a suitable method to evaluate insulin secretion rates in patients with diabetes.

C-peptide was first described as a “byproduct” of insulin biosynthesis (Steiner et al., 1967; Steiner and Oyer, 1967). It consists of 31 amino acids and is secreted in equimolar amounts with insulin in circulation. The first antibodies for C-peptide immunoassays were generated between 1973 and 1976, after C-peptide could be chemically synthesized (Naithani et al., 1975; Yanaihara et al., 1975). Since then, these immunoassays have been used in large numbers in numerous clinical trials and C-peptide determination is now widely accepted as a surrogate marker of insulin secretion in diabetes. Despite great efforts in the field of immunoassays over the last several decades, comparison analyses between different assay systems (using both insulin and C-peptide) and between laboratories still showed significant differences at that time (Wiedmeyer et al., 2007).

5.3. Harmonization and implementation

As described before, the increasing and widespread use of C-peptide and insulin immunoassays and the continuing discordant comparison results made it necessary to implement an international standard to achieve harmonization of these assays. In the following section, we focus on the efforts to standardize and harmonize C-peptide and insulin immunoassays.

C-peptide: A WHO international reference reagent (IRR) for human C-peptide (NIBSC code 84/510), as it is required for a reference measurement system (compare Fig. 1), has been available since 1977. However, persistent substantial differences remained and made it necessary to improve C-peptide immunoassays. In 2002, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) established a C-peptide standardization committee and organized an international C-peptide comparison study with focus on the comparability of C-peptide results between methods and between laboratories (Wiedmeyer et al., 2007). This report revealed that the C-peptide determinations were not sufficiently comparable. However in another study, using for the first time a LC-MS based reference method for recalibration, significant improvements of the coefficients of variation between methods and between laboratories could be shown (Little et al., 2008). Furthermore in 2014, the LC-MS reference methods described by Kinumi and colleagues (Kinumi et al., 2014) and by Stoyanov and colleagues (Stoyanov et al., 2013) were successfully listed in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database, which is necessary for the re-calibration of C-peptide assays by the manufacturers. In addition, pooled serum samples have been established as secondary reference materials. However, the manufacturers still need to re-calibrate the assays in order to see the full impact on the harmonization process. In 2017, 30 years after the first C-peptide standard had been established and which is now exhausted, the first international standard was prepared and evaluated (NIBSC code 13/146) (Moore et al., 2017). The WHO Expert Committee on Biological Standardization (ECBS) has approved this material for human C-

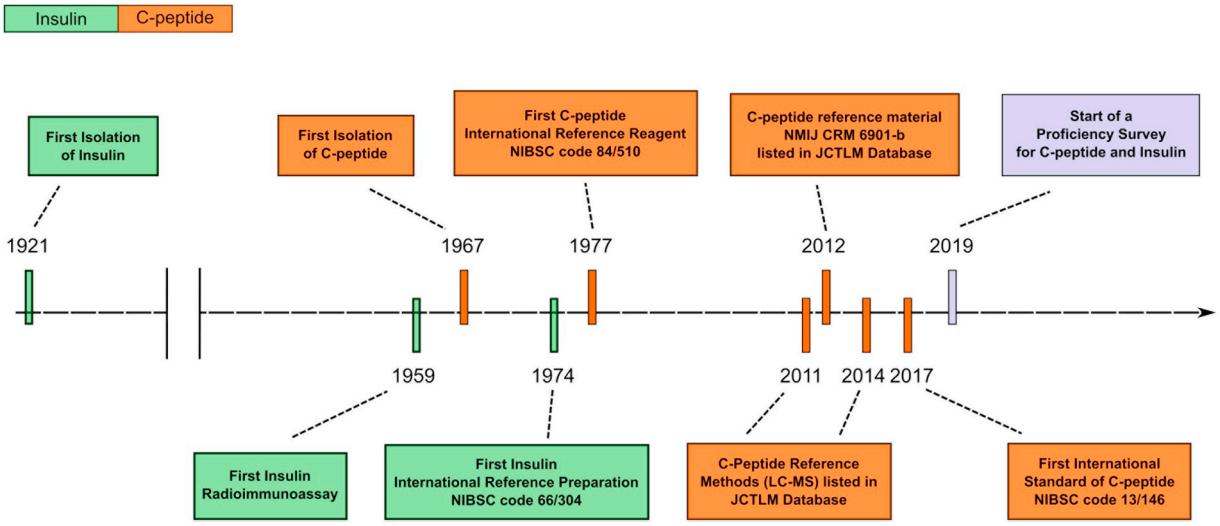

Fig. 9. Timeline of the standardization and harmonization process of C-peptide and insulin immunoassays. Important landmarks in the history of C-peptide (orange) and insulin (green), starting with the discovery or first description of both parameters and the subsequent steps towards international reference materials and reference methods. Details of these efforts are described in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peptide assays. However, the NIBSC material was produced for the calibration of C-peptide immunoassays and not as primary reference material in the ID-MS reference measurement system. It is therefore not appropriate to standardize C-peptide assays. In addition to NIBSC 13/146, another reference material is available. The National Metrology Institute of Japan (NMIJ) provides a C-peptide certified reference material (NMIJ CRM 6901-b; (Kinumi et al., 2012)), which is listed in the JCTLM database and is suitable for the ID-MS reference management system (Little et al., 2017a; Little et al., 2017b). Therefore, according to the minutes of the C-peptide standardization committee, the NMIJ certified reference material 6901-b should be used as primary reference material for the traceability chain. This procedure was well described in a recent report by Little and colleagues proposing a traceability chain, based on the in vitro diagnostic medical devices ISO 17511:2003 (ISO, 2003), for the implementation of a reference measurement system for C-peptide (Little et al., 2017b). Despite this tremendous success, there are competing ongoing efforts in parallel among the participating laboratories in different countries. Increased cooperation among these laboratories, together with the outlined scheme, can make this long-term project a real success.

Insulin: Despite its limited indications in clinical practice, accurate and reliable determination of insulin concentrations is crucial for research studies and special clinical applications, in particular for the determination of insulin resistance as part of multicenter studies. The first human insulin IRP (NIBSC code 66/304) was established in 1974. Prior to that reference, each laboratory prepared their own insulin standards producing variable assay results due to the diverse immunogenic and biologic properties of the applied antibodies. Several decades later, a task force from the ADA on the standardization of insulin assays investigated the analytical performance of insulin assays and reported significantly different values across laboratories and methods (Robbins et al., 1996). These differences were also reported in a later ADA Workgroup study from 2004 to 2006 (Marcovina et al., 2007). To improve these issues the ADA working group recommended: a) the preparation of a new reference material, b) the validation of the preparation and c) the development of a reference method. At that time there had been no approved reference method for the determination of insulin. In the following years, an isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/MS) procedure was reported by Miller and colleagues leading to a slightly improved comparability between methods (Miller et al., 2009). However, at the present time, there is no reference material or reference method listed in the JCTLM database for insulin. These efforts are underway by the IFCC Working Group on Standardization of Insulin Assays (SWG-IA). In summary, there is clearly an ongoing need for improvement in standardization and harmonization of insulin assay results. In contrast to the processes that have been reached for C-peptide assays, standardization and harmonization of insulin assays still requires much effort. The proposed traceability chain by Little and colleagues for the implementation of an RMP to C-peptide assays can therefore be assumed as a model for insulin standardization (Little et al., 2017b).

Regarding both biomarkers, an external quality assessment is useful in comparing measurement results between laboratories and in demonstrating the success of the harmonization process. Results of C-peptide measurements included in the German external quality assessment study show high agreement between methods and laboratories, suggesting effectiveness of the previous efforts (RfB, 2019). In contrast, insulin external quality assessment results showed significant and relevant differences for the same reference material between methods and laboratories (INSTAND e. V., 2019). It is therefore important to continue efforts in the harmonization and implementation process of insulin to guarantee reliable, comparable, and reproducible measurement results. In 2019, the College of American Pathologists will begin an accuracy-based program including insulin and C-peptide to assess the differences among methods for each analyte. This may help to evaluate the current progress of the harmonization process.

