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ARTICLE INFO

ABSTRACT

Background: Despite international efforts to standardize C-peptide and insulin calibrators and immunoassays, platform dependent differences still exist, and platform specific reference intervals are hence needed for correct interpretation. We therefore wanted to establish traceable reference intervals for C-peptide and insulin.

Methods: In 623 consecutively recruited participants, insulin and C-peptide were measured using the Cobas e411 (Roche Diagnostics, Switzerland). Participants with diabetes were excluded (fasting Glucose ≥ 7.0 mmol/L or HbA1c ≥ 6.5%/ ≥ 48 mmol/L) and reference intervals were calculated with and without the inclusion of persons who were prediabetic, according to two definitions (The World Health Organization (WHO) and American Diabetes Association (ADA)). To ensure the correctness of calibration, the control pools were analyzed by a reference laboratory. The reference intervals were calculated according to the IFCC guidelines, using the RefVal software (Solberg, Oslo, Norway).

Results: Comparison of our results with those from the reference laboratory revealed equivalence for C-peptide results whereas the insulin determined on the Cobas e411 assay were 15–20% higher. The difference is attributed to an incorrect conversion factor for converting from activity to metric units. The Cobas e411 assay uses the factor 6.945 for converting from U/mL to pmol/L. This is in disagreement with the biological activity of insulin which is 166.8×10^6 IU/mol or 6.00 nmol/IU.

Conclusion: We successfully established reference intervals for C-peptide and insulin for non-diabetic and prediabetic participants. The reference intervals for fasting C-peptide and fasting insulin are ready for implementation. A recertification of the insulin standards is needed.

1. Introduction

Human insulin is a 5.8 kDa polypeptide produced in the islets of Langerhans, the endocrine part of the pancreas. Glucose is the most important regulator of insulin secretion. Insulin is an anabolic hormone that lowers blood glucose through stimulation of synthesis of glycogen, proteins and lipids and inhibition of lipolysis [1]. The 81 amino acid proinsulin peptide consists of three domains: an amino-terminal B-chain, a carboxy-terminal A-chain and a connecting peptide in the middle, known as the *C-peptide*. During passage through the endoplasmic reticulum, the precursor folds and the prohormone convertases PC1/3 and -2 subsequently excise the C-peptide, generating the mature form of insulin and C-peptide [2]. C-peptide is stored in beta cells and released by active secretion in equimolar amounts to insulin [3]. Only fully matured insulin from which C-peptide has been removed can interact with the insulin receptor [4]. The conversion of proinsulin is almost complete, thus only a small amount of proinsulin is found in the blood. Insulin is primarily degraded in the liver, whereas 85% of C-peptide, in contrast, is cleared from circulation by renal filtration [5]. Furthermore, the half-life of C-peptide is longer than insulin (20–30 and 3–5 min, respectively). Hence, the concentration of C-peptide is three to six times

higher than the concentration of insulin. The rate of elimination of insulin depends on multiple metabolic processes leading to great variety in plasma level, whereas the plasma concentration of C-peptide varies less. Therefore, the plasma C-peptide concentration is a better measure for assessing insulin secretion [1].

In the clinic, measurements of C-peptide are mainly used to distinguish between diabetes mellitus (DM) type 1 (DM1) and 2 (DM2). DM is defined as "*a group of diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both*" [6]. DM1 is caused by beta-cell destruction and leads to absolute insulin deficiency. In contrast, DM2 results from insulin resistance and a progressive insulin secretory defect [7]. Prediabetes (PDM) indicates an increased risk of developing diabetes. Prediabetic patients by definition have impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) which are associated with the metabolic syndrome (MetS) [8]. MetS is a group of risk factors together linked to an increased risk of DM2 and cardiovascular disease: obesity/central obesity, insulin resistance, hypertension and dyslipidemia (hypertriglyceridemia) [9]. Abdominal obesity or adiposity is the key clinical feature in MetS and seems to precede the other risk factors [10].