5.4. Does the harmonization of insulin and C-peptide improve patient care and safety?

In the past decades substantially improvements have been made in the standardization of immunoassays for C-peptide and insulin. Fig. 9 shows important steps towards a standardization of these immunoassays. However, this process is still ongoing and especially in case of insulin immunoassays further efforts are still needed. Standardization is crucial to compare results from different research studies and clinical trials. Although insulin is the biological active hormone, its indications in clinical practice are limited. Determination and interpretation of insulin resistance and sensitivity is still restricted to research studies. Furthermore preanalytical issues for insulin determination, such as a shorter half-life compared to C-peptide and a higher affection by hemolysis limits its widespread use. The establishment and implementation of traceable reference intervals for insulin is also challenging (Larsen et al., 2017). However, cutoff values of insulin

concentrations and insulin sensitivity are needed to implement insulin determination in routine clinical practice (Staten et al., 2010). In contrast, C-peptide is recommended and widely used for diabetes classification and as a surrogate marker for insulin secretion (Palmer et al., 2004), especially for insulin-treated patients (Jones and Hattersley, 2013). C-peptide exhibits a longer half-life in vivo and the standardization and harmonization process is far advanced in comparison to insulin (Bonser and Garcia-Webb, 1984; Little et al., 2017b). This enables reliable and correct decisions concerning diabetes diagnosis, distinction between diabetes mellitus type 1 and 2 (Leighton et al., 2017) and therapy, thereby directly increasing the patient's safety. Furthermore, reliable comparison of C-peptide is also essential for the recently introduced novel classification of diabetic subgroups suggested by a Scandinavian consortium (Ahlqvist et al., 2018). This classification is based on both anthropomorphic data and on biomarkers in particular, including HbA1c, beta cell autoantibody, GAD, and C-peptide to calculate HOMA indices for assessing insulin secretion and insulin resistance. This novel classification leads to more distinct classification of type 2 diabetes indicting e.g. if the diabetic patient is more prone to diabetic retinopathy or nephropathy. Since this novel classification is mainly based on laboratory biomarkers wrong clinical decisions may be made if the respective biomarkers are not harmonized or standardized.

6. Autoantibodies to beta cell proteins

Autoantibodies to antigens of the pancreatic islet beta cells are a hallmark of type 1 diabetes pathogenesis (Ziegler and Nepom, 2010). They precede diabetes onset in > 95% of children who develop disease and are frequent in insulin-requiring diabetes in adults. Bottazzo and colleagues first identified islet cell antibodies (ICA) in sera of type 1 diabetic patients who had polyendocrine autoimmune deficiencies associated with organ-specific autoimmunity (Bottazzo et al., 1974). These antibodies were detected by indirect immunofluorescence tested on sections of fresh frozen human pancreas bound to cytoplasmic antigens. ICA were subsequently found at high frequency in children with newly diagnosed type 1 diabetes, and it was recognized that these antibodies are often present in the serum from non-diabetic relatives of individuals with type 1 diabetes indicating a prediabetic period with an autoimmune serology (Gorsuch et al., 1981). ICA are heterogeneous between individuals with respect to their molecular targets, thus tests are difficult to standardize. Today, ICA testing is being superseded by testing for autoantibodies against biochemically defined islet antigens. While a variety of molecules have been reported (Lampasona and Liberati, 2016), only the following have been confirmed as major targets of autoantibodies in type 1 diabetes.

6.1. Insulin autoantibodies

It had long been recognized that treatment with exogenous forms of insulin could induce antibodies directed against insulin peptides when in 1983, Palmer and colleagues first described insulin autoantibodies (IAA) in type 1 diabetic patients before they received insulin therapy (Palmer et al., 1983). Insulin and proinsulin are early targets of islet autoimmunity, and IAA are frequent (> 70%) in childhood diabetes and less prominent markers of type 1 diabetes with clinical onset after adolescence. The first occurrence of IAA is marked at around 1–2 years of age (Krischer et al., 2015; Parikka et al., 2012; Ziegler et al., 2012), and there are distinct immunization patterns with respect to the affinity and epitope specificities of IAA (Achenbach et al., 2004). In general, the high-affinity IAA are more predictive for type 1 diabetes and share certain characteristics, including association with HLA DRB1*04, subsequent progression to autoantibodies against multiple beta cell antigens, binding to human insulin A chain residues 8–13, and binding to proinsulin. In contrast, children with IAA of lower affinity rarely progress to type 1 diabetes (Achenbach et al., 2010).

6.2. GAD autoantibodies

In 1990, Baekkeskov and colleagues identified a 65-kDa isoform of glutamate decarboxylase (GAD65) as major type 1 diabetes autoantigen (Baekkeskov et al., 1990). Autoantibodies to GAD (GADA) are frequent (> 70%) in type 1 diabetes seen at all ages, and the typical marker of adult-onset type 1 diabetes including the so-called latent autoimmune diabetes in adults (LADA) (Tuomi et al., 1993). GADA are also detected in neurological disorders, especially in stiff man syndrome and in conditions unrelated to diabetes (Baekkeskov et al., 1990). Affinity and epitope specificity of GADA can stratify the progression to type 1 diabetes (Mayr et al., 2007; Williams et al., 2015) and predict insulin therapy in individuals with adult-onset diabetes (Achenbach et al., 2018; Krause et al., 2014).

6.3. IA-2 and IA-2β autoantibodies

In 1994, Rabin and colleagues recognized islet cell antigen 512 (ICA512) as diabetes-specific autoantigen related to protein tyrosine phosphatases (Rabin et al., 1994). Before, two tryptic digest fragments of islet antigens had been characterized from individuals with type 1 diabetes (Christie et al., 1993). A 40-kDa fragment from the intracellular portion of a tyrosine phosphatase-like protein (*PTPRN* gene) is now referred to as IA-2ic or ICA512ic (Payton et al., 1995). Autoantibodies to IA-2 (IA-2A) are highly specific and predictive for type 1 diabetes (Decochez et al., 2002), and frequently detected (> 70%) at clinical onset. They usually occur together with other beta cell autoantibodies. The other 37-kDa tryptic fragment was identified as the IA-2-related protein IA-2β or phogrin (Hawkes et al., 1996; Lu et al., 1996). Since almost all individuals with autoantibodies against IA-2β also have IA-2A, IA-2β autoantibodies are not commonly used as a first line test, although they can identify individuals at high risk of disease progression (Achenbach et al., 2008; De Grijse et al., 2010).

6.4. ZnT8 autoantibodies

In 2007, Hutton and colleagues identified zinc transporter-8 (ZnT8) as type 1 diabetes autoantigen by screening for highly expressed islet beta cell-specific molecules (Wenzlau et al., 2007). Autoantibodies to ZnT8 (ZnT8A) are found in about 70% of patients with type 1 diabetes and improve prediction of disease. As for IA-2A; they usually occur together with other beta cell autoantibodies. A principle epitope targeted by ZnT8A is influenced by the single amino acid at position 325 encoded as arginine, tryptophan, or glutamine by different polymorphic variants of the ZnT8-encoding gene *SLC30A8* (Wenzlau et al., 2008). Autoimmunity against the COOH-terminal region of ZnT8 is highly prognostic for progression to clinical diabetes (Achenbach et al., 2009; Andersson et al., 2011; De Grijse et al., 2010).

6.5. Clinical utility

Beta cell autoantibodies circulate in blood, signalling an active and disease-specific B lymphocyte response, they can be detected very reproducibly and are therefore excellent and available immune biomarkers for type 1 diabetes. In individuals with clinically manifest diabetes, these autoantibodies are used for disease classification, distinguishing between autoimmune (type 1) and non-autoimmune (mainly type 2) diabetes, and for prediction of the need for insulin treatment (Bingley, 2010). Traditionally clinicians have differentiated between type 1 and type 2 diabetes based on phenotypic characteristics, including age at onset, abruptness of onset of hyperglycemia, ketosis-proneness, degree of obesity, prevalence of other autoimmune disease, and the need for insulin replacement therapy. The presence or absence of beta cell autoantibodies in the blood is an important additional criterion if the diagnosis is not clear by clinical symptoms or other laboratory biomarkers. In particular, a subset of patients with adult-onset

diabetes present clinically without ketoacidosis and weight loss, require no insulin therapy initially but develop insulin dependence more rapidly than typical type 2 diabetic patients, and autoantibodies (mainly GADA) are detectable. This slowly developing form of autoimmune type 1 diabetes, also called LADA, found in people over 30 years old, is often misdiagnosed and treated as type 2 diabetes (Leslie et al., 2016). Thus the different etiology of diabetes affects the clinical phenotype of patients and can be uncovered by autoantibody testing, to the benefit of therapy.