Both insulin and C-peptide are usually measured using antibody based assays. In fact, the radioimmunoassay (RIA) - invented by Yalow and Berson more than fifty years ago - was first used to quantify insulin [11]. Despite this, the standardization of the calibrators, assays and standardization of reference intervals is still not complete [12]. An Insulin Standardization Workgroup was established by ADA in 2004 to address the issue [13]. Furthermore the preanalytical conditions affecting insulin measurements have been carefully reviewed [14]. The co-determination of exogenous insulin depends on the type of insulin injected and must be determined for each product and assay. C-peptide is often measured in preference of insulin, especially in the case of injection of exogenous insulin. Measurement of plasma insulin aims to assess insulin production by the beta cells in diagnosis of insulin producing tumours (insulinoma) or other causes of low blood glucose. In addition, insulin analysis is used to characterize insulin resistance or to judge when type 2 diabetics need insulin as supplement to their oral therapy [1]. Standardization and harmonization is therefore pivotal for clinical practice in diagnosing and treatment of DM. We wanted to use the Cobas e411 (Roche®) to establish traceable reference intervals for C-peptide and fasting insulin.

2. Methods

2.1. Definitions

While the definition of diabetes is the same for the WHO and the American Diabetes association (ADA) [7,15], the WHO and the ADA definitions of prediabetes (PDM) differ from each other. The definition by ADA uses lower limits for fasting plasma glucose (FPG) and HbA1c than WHO. The following reference intervals are calculated for both non-diabetics as well as prediabetics, according to both WHO and ADA definitions (Table 1).

2.2. Study population

The participants were recruited from a follow-up of the health survey "Helbred2006" (*Health2006*). The main study was launched in the summer of 2006 and included 3471 randomly chosen citizens

Table 1
The definitions of pre-diabetes and diabetes used define the groups of the participants.

Diabetes	Fasting plasma glucose (FPG) of ≥7 mmol/L and/or HbA1c ≥ 6.5%/≥48 mmol/L.
Prediabetes (WHO)	FPG ≥ 6.1 mmol/L and/or HbA1c ≥ 6.0%/≥42 mmol/L [15].
Prediabetes (ADA)	FPG ≥ 5.6 mmol/L and/or HbA1c ≥ 5.7% [7].

(participation rate of 44.7%) living in the suburbs of Copenhagen, Denmark. Five years later the participants in the former survey was re-invited and a total of 2308 individuals agreed to a re-examination in between November 2011 and 2012 [16,17]. Inclusion of the participants from whom we obtained blood samples was done consecutively, though at the end of recruitment period we tried to achieve a gender balance. Blood samples were analyzed between March and August 2012. Pregnant women were excluded from participation, and users of antihistamine were asked to refrain from these medications three days prior to the examination, if possible. This also applied to use of inhalers for lung disease. The Ethics Committee of the Capital Region of Denmark (code H-3-2011-081) approved the study. The health examinations are thoroughly described by Thuesen et al. [17].

2.3. Sample handling

All participants fasted from midnight and the following morning a venous blood sample was drawn [17]. The samples were initially stored at −20 °C for one to four weeks while waiting analysis (expected to be days/at most 1–2 weeks) and subsequently transferred from −20° to −80 °C during extended storage time up to six months. The blood samples were spun immediately after sampling, the plasma collected, refrigerated and stored at −20 °C. Fasting insulin and –C-peptide were analyzed at Steno Diabetic Center (SDC), Gentofte, Denmark. In addition measurements of fasting glucose, HbA1c and other biochemical variables were performed. These included cholesterol and triglycerides, measured on VITROS® 5600 (Ortho Clinical Diagnostics, Raritan, NJ). Furthermore, participants' weight, height, waist- and hip circumference was obtained. Body fat percentage was measured using a Tanita BC-420 MA segmental body composition monitor (Tanita, Tokyo, Japan).

2.4. Insulin and C-peptide assays

C-peptide and insulin were measured using a Cobas e411 (Roche Diagnostics, Mannheim, Germany) and on an AutoDELFIA (Perkin Elmer, Turku, Finland) instrument at SDC. Characteristics of both the C-peptide and fasting insulin assays on Cobas e411 and AutoDELFIA are listed in Table 2.

2.5. Insulin assays

The samples were analyzed on the Cobas e411 and on the AutoDELFIA. For the Cobas e411 analysis of insulin, the following reagents were used: cat. #12017504122, Insulin CalSet, cat. #05341787190, PreciControl Multimarker, cat. #03609979190 PreciControl Multianalyte, cat. #11731416190 and #11731416160 PreciControl. A detection interval from 0.200–1000 μU/mL or 1.39–6945 pmol/L was defined by the manufacturer [18]. The assays used for the measurements on AutoDELFIA were B080–101 with a detection limit of 3 pmol/L and measurement interval 3–1000 pmol/L [19].

2.6. C-Peptide assays

The analytical measurement range on the Cobas e411 C-peptide assay was 0.003–13.3 nmol/L or 0.010–40.0 μg/L. The following reagents were used: cat. #03184919190, C-peptide CalSet, cat. #05341787190, PreciControl Multimarker, cat. #03609979190, PreciControl Multianalyte, cat. #03609987190 Diluent MultiAssay [20]. The AutoDELFIA assay used was B-081-101 with an analytical measurement range of 10–6000 pmol/L [21].

2.7. Reference measurements

To ensure correctness of calibration, three reference pools were analyzed both on the AutoDelfia at SDC and on Cobas e411 at the Department of Laboratory Medicine and Pathology, University of Minnesota,

Table 2
Analytical characteristics of the insulin and C-peptide assays.
The characteristics are those obtained during the study and analysis period.

Asssay	Manufacturer	Traceability	Analytical imprecision, SDC	
			Level, pmol/L	CV %
C-Peptide	Cobas e411, Roche Diagnostics®	WHO International Reference Reagent, IRR 84/510	130	3.0
			1100	2.4
			2575	3.6
Insulin	Cobas e411, Roche Diagnostics®	WHO IRP 66/304	33	5.0
			128	4.4
			739	3.9
C-Peptide	AutoDELFIA, Perkin Elmer®	WHO 84/510	113	3.7
			1092	2.8
			2489	2.3
Insulin	AutoDELFIA, Perkin Elmer®	WHO IRP 66/304	21	7.0
			74	4.7
			301	3.8

Minneapolis (UofMN), Minnesota, USA. These assays have been calibrated against the reference methods currently operated by Professor Daniel T. Stein, Albert Einstein College of Medicine, New York City, New York, USA.

2.8. Statistics

The RefVal 4.11 software (Solberg, Oslo, Norway) was used to determine the reference interval according to the International Federation of Clinical Chemistry (IFCC) guidelines [22]. Reference intervals were calculated using non-parametric method (bootstrapping).

3. Results

3.1. The study population

A total of 2308 participants were included in the follow-up of the population survey Health2006. Blood samples from 623 of these were used in this study. Of the 623 participants, 33 were excluded due to DM; 8 women and 25 men. From the remaining 590 participants, 85 had PDM according to the WHO definition, and 276 with reference to the ADA definition (Fig. 1). There was a nearly equal gender distribution

(men, n = 287 (48.6%), women, n = 303 (51.4%)). Most participants were 40–69 years old (mean age 54 years).

3.2. Body composition and assessment of nutritional status of the study population

To characterize the participants according to nutritional status we calculated BMI and waist hip ratio (WHR). Participants who were not diabetic nor prediabetic according to the definition by ADA had a median BMI 24.5 kg/m². Mean BMI was 25 kg/m². In summary our study population, both men and women, were overweight and the BMI of the non-diabetic and non-prediabetic participants was slightly lower. Anthropometric indicators of body fat distribution, waist circumference (WC) and waist hip ratio (WHR) [23] are also associated with risk of diabetes and could be better indicators to discriminate the participants with diabetes from those without. Still though, both BMI, WC and WHR were all useful parameters [24]. Cut-off values of WHR vary among countries, but more variation is found for WC than WHR. In this study WHR was considered elevated when WHR > 0.85 in women, and WHR > 0.90 in men [25]. In women the minimum WHR was 0.68, the median value 0.82 and maximum WHR 1.03. In men the minimum WHR was 0.78, median 0.93 and maximum 1.13.

We used the intervals 20.1–24.9% of body fat (BF) percentage for men and 30.1–34.9% for women to define overweight; correspondently BF% ≥ 25 in men and ≥35 in women was defined as obesity [23]. The overall BF% for all participants ranged from 10.2 to 50.9 with a mean BF% of 35.5 for women, equaling obesity. The male population was leaner with a median BF% below 25. Still though, the median amount of body fat found corresponds to overweight; 22.3%. In summary, analyses of the body composition of the participant using BMI, WHR and BF% revealed that the majority of our population is either overweight or obese.