Moreover, beta cell autoantibodies are excellent biomarkers for identifying early immunization to beta cell antigens and monitoring progression of islet autoimmunity in preclinical diabetes. Although beta cell autoantibodies are not considered effectors of beta cell damage, they are used for identification of individuals at risk for developing type 1 diabetes, and as end-points in observational and intervention studies (Bonifacio, 2015; Ziegler et al., 2013). Clinical trials are currently in progress to identify ways to prevent or reverse the autoimmunity of type 1 diabetes (Battaglia et al., 2017). These trials commonly use autoantibody positivity as an inclusion criterion for participants. More recently, beta cell autoantibodies are used for diagnosing asymptomatic early stage type 1 diabetes (Insel et al., 2015; Raab et al., 2016).

However, particularly single positive autoantibody results require critical interpretation since 1–7% of individuals without diabetes and the same number of individuals with type 2 diabetes have false positive (i.e. not type 1 diabetes-related, mainly low-affinity) autoantibodies depending on the cut-off for positivity, and the assay method used (Thomas et al., 2018). It is therefore obvious that the assays for the determination of beta cell autoantibodies need to be harmonized and improved, particularly in their specificity for type 1 diabetes. The latter could be achieved by modifying the target antigen to reduce unspecific binding sites. For example, the presence of autoantibodies to N-terminally truncated GAD65 (consisting of amino acids 96–585) is associated with the clinical phenotype of autoimmune type 1 diabetes and predicts insulin therapy in adult-onset diabetes, whereas restricted antibody binding to N-terminal GAD65 epitopes is associated with similar clinical phenotype to GADA-negative, type 2 diabetic individuals (Achenbach et al., 2018).

6.6. Definition of the biomarker

Unlike other biomarker immunoassays, the assays for detection of beta cell autoantibodies are not using antibodies for measuring the biomarker (i.e. the specific autoantibody) but rather determining the quantity of autoantibodies against specific beta cell proteins. Since the molecular nature of the main target antigens in type 1 diabetes is known, primary reference material for immunoassays can be defined, and human insulin, GAD, IA-2, and ZnT8 protein can be prepared by recombinant procedures. On the other hand, the biomarker autoantibodies are less well defined since a) the autoantibodies present in a person may shift between immunoglobulin isotypes and subtypes and target epitopes over time and b) the epitope recognition vary substantially between individuals. This biological variability makes the molecular definition and, therefore, standardization of a unique autoantibody-immunoglobulin impossible. Another challenge for reducing the variability of assay results is that most epitopes recognized by beta cell autoantibodies are conformational epitopes requiring the correct protein structure. Thus, single epitope assays are hard to achieve.

Today, most research laboratories use various forms of non-commercial fluid phase radio-binding assays (RBAs) for detecting autoantibodies. Some commercial ELISAs now perform as well or better than RBAs in detecting GADA (Torn et al., 2008). While IAA are considered important autoantibodies for predicting type 1 diabetes in children, current assays produce highly variable results, and only a few clinical laboratories consistently perform RBAs with high sensitivity and specificity for the disease (Schlosser et al., 2010). Recently, high-performing and low serum volume-requiring IAA assays have been developed based on luciferase immunoprecipitation system (LIPS) and electrochemiluminescence (ECL) technology, These assays are suitable for large-scale capillary blood screening of young children (Liberati et al., 2018; Yu et al., 2013).

6.7. Harmonization and implementation

Harmonizing the assays for the detection of beta cell autoantibodies is challenging but essential in order to obtain results that are comparable among laboratories, particularly for multicenter studies. This in mind, the first standardization workshops were established with focus on cytoplasmic islet cell antibodies (Boitard et al., 1988; Bonifacio et al., 1990). Later on, the workshop also referred to other beta cell autoantibodies; the aim was to establish reference materials and to improve laboratory methods measuring diabetes-associated autoantibodies (Verge et al., 1998). The WHO adopted a serum reference standard for GADA and IA-2A assays, and these standards have assigned values of 250 units/mL for each autoantibody (Mire-Sluis et al., 2000). An international reference standard for IAA and ZnT8A does not yet exist.

Another step forward to harmonization of beta cell autoantibody assays was promoted by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). To this end, an Islet Autoantibody Harmonization Committee was established to enable comparison of autoantibodies between different laboratories included in multicenter studies (Bonifacio et al., 2010). A NIDDK working calibrator was prepared and introduced, which was made from a mixture of numerous sera to reduce bias. This is in contrast to the WHO reference material, which was made out of a single serum preparation. Calibration was done against the WHO reference material to ensure that units obtained using the NIDDK calibrator, i.e. NIDDK units, are comparable to WHO units. The harmonized assays showed higher comparability and disagreements in total values could be reduced.

In 2000, the Immunology of Diabetes Society (IDS) and the U.S. Centers for Disease Control and Prevention (CDC) established the Diabetes Antibody Standardization Program (DASP) to improve comparability and to act as an outlet mechanism to evaluate new autoantigens and test methodologies (Bingley et al., 2003). Since 2012, this program has been continued as Islet Autoantibody Standardization Program (IASP), funded by the U.S. National Institutes of Health (NIH), supervised by the IDS and organized by the TrialNet Islet Cell Autoantibody Core Laboratory at the University of Florida. The goal of DASP/IASP is to improve detection and diagnosis of autoimmune diabetes by: a) providing technical support, training, and information about the best methods; b) providing proficiency testing to evaluate laboratory performance; c) supporting development of highly sensitive and specific measurement technologies; and d) developing reference materials. Since its inception, every 18 months DASP/IASP has conducted international workshops in which laboratories worldwide assay blinded samples from 50 patients with new-onset type 1 diabetes and up to 100 controls. This format provides an evaluation of the sensitivity and specificity of each test and enables DASP/IASP to assess implementation of assay methods and to document any improvement in performance. Among the major accomplishments to date, DASP/IASP has demonstrated that assays in which antibody binding occurs at the interface of a solid surface coated with antigen, like direct ELISAs, do not allow for sensitive and specific detection of beta cell autoantibodies, in contrast to assay formats in which the interaction of antibodies with antigen happens completely or partially in fluid phase such as RBA, and bridge-ELISA (Lampasona and Liberati, 2016). Other DASP/IASP activities include the evaluation of the stability of the WHO GADA and IA-2 autoantibody standard; the evaluation of standard method protocols for GADA and IA-2A, and the validation of new autoantibody markers (Achenbach et al., 2007; Bingley et al., 2003; Lampasona et al., 2011; Williams et al., 2015). As a general recommendation, before using an autoantibody assay for type 1 diabetes risk assessment and diagnostic

purpose, its performance in the IDS-based international workshops should be ascertained.

6.8. Does the harmonization of diabetic autoantibodies improve patient care and safety?

Exact and reproducible autoantibody measurement is a pre-requisite for accurate prediction of type 1 diabetes and diagnostic autoantibody testing in patients. The development of autoantibodies against multiple beta cell antigens has long been recognized as a critical step in pathogenesis, associated with a significantly higher diabetes risk than the presence of just a single autoantibody (Bingley et al., 1994; Verge et al., 1996). More recently, it became evident that children who have developed two or more of the beta cell autoantibody types almost inevitably progress to clinically symptomatic diabetes (Ziegler et al., 2013). These findings have led to a new staging of type 1 diabetes, which classifies the presence of advanced beta cell autoimmunity (multiple autoantibodies) in individuals without diabetic symptoms as an early stage of disease (Insel et al., 2015). Since 2015, the Fr1da study in Bavaria, Germany uses capillary blood beta cell autoantibody screening for identifying asymptomatic early stage type 1 diabetes in a total of 100,000 children aged 2–5 years (Raab et al., 2016). This study aims to assess whether early staging of type 1 diabetes: a) is feasible at a population-based level; b) prevents severe metabolic decompensation observed at the clinical manifestation of type 1 diabetes; and c) reduces psychological distress through preventive teaching and care. At a time when there is an effective treatment available to reintroduce tolerance in autoimmune diabetes, high-quality, high-throughput, and harmonized autoantibody tests will become essential for identifying individuals who can benefit from such treatment. Today, the presence of beta cell autoantibodies is already used as a criterion for recruiting individuals into prevention studies, or as study end point (Battaglia et al., 2017; Bonifacio, 2015). Furthermore, a harmonized protocol is already implemented for measuring autoantibody responses in large multicenter prospective natural history studies of type 1 diabetes, such as the The Environmental Determinants of Diabetes in the Young (TEDDY) study (Krischer et al., 2015).

7. Critique and outlook

The general characteristics of important biomarkers in diabetology discussed above are summarized in Table 1. After the discovery of HbA1c in 1969 and the recognition of its potential clinical use as biomarker for diagnosis of diabetes and metabolic monitoring, numerous assays based on various different principles have been introduced. However, the different assays yielded variable results causing widespread confusion among the users. It took > 30 years until a worldwide accepted standardization was achieved. Even worse, although insulin was discovered in 1921, standardization of insulin immunoassays is still in process after its first description a century ago and recognition of its central physiological role in metabolism. Accordingly, it took > 50 years following the discovery of C-peptide in 1967 until the standardization procedure for C-peptide immunoassays has been proposed in 2017. The situation concerning autoantibodies (detected in 1974) is different since autoantibodies are highly variable in individual patients and may also vary over time. Here, after a long initiation period international efforts have achieved substantial improvement in harmonization over the last decades. Taken together, the described history of harmonization of these important biomarkers in diabetology teaches us to initiate international harmonization efforts immediately after the clinical use of newly discovered biomarkers has been demonstrated, e.g. by establishing international working groups.

The importance of proper determination of the discussed biomarkers in diabetology is obvious considering the classification of diabetes subtypes. As outlined in Fig. 10 plasma concentrations of insulin or C-peptide together with the occurrence of autoantibodies are

Table 1
General characteristics of immunoassays as biomarkers in diabetology.

Biomarker	Discovery/first descripton	Formation process – physiological role	Clinical utility	Measurement methods (most used)	Status of harmonization and standardization
HbA1c	1969	• metabolic byproduct • non-enzymatic addition of glucose to N-terminal valine of the hemoglobin β-chain	• diagnosis of DM • therapy monitoring • long-term glycemic control • correlation with long-term diabetic complications	Immunoassay HPLC	• higher-order reference material and reference measurement methods listed in the JCTLM database
Insulin	1921	• endogenous hormone • synthesized and generated from proinsulin in the pancreatic beta-cells • secreted upon stimulation	• evaluation of beta-cell function/insulin reserve • during a fasting test • determination of insulin sensitivity and resistance	Immunoassay	• JCTLM listed reference material and reference methods not available • Standardization working group established
C-peptide	1967	• byproduct from limited proteolysis of proinsulin • secreted together with insulin	• differential diagnosis of DM • during a fasting test • determination of insulin sensitivity and resistance	Immunoassay	• higher-order reference material and reference measurement methods listed in the JCTLM database
Autoantibodies	1974	• autoimmune pathogenesis • promoting the autoimmune response against beta cell antigens • antibodies with high biological variability	• differential diagnosis of DM • prediction of the need for insulin treatment • identification of individuals at risk for developing type 1 DM • diagnosing asymptomatic early type 1 DM	Immunoassay	• JCTLM listed reference material and reference methods not available • WHO reference material for GADA and IA-2A available • NIDDK working calibrator for GADA and IA-2A available

HbA1c, Insulin, C-peptide and diabetes associated autoantibodies are laboratory parameters supporting the diagnosis, prognosis and classification of diabetes mellitus (DM).

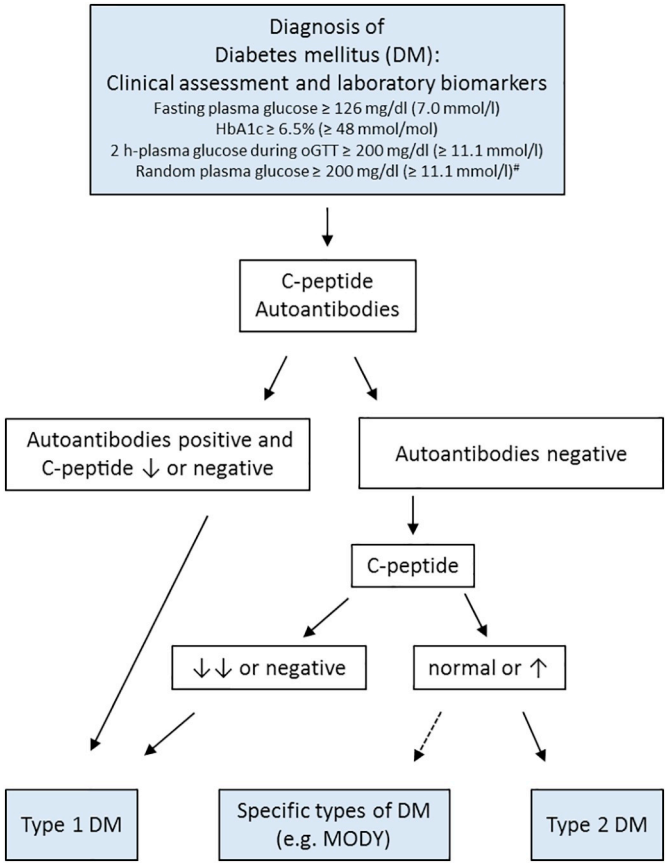

Fig. 10. Clinical algorithm for classification of Diabetes mellitus.
Diabetes mellitus can be diagnosed according to the criteria for plasma glucose or HbA1c measurements together with clinical symptoms (# in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis). Type 1 and type 2 diabetes is traditionally classified based on phenotypic characteristics (age at onset, abruptness of onset of hyperglycemia, ketosis-proneness, degree of obesity and the need for insulin replacement therapy). In case of uncertain classification the determination of diabetes-associated autoantibodies and C-peptide can be beneficial. Positive testing for autoantibodies accompanied with low or negative C-peptide is indicative for type 1 diabetes mellitus. In case of negative autoantibodies, C-peptide can be used for classification, e.g. in lean patients or patients with poor response to sulfonylureas or metformin. Strongly decreased or absent C-peptide in these patients indicates type 1 diabetes. Evidence for sufficient C-peptide is in accordance with type 2 diabetes diagnosis or in rare cases with specific types of diabetes. If diagnosis of type 2 Diabetes is not appropriate further testing, e.g. genetic analyses, is recommended. The standardization and harmonization of the indicated biomarkers is essential for the proposed algorithm to guide correct diabetes classification and therapy.

essential for the classification of type 1 and type 2 diabetes if the clinical diagnosis is uncertain. Furthermore, the illustrated clinical algorithm may help to indicate if a specific, less common type of diabetes may be present in an individual patient. Following this scheme, laboratory biomarkers need to be standardized and harmonized to avoid misclassification and in consequence possibly wrong clinical decisions.