3.3. Cholesterol and triglyceride levels

A total blood cholesterol <5.2 mmol/L (<200 mg/dL) is considered desirable, between 5.2 and 6.2 mmol/L (200–239 mg/dL borderline high, and >6.2 mmol/L (>240 mg/dL) as high [26]. Triglycerides below 1.7 mmol/L (<150 mg/dL) is recommended by the American Heart Association, whereas 1.7–2.3 mmol/L (150–199 mg/dL) is borderline
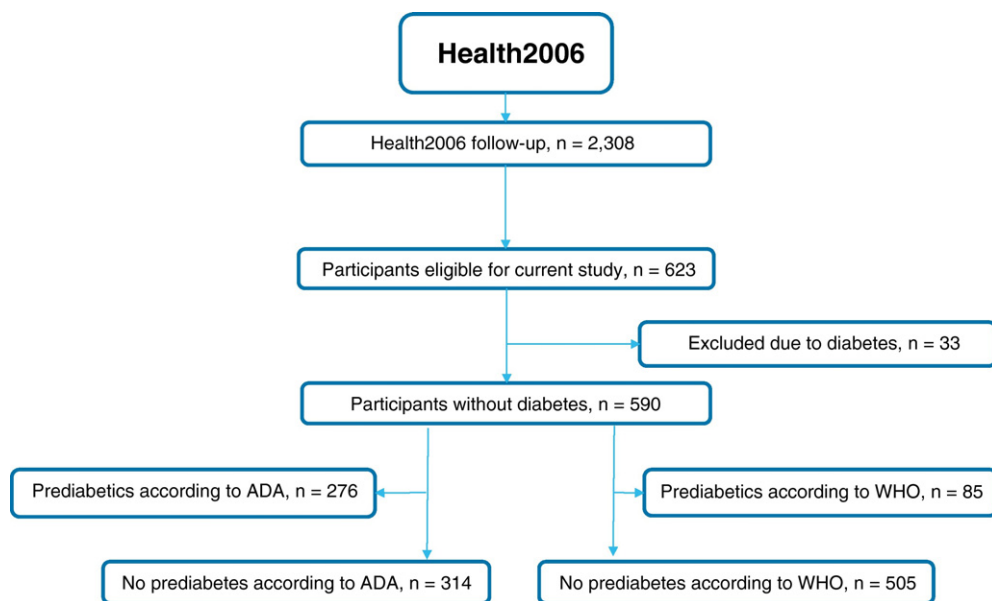

Fig. 1. Flow-chart outlining the study design and number of participants used for calculating the reference intervals. With reference to definitions listed elsewhere, participants were grouped/excluded according to their FPG and HbA1c-level. Of the 590 participants without DM, almost half of them (47%) had PDM with reference to ADA and only 85 (14%) according to WHO.

Table 3
Correctness of the calibration.

Pool	C-Peptide (UofMN) (pmol/L)	C-Peptide (SDC) (pmol/L)	Insulin (UofMN) (pmol/L)	Insulin (SDC) (pmol/L) CF 6.0	Insulin (SDC) (pmol/L) CF 6.945
Low	126	131	27	29	33
Middle	1060	1110	107	111	129
High	2510	2614	614	641	742

SDC and UofMN reported similar results for C-peptide for all calibration levels with duplicate measurements of each sample. Using the default conversion factor listed in the Cobas insert kit yielded greater values at SDC at all levels. A conversion factor of 6.00 almost eliminated this difference.

high, 2.3–5.6 (200–499 mg/dl) high and ≥5.6 (≥500 mg/dl) very high [27]. In only 41% of non-diabetic men and 42% of non-diabetic women total cholesterol was <5.2 mmol/L and in 17 and 16% it was high. The majority of both non-diabetic men and women had blood triglyceride level below the cut off (78% of men, 87% of women). The percentage of the reference population with elevated levels of triglycerides decreased with exclusion of diabetics and prediabetics, from 30.2 to 5.8% in women and from 21.5 to 15.5% in men. Overall, the reference population is overweight /obese and dyslipidemic, which match findings from a general Danish population survey [28].

3.4. Trueness of instrument calibration

To ascertain the trueness of the calibration we exchanged three pools with the laboratory at UofMN, USA which also uses the Roche assay but have calibrated this against the reference methods currently operated by Randy Little and Daniel T. Stein [29,30].