The transfer of these theoretical considerations into routine laboratory measurements is not without problems since different manufacturers use different assay principles. This is particularly true for the measurement of HbA1c. The offered assays may be based on chromatography, electrophoresis, affinity to boronate or on specific antibodies. According to the survey 2018b of the College Of American Pathologists (http://www.ngsp.org/capdata.asp) immunoassays are the most widely used assays (e.g. Roche, Basel, Switzerland and Siemens Healthineers, Erlangen, Germany) followed by ion chromatography using HPLC (e.g. Bio-Rad, Hercules, USA and Tosoh, Tokio, Japan). Many manufacturers of immunoassays for insulin and C-peptide are on the market which is quite diverse for these biomarkers and therefore it is not clear which assay is the most widely used worldwide. For the determination of autoantibodies to GAD, IA-2 and ZnT8 the solid-phase/liquid-phase bridge ELISA provided by RSR Ltd. (Cardiff, United Kingdom) is used by most laboratories. Other manufacturers produce immunoassays based on the antigens used in these ELISAs. Taken together this diversity emphasizes the need not only for harmonization of the assays of biomarkers but also the need for implementation of the whole standardization procedure worldwide.

8. Conclusions

Harmonization of laboratory tests is a pre-requisite for the comparability of biomarkers contributing to the diagnosis, classification, and monitoring of individuals. The increasing use of clinical practice guideline based on outcome studies requires biomarkers to be harmonized or, if possible, standardized. Besides glucose, HbA1c is the major laboratory biomarker supporting the diagnosis of diabetes mellitus while insulin and C-peptide and autoantibodies against beta cell epitopes are important biomarkers for the classification of diabetes subtypes. HbA1c has been standardized worldwide and standardization of C-peptide is close to be finalized but standardization procedure for insulin is still in process. Most important for the prediction of type 1 diabetes in infants and young individuals worldwide the harmonization of autoantibodies against beta cell epitopes is well underway. Further progress in harmonization and standardization will increase the comparability of laboratory tests and therefore support the reliability of biomarkers for clinical decisions, finally improving patient care and safety.

Acknowledgements

We thank I. Rettig for critical reading of the manuscript.

Funding

The present study was supported in part by a grant (01GI0925) from the German Federal Ministry of Education and Research to the German Center for Diabetes Research (DZD e.V.)

Conflict of interest

No conflicts of interest were reported by S.H., P.A., E.S. and A.P.

References

Achenbach, P., Koczwara, K., Knopff, A., Naserke, H., Ziegler, A.G., Bonifacio, E., 2004. Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. J. Clin. Invest. 114 (4), 589–597.
Achenbach, P., Schlosser, M., Williams, A.J., Yu, L., Mueller, P.W., Bingley, P.J., Bonifacio, E., 2007. Combined testing of antibody titer and affinity improves insulin autoantibody measurement: diabetes antibody Standardization program. Clin. Immunol. 122 (1), 85–90.
Achenbach, P., Bonifacio, E., Williams, A.J., Ziegler, A.G., Gale, E.A., Bingley, P.J., Group, E., 2008. Autoantibodies to IA-2beta improve diabetes risk assessment in high-risk relatives. Diabetologia 51 (3), 488–492.
Achenbach, P., Lampasona, V., Landherr, U., Koczwara, K., Krause, S., Grallert, H., Winkler, C., Pfluger, M., Illig, T., Bonifacio, E., Ziegler, A.G., 2009. Autoantibodies to zinc transporter 8 and SLC30A8 genotype stratify type 1 diabetes risk. Diabetologia 52 (9), 1881–1888.
Achenbach, P., Guo, L.H., Gick, C., Adler, K., Krause, S., Bonifacio, E., Colman, P.G., Ziegler, A.G., 2010. A simplified method to assess affinity of insulin autoantibodies. Clin. Immunol. 137 (3), 415–421.
Achenbach, P., Hawa, M.I., Krause, S., Lampasona, V., Jerram, S.T., Williams, A.J.K., Bonifacio, E., Ziegler, A.G., Leslie, R.D., Action, L.c., 2018. Autoantibodies to N-terminally truncated GAD improve clinical phenotyping of individuals with adult-onset diabetes: action LADA 12. Diabetologia 61 (7), 1644–1649.
Ahlqvist, E., Storm, P., Karajamaki, A., Martinell, M., Dorkhan, M., Carlsson, A., Vikman, P., Prasad, R.B., Aly, D.M., Almgren, P., Wessman, Y., Shaat, N., Spegel, P., Mulder, H., Lindholm, E., Melander, O., Hansson, O., Malmqvist, U., Lernmark, A., Lahti, K.,

Forsen, T., Tuomi, T., Rosengren, A.H., Groop, L., 2018. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol. 6 (5), 361–369.
American Diabetes Association, A, 2018. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes-2018. Diabetes Care 41 (Suppl. 1), S13–S27.
Andersson, C., Larsson, K., Vaziri-Sani, F., Lynch, K., Carlsson, A., Cedervall, E., Jonsson, B., Neiderud, J., Mansson, M., Nilsson, A., Lernmark, A., Elding Larsson, H., Ivarsson, S.A., 2011. The three ZNT8 autoantibody variants together improve the diagnostic sensitivity of childhood and adolescent type 1 diabetes. Autoimmunity 44 (5), 394–405.
Andreis, E., Kullmer, K., Appel, M., 2014. Application of the reference method isotope dilution gas chromatography mass spectrometry (ID/GC/MS) to establish metrological traceability for calibration and control of blood glucose test systems. J. Diabetes Sci. Technol. 8 (3), 508–515.
Artunc, F., Schleicher, E., Weigert, C., Fritsche, A., Stefan, N., Haring, H.U., 2016. The impact of insulin resistance on the kidney and vasculature. Nat. Rev. Nephrol. 12 (12), 721–737.
Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., De Camilli, P., 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347 (6289), 151–156.
Battaglia, M., Anderson, M.S., Buckner, J.H., Geyer, S.M., Gottlieb, P.A., Kay, T.W.H., Lernmark, A., Muller, S., Pugliese, A., Roep, B.O., Greenbaum, C.J., Peakman, M., 2017. Understanding and preventing type 1 diabetes through the unique working model of TrialNet. Diabetologia 60 (11), 2139–2147.
Bingley, P.J., 2010. Clinical applications of diabetes antibody testing. J. Clin. Endocrinol. Metab. 95 (1), 25–33.
Bingley, P.J., Christie, M.R., Bonifacio, E., Bonfanti, R., Shattock, M., Fonte, M.T., Bottazzo, G.F., Gale, E.A., 1994. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. Diabetes 43 (11), 1304–1310.
Bingley, P.J., Bonifacio, E., Mueller, P.W., 2003. Diabetes antibody standardization program: first assay proficiency evaluation. Diabetes 52 (5), 1128–1136.
Boitard, C., Bonifacio, E., Bottazzo, G.F., Gleichmann, H., Molenaar, J., 1988. Immunology and diabetes workshop: report on the third international (stage 3) workshop on the standardisation of cytoplasmic islet cell antibodies. Held in New York, New York, October 1987. Diabetologia 31 (7), 451–452.
Bonifacio, E., 2015. Predicting type 1 diabetes using biomarkers. Diabetes Care 38 (6), 989–996.
Bonifacio, E., Boitard, C., Gleichmann, H., Shattock, M.A., Molenaar, J.L., Bottazzo, G.F., 1990. Assessment of precision, concordance, specificity, and sensitivity of islet cell antibody measurement in 41 assays. Diabetologia 33 (12), 731–736.
Bonifacio, E., Yu, L., Williams, A.K., Eisenbarth, G.S., Bingley, P.J., Marcovina, S.M., Adler, K., Ziegler, A.G., Mueller, P.W., Schatz, D.A., Krischer, J.P., Steffes, M.W., Akolkar, B., 2010. Harmonization of glutamic acid decarboxylase and islet antigen-2 autoantibody assays for national institute of diabetes and digestive and kidney diseases consortia. J. Clin. Endocrinol. Metab. 95 (7), 3360–3367.
Bonser, A.M., Garcia-Webb, P., 1984. C-peptide measurement: methods and clinical utility. Crit. Rev. Clin. Lab. Sci. 19 (4), 297–352.
Bottazzo, G.F., Florin-Christensen, A., Doniach, D., 1974. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 2 (7892), 1279–1283.
Bunn, H.F., 1981. Evaluation of glycosylated hemoglobin diabetic patients. Diabetes 30 (7), 613–617.
Bunn, H.F., Haney, D.N., Gabbay, K.H., Gallop, P.M., 1975. Further identification of the nature and linkage of the carbohydrate in hemoglobin A1c. Biochem. Biophys. Res. Commun. 67 (1), 103–109.
Bunn, H.F., Haney, D.N., Kamin, S., Gabbay, K.H., Gallop, P.M., 1976. The biosynthesis of human hemoglobin A1c. Slow glycosylation of hemoglobin in vivo. J. Clin. Invest. 57 (6), 1652–1659.
Christie, M.R., Hollands, J.A., Brown, T.J., Michelsen, B.K., Delovitch, T.L., 1993. Detection of pancreatic islet 64,000 M(r) autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. J. Clin. Invest. 92 (1), 240–248.
Cryer, P.E., Axelrod, L., Grossman, A.B., Heller, S.R., Montori, V.M., Seaquist, E.R., Service, F.J., Endocrine, S., 2009. Evaluation and management of adult hypoglycemic disorders: an Endocrine Society clinical practice guideline. J. Clin. Endocrinol. Metab. 94 (3), 709–728.
De Grijse, J., Asanghanwa, M., Nouthe, B., Albrecher, N., Goubert, P., Vermeulen, I., Van Der Meeren, S., Decochez, K., Weets, I., Keymeulen, B., Lampasona, V., Wenzlau, J., Hutton, J.C., Pipeleers, D., Gorus, F.K., Belgian Diabetes, R., 2010. Predictive power of screening for antibodies against insulinoma-associated protein 2 beta (IA-2beta) and zinc transporter-8 to select first-degree relatives of type 1 diabetic patients with risk of rapid progression to clinical onset of the disease: implications for prevention trials. Diabetologia 53 (3), 517–524.
Decochez, K., De Leeuw, I.H., Keymeulen, B., Mathieu, C., Rottiers, R., Weets, I., Vandemeulebroucke, E., Truyen, I., Kaufman, L., Schuit, F.C., Pipeleers, D.G., Gorus, F.K., Belgian Diabetes, R., 2002. IA-2 autoantibodies predict impending type I diabetes in siblings of patients. Diabetologia 45 (12), 1658–1666.
European HbA1c Trial, 2018. EurA1c: the European HbA1c Trial to investigate the performance of HbA1c assays in 2166 laboratories across 17 countries and 24 manufacturers by use of the IFCC model for quality targets. Clin. Chem. 64 (8), 1183–1192.
Finke, A., Kobold, U., Hoelzel, W., Weykamp, C., Miedema, K., Jeppsson, J.O., 1998. Preparation of a candidate primary reference material for the international standardisation of HbA1c determinations. Clin. Chem. Lab. Med. 36 (5), 299–308.
Gillery, P., 2013. A history of HbA1c through clinical chemistry and laboratory medicine. Clin. Chem. Lab. Med. 51 (1), 65–74.
Gorsuch, A.N., Spencer, K.M., Lister, J., McNally, J.M., Dean, B.M., Bottazzo, G.F., Cudworth, A.G., 1981. Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. Lancet 2 (8260–61), 1363–1365.
Hannestad, U., Lundblad, A., 1997. Accurate and precise isotope dilution mass spectrometry method for determining glucose in whole blood. Clin. Chem. 43 (5), 794–800.
Hawkes, C.J., Wasmeier, C., Christie, M.R., Hutton, J.C., 1996. Identification of the 37-kDa antigen in IDDM as a tyrosine phosphatase-like protein (phogrin) related to IA-2. Diabetes 45 (9), 1187–1192.
Hoelzel, W., Weykamp, C., Jeppsson, J.O., Miedema, K., Barr, J.R., Goodall, I., Hoshino, T., John, W.G., Kobold, U., Little, R., Mosca, A., Mauri, P., Paroni, R., Susanto, F., Takei, I., Thienpont, L., Umemoto, M., Wiedmeyer, H.M., Standardization, I.W.G.o.H.c., 2004. IFCC reference system for measurement of hemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. Clin. Chem. 50 (1), 166–174.
Holmquist, W.R., Schroeder, W.A., 1966. A new N-terminal blocking group involving a Schiff base in hemoglobin AIc. Biochemistry 5 (8), 2489–2503.
Insel, R.A., Dunne, J.L., Atkinson, M.A., Chiang, J.L., Dabelea, D., Gottlieb, P.A., Greenbaum, C.J., Herold, K.C., Krischer, J.P., Lernmark, A., Ratner, R.E., Rewers, M.J., Schatz, D.A., Skyler, J.S., Sosenko, J.M., Ziegler, A.G., 2015. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care 38 (10), 1964–1974.
INSTAND e. V., Munich, Germany, 2019. C-peptide survey. Summary Eval. Year 2018. https://www.instand-ev.de/no_cache/en/eqas-online/service-for-eqa-tests/#rvp//300/-2018/C-PE, Accessed date: 14 January 2019.
International Organization for Standardization, 2003. ISO 17511:2003 – In vitro diagnostic medical devices – Measurement of Quantities in Biological Samples – Metrological Traceability of Values Assigned to Calibrators and Control Materials. https://www.iso.org/standard/30716.html.
Jeppsson, J.O., Kobold, U., Barr, J., Finke, A., Hoelzel, W., Hoshino, T., Miedema, K., Mosca, A., Mauri, P., Paroni, R., Thienpont, L., Umemoto, M., Weykamp, C., International Federation of Clinical, C, Laboratory, M., 2002. Approved IFCC reference method for the measurement of HbA1c in human blood. Clin. Chem. Lab. Med. 40 (1), 78–89.
Jones, A.G., Hattersley, A.T., 2013. The clinical utility of C-peptide measurement in the care of patients with diabetes. Diabet. Med. 30 (7), 803–817.
Kinumi, T., Goto, M., Eyama, S., Kato, M., Kasama, T., Takatsu, A., 2012. Development of SI-traceable C-peptide certified reference material NMIJ CRM 6901-a using isotope-dilution mass spectrometry-based amino acid analyses. Anal. Bioanal. Chem. 404 (1), 13–21.
Kinumi, T., Mizuno, R., Takatsu, A., 2014. Quantification of serum C-peptide by isotope-dilution liquid chromatography-tandem mass spectrometry: enhanced detection using chemical modification and immunoaffinity purification. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 953-954, 138–142.
Kobold, U., Jeppsson, J.O., Dulffer, T., Finke, A., Hoelzel, W., Miedema, K., 1997. Candidate reference methods for hemoglobin A1c based on peptide mapping. Clin. Chem. 43 (10), 1944–1951.
Koenig, R.J., Blobstein, S.H., Cerami, A., 1977. Structure of carbohydrate of hemoglobin AIc. J. Biol. Chem. 252 (9), 2992–2997.
Krause, S., Landherr, U., Agardh, C.D., Hausmann, S., Link, K., Hansen, J.M., Lynch, K.F., Powell, M., Furmaniak, J., Rees-Smith, B., Bonifacio, E., Ziegler, A.G., Lernmark, A., Achenbach, P., 2014. GAD autoantibody affinity in adult patients with latent autoimmune diabetes, the study participants of a GAD65 vaccination trial. Diabetes Care 37 (6), 1675–1680.
Krischer, J.P., Lynch, K.F., Schatz, D.A., Ilonen, J., Lernmark, A., Hagopian, W.A., Rewers, M.J., She, J.X., Simell, O.G., Toppari, J., Ziegler, A.G., Akolkar, B., Bonifacio, E., Groups, T.S., 2015. The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. Diabetologia 58 (5), 980–987.
Lampasona, V., Liberati, D., 2016. Islet Autoantibodies. Curr. Diab. Rep 16 (6), 53.
Lampasona, V., Schlosser, M., Mueller, P.W., Williams, A.J., Wenzlau, J.M., Hutton, J.C., Achenbach, P., 2011. Diabetes antibody standardization program: first proficiency evaluation of assays for autoantibodies to zinc transporter 8. Clin. Chem. 57 (12), 1693–1702.
Larsen, P.B., Linneberg, A., Hansen, T., Friis-Hansen, L., 2017. Reference intervals for C-peptide and insulin derived from a general adult Danish population. Clin. Biochem. 50 (7–8), 408–413.
Leighton, E., Sainsbury, C.A., Jones, G.C., 2017. A practical review of C-peptide testing in Diabetes. Diabetes Ther. 8 (3), 475–487.
Lenters-Westra, E., Schindhelm, R.K., Bilo, H.J., Slingerland, R.J., 2013. Haemoglobin A1c: historical overview and current concepts. Diabetes Res. Clin. Pract. 99 (2), 75–84.
Leslie, R.D., Palmer, J., Schloot, N.C., Lernmark, A., 2016. Diabetes at the crossroads: relevance of disease classification to pathophysiology and treatment. Diabetologia 59 (1), 13–20.
Liberati, D., Wyatt, R.C., Brigatti, C., Marzinotto, I., Ferrari, M., Bazzigaluppi, E., Bosi, E., Gillard, B.T., Gillespie, K.M., Gorus, F., Weets, I., Balti, E., Piemonti, L., Achenbach, P., Williams, A.J.K., Lampasona, V., 2018. A novel LIPS assay for insulin autoantibodies. Acta Diabetol. 55 (3), 263–270.
Lillioja, S., Mott, D.M., Spraul, M., Ferraro, R., Foley, J.E., Ravussin, E., Knowler, W.C., Bennett, P.H., Bogardus, C., 1993. Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. N. Engl. J. Med. 329 (27), 1988–1992.
Little, R.R., Rohlfing, C.L., Tennill, A.L., Madsen, R.W., Polonsky, K.S., Myers, G.L., Greenbaum, C.J., Palmer, J.P., Rogatsky, E., Stein, D.T., 2008. Standardization of C-peptide measurements. Clin. Chem. 54 (6), 1023–1026.
Little, R.R., Rohlfing, C.L., Sacks, D.B., National Glycohemoglobin Standardization Program Steering, C, 2011. Status of hemoglobin A1c measurement and goals for improvement: from chaos to order for improving diabetes care. Clin. Chem. 57 (2),