We found that C-peptide measurements were equivalent but 21% higher values for insulin were determined on AutoDELFIA. We recalculated with a conversion factor of 6.0 equaling the biological activity of insulin [31] and now similar results were found (Table 3). Bias was greater when using the 6.945 conversion factor than 6.0: 32 versus 14% (Fig. 2).

3.5. C-Peptide reference intervals

We calculated the reference intervals using bootstrapping as the distribution of C-peptide measurements were not Gaussian (data not shown). Reference intervals for C-peptide based on measurements on Cobas e411 are listed in Table 4.

3.6. Insulin reference intervals

The distribution of insulin concentrations was not Gaussian either, so again we used bootstrapping to calculate the reference intervals. The results of the insulin measurements using both conversion factors are shown in Table 5, 6 and 7. The Cobas e411 uses a factor 6.945 for converting ($\mu U/mL \times 6.945 = pmol/L$) [19] (Table 5). Using the conversion factor of 6.00 gave other results (Table 6). We also calculated reference interval from measurements on AutoDELFIA (Table 7). Detection limit of the AutoDELFIA was 3 pmol/L, however functional detection limit of 10 pmol/L was used and results lower than 10 pmol/L were reported as <10 pmol/L. We calculated different reference intervals for insulin based on the AutoDELFIA measurements (Table 7). The Insulin reference intervals become narrower and upper limits decreased with exclusion of prediabetic participants from the reference population. Reference intervals for insulin on Cobas e411 determined with a conversion factor of $\mu U/mL \times 6.0 = pmol/L$ were much closer to the reference intervals from the AutoDELFIA measurements than calculations with conversion factor 6.945.

4. Discussion

In this study we determined reference intervals for C-peptide and insulin, measured on the Roche Cobas e411 for people without DM and PDM using bootstrapping on a sample of general population of men and women 18–76 years old who were slightly overweight and hyperlipidemic. We also confirmed the previously reported problem when using the built in factory recommended conversion factor for reporting the insulin concentration in pmol/L [13,30–33].

The quality of assays is important for both the daily usage and for portability of the test results. The platform dependency of test results

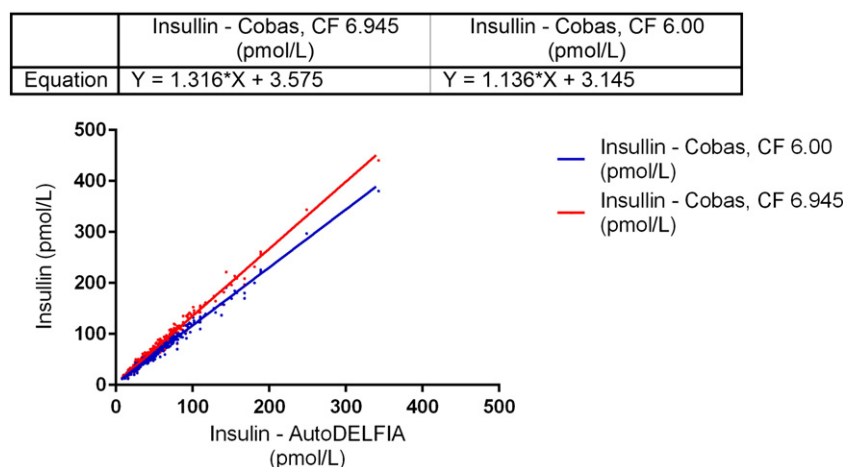|	Insullin - Cobas, CF 6.945 (pmol/L)	Insullin - Cobas, CF 6.00 (pmol/L)
Equation	Y = 1.316*X + 3.575	Y = 1.136*X + 3.145

Fig. 2. Method comparison between AutoDELFIA and Cobas e411 plasma insulin measurements. Plasma insulin measurements generated using the AutoDELFIA were compared to those obtained on the Cobas platform using the conversion factor (CF) 6.945 (32% higher results) or a CF of 6.00 (14% higher results).

Table 4
Reference intervals for C-peptide (Cobas e411).
The C-peptide reference intervals depend on whether prediabetic populations are included or not. With exclusion of prediabetic participants the reference interval narrows.

C-Peptide (pmol/L)	2,5% limit (90% CI