205–214.
Little, R.R., Kinumi, T., Connolly, S., Kabytaev, K., 2017a. Implementing a reference measurement system for C-peptide: an addendum. Clin. Chem. 63 (12), 1904–1905.
Little, R.R., Wielgosz, R.I., Josephs, R., Kinumi, T., Takatsu, A., Li, H., Stein, D., Burns, C., 2017b. Implementing a reference measurement system for C-peptide: successes and lessons learned. Clin. Chem. 63 (9), 1447–1456.
Lu, J., Li, Q., Xie, H., Chen, Z.J., Borovitskaya, A.E., Maclaren, N.K., Notkins, A.L., Lan, M.S., 1996. Identification of a second transmembrane protein tyrosine phosphatase, IA-2beta, as an autoantigen in insulin-dependent diabetes mellitus: precursor of the 37-kDa tryptic fragment. Proc. Natl. Acad. Sci. U. S. A. 93 (6), 2307–2311.
Marcovina, S., Bowsher, R.R., Miller, W.G., Staten, M., Myers, G., Caudill, S.P., Campbell, S.E., Steffes, M.W., Insulin Standardization, W., 2007. Standardization of insulin immunoassays: report of the American Diabetes Association Workgroup. Clin. Chem. 53 (4), 711–716.
Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., Turner, R.C., 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28 (7), 412–419.
Mayr, A., Schlosser, M., Grober, N., Kenk, H., Ziegler, A.G., Bonifacio, E., Achenbach, P., 2007. GAD autoantibody affinity and epitope specificity identify distinct immunization profiles in children at risk for type 1 diabetes. Diabetes 56 (6), 1527–1533.
Miedema, K., 2004. Towards worldwide standardisation of HbA1c determination. Diabetologia 47 (7), 1143–1148.
Miller, W.G., Thienpont, L.M., Van Uytfanghe, K., Clark, P.M., Lindstedt, P., Nilsson, G., Steffes, M.W., Insulin Standardization Work, G., 2009. Toward standardization of insulin immunoassays. Clin. Chem. 55 (5), 1011–1018.
Miller, W.G., Tate, J.R., Barth, J.H., Jones, G.R., 2014. Harmonization: the sample, the measurement, and the report. Ann. Lab. Med. 34 (3), 187–197.
Mire-Sluis, A.R., Gaines Das, R., Lernmark, A., 2000. The World Health Organization International Collaborative Study for islet cell antibodies. Diabetologia 43 (10), 1282–1292.
Moore, M., Dougall, T., Ferguson, J., Rigsby, P., Burns, C., 2017. Preparation, calibration and evaluation of the First International Standard for human C-peptide. Clin. Chem. Lab. Med. 55 (8), 1224–1233.
Naithani, V.K., Dechesne, M., Markussen, J., Heding, L.G., 1975. Studies on polypeptides. V. Improved synthesis of human proinsulin C-peptide and its benzyloxycarbonyl derivative. Circular dichroism and immunological studies of human C-peptide. Hoppe Seylers Z Physiol Chem 356 (6), 997–1010.
Nathan, D.M., Control, D., Complications Trial Research, G., Genuth, S., Lachin, J., Cleary, P., Crofford, O., Davis, M., Rand, L., Siebert, C., 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N. Engl. J. Med. 329 (14), 977–986.
NCD-RisC, N.C.D.R.F.C., 2016. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet 387 (10027), 1513–1530.
Oram, R.A., Jones, A.G., Besser, R.E., Knight, B.A., Shields, B.M., Brown, R.J., Hattersley, A.T., McDonald, T.J., 2014. The majority of patients with long-duration type 1 diabetes are insulin microsecretors and have functioning beta cells. Diabetologia 57 (1), 187–191.
Palmer, J.P., Asplin, C.M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P.K., Paquette, T.L., 1983. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 222 (4630), 1337–1339.
Palmer, J.P., Fleming, G.A., Greenbaum, C.J., Herold, K.C., Jansa, L.D., Kolb, H., Lachin, J.M., Polonsky, K.S., Pozzilli, P., Skyler, J.S., Steffes, M.W., 2004. C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve beta-cell function: report of an ADA workshop, 21-22 October 2001. Diabetes 53 (1), 250–264.
Panteghini, M., John, W.G., Division, I.S., 2007. Implementation of haemoglobin A1c results traceable to the IFCC reference system: the way forward. Clin. Chem. Lab. Med. 45 (8), 942–944.
Parikka, V., Nanto-Salonen, K., Saarinen, M., Simell, T., Ilonen, J., Hyoty, H., Veijola, R., Knip, M., Simell, O., 2012. Early seroconversion and rapidly increasing autoantibody concentrations predict prepubertal manifestation of type 1 diabetes in children at genetic risk. Diabetologia 55 (7), 1926–1936.
Payton, M.A., Hawkes, C.J., Christie, M.R., 1995. Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). J. Clin. Invest. 96 (3), 1506–1511.
Raab, J., Haupt, F., Scholz, M., Matzke, C., Warncke, K., Lange, K., Assfalg, R., Weininger, K., Wittich, S., Lobner, S., Beyerlein, A., Nennstiel-Ratzel, U., Lang, M., Laub, O., Dunstheimer, D., Bonifacio, E., Achenbach, P., Winkler, C., Ziegler, A.G., Fr1da Study, G, 2016. Capillary blood islet autoantibody screening for identifying pre-type 1 diabetes in the general population: design and initial results of the Fr1da study. BMJ Open 6 (5), e011144.
Rabin, D.U., Pleasic, S.M., Shapiro, J.A., Yoo-Warren, H., Oles, J., Hicks, J.M., Goldstein, D.E., Rae, P.M., 1994. Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. J. Immunol. 152 (6), 3183–3188.
Rahbar, S., Blumenfeld, O., Ranney, H.M., 1969. Studies of an unusual hemoglobin in patients with diabetes mellitus. Biochem. Biophys. Res. Commun. 36 (5), 838–843.
Reference Institute for Bioanalytics, Bonn, Germany, 2019. Insulin Survey, Summary Evaluation HP4/18. https://www.rfb.bio/cgi/displayAnaStats?rv_type=all&rvTypeForDetails=HP&year=2018&rv_num=4&analyte=insulin&searchType=analyte&anaV=7, Accessed date: 14 January 2019.
Rewers, M., Zaccaro, D., D'Agostino, R., Haffner, S., Saad, M.F., Selby, J.V., Bergman, R., Savage, P., Insulin Resistance Atherosclerosis Study, I, 2004. Insulin sensitivity, insulinemia, and coronary artery disease: the Insulin resistance atherosclerosis study. Diabetes Care 27 (3), 781–787.
Robbins, D.C., Andersen, L., Bowsher, R., Chance, R., Dinesen, B., Frank, B., Gingerich, R., Goldstein, D., Widemeyer, H.M., Haffner, S., Hales, C.N., Jarett, L., Polonsky, K., Porte, D., Skyler, J., Webb, G., Gallagher, K., 1996. Report of the American Diabetes Association's Task Force on standardization of the insulin assay. Diabetes 45 (2), 242–256.
Rubenstein, A.H., Melani, F., Pilkis, S., Steiner, D.F., 1969. Proinsulin. Secretion, metabolism, immunological and biological properties. Postgrad. Med. J. 45 (Suppl), 476–481.
Schlosser, M., Mueller, P.W., Torn, C., Bonifacio, E., Bingley, P.J., Participating, L., 2010. Diabetes antibody standardization program: evaluation of assays for insulin autoantibodies. Diabetologia 53 (12), 2611–2620.
Schroer, J.A., Bender, T., Feldmann, R.J., Kim, K.J., 1983. Mapping epitopes on the insulin molecule using monoclonal antibodies. Eur. J. Immunol. 13 (9), 693–700.
Staten, M.A., Stern, M.P., Miller, W.G., Steffes, M.W., Campbell, S.E., Insulin Standardization, W., 2010. Insulin assay standardization: leading to measures of insulin sensitivity and secretion for practical clinical care. Diabetes Care 33 (1), 205–206.
Steiner, D.F., Oyer, P.E., 1967. The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. Proc. Natl. Acad. Sci. U. S. A. 57 (2), 473–480.
Steiner, D.F., Cunningham, D., Spigelman, L., Aten, B., 1967. Insulin biosynthesis: evidence for a precursor. Science 157 (3789), 697–700.
Stoyanov, A.V., Rogatsky, E., Stein, D., Connolly, S., Rohlfing, C.L., Little, R.R., 2013. Isotope dilution assay in peptide quantification: the challenge of microheterogeneity of internal standard. Proteomics Clin. Appl. 7 (11 – 12), 825–828.
Stumvoll, M., Mitrakou, A., Pimenta, W., Jenssen, T., Yki-Jarvinen, H., Van Haeften, T., Renn, W., Gerich, J., 2000. Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. Diabetes Care 23 (3), 295–301.
Temple, R., Clark, P.M., Hales, C.N., 1992. Measurement of insulin secretion in type 2 diabetes: problems and pitfalls. Diabet. Med. 9 (6), 503–512.
Thomas, N.J., Jones, S.E., Weedon, M.N., Shields, B.M., Oram, R.A., Hattersley, A.T., 2018. Frequency and phenotype of type 1 diabetes in the first six decades of life: a cross-sectional, genetically stratified survival analysis from UK Biobank. Lancet Diabetes Endocrinol. 6 (2), 122–129.
Tietz, N.W., 1979. A model for a comprehensive measurement system in clinical chemistry. Clin. Chem. 25 (6), 833–839.
Torn, C., Mueller, P.W., Schlosser, M., Bonifacio, E., Bingley, P.J., Participating, L., 2008. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. Diabetologia 51 (5), 846–852.
Trivelli, L.A., Ranney, H.M., Lai, H.T., 1971. Hemoglobin components in patients with diabetes mellitus. N. Engl. J. Med. 284 (7), 353–357.
Tuomi, T., Groop, L.C., Zimmet, P.Z., Rowley, M.J., Knowles, W., Mackay, I.R., 1993. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 42 (2), 359–362.
UKPDS Group, 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 352 (9131), 837–853.
Verge, C.F., Gianani, R., Kawasaki, E., Yu, L., Pietropaolo, M., Jackson, R.A., Chase, H.P., Eisenbarth, G.S., 1996. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes 45 (7), 926–933.
Verge, C.F., Stenger, D., Bonifacio, E., Colman, P.G., Pilcher, C., Bingley, P.J., Eisenbarth, G.S., 1998. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. Diabetes 47 (12), 1857–1866.
Vogt, B.W., Schleicher, E.D., Wieland, O.H., 1982. Epsilon-amino-lysine-bound glucose in human tissues obtained at autopsy. Increase in diabetes mellitus. Diabetes 31 (12), 1123–1127.
Wenzlau, J.M., Juhl, K., Yu, L., Moua, O., Sarkar, S.A., Gottlieb, P., Rewers, M., Eisenbarth, G.S., Jensen, J., Davidson, H.W., Hutton, J.C., 2007. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc. Natl. Acad. Sci. U. S. A. 104 (43), 17040–17045.
Wenzlau, J.M., Liu, Y., Yu, L., Moua, O., Fowler, K.T., Rangasamy, S., Walters, J., Eisenbarth, G.S., Davidson, H.W., Hutton, J.C., 2008. A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes. Diabetes 57 (10), 2693–2697.
Weyer, C., Tataranni, P.A., Bogardus, C., Pratley, R.E., 2001. Insulin resistance and insulin secretory dysfunction are independent predictors of worsening of glucose tolerance during each stage of type 2 diabetes development. Diabetes Care 24 (1), 89–94.
Weykamp, C., 2013. HbA1c: a review of analytical and clinical aspects. Ann. Lab. Med. 33 (6), 393–400.
Weykamp, C., John, W.G., Mosca, A., Hoshino, T., Little, R., Jeppsson, J.O., Goodall, I., Miedema, K., Myers, G., Reinauer, H., Sacks, D.B., Slingerland, R., Siebelder, C., 2008. The IFCC reference measurement system for HbA1c: a 6-year progress report. Clin. Chem. 54 (2), 240–248.
Wiedmeyer, H.M., Polonsky, K.S., Myers, G.L., Little, R.R., Greenbaum, C.J., Goldstein, D.E., Palmer, J.P., 2007. International comparison of C-peptide measurements. Clin. Chem. 53 (4), 784–787.
Williams, A.J., Lampasona, V., Schlosser, M., Mueller, P.W., Pittman, D.L., Winter, W.E., Akolkar, B., Wyatt, R., Brigatti, C., Krause, S., Achenbach, P., Participating, L., 2015. Detection of antibodies directed to the N-terminal region of GAD is dependent on assay format and contributes to differences in the specificity of GAD autoantibody assays for type 1 Diabetes. Diabetes 64 (9), 3239–3246.
Yalow, R.S., Berson, S.A., 1959. Assay of plasma insulin in human subjects by

immunological methods. Nature 184 (Suppl. 21), 1648–1649.
Yanaihara, N., Sakura, N., Yanaihara, C., Hashimoto, T., Rubenstein, A.H., Steiner, D.F., 1975. Syntheses and immunological evaluation of bovine proinsulin C-peptide analogues. Nature 258 (5533), 365–366.
Yu, L., Dong, F., Miao, D., Fouts, A.R., Wenzlau, J.M., Steck, A.K., 2013. Proinsulin/Insulin autoantibodies measured with electrochemiluminescent assay are the earliest indicator of prediabetic islet autoimmunity. Diabetes Care 36 (8), 2266–2270.
Zheng, Y., Ley, S.H., Hu, F.B., 2018. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat. Rev. Endocrinol. 14 (2), 88–98.
Ziegler, A.G., Nepom, G.T., 2010. Prediction and pathogenesis in type 1 diabetes. Immunity 32 (4), 468–478.
Ziegler, A.G., Bonifacio, E., Group, B.-B.S., 2012. Age-related islet autoantibody incidence in offspring of patients with type 1 diabetes. Diabetologia 55 (7), 1937–1943.
Ziegler, A.G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E., Eisenbarth, G.S., 2013. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 309 (23), 2473–2479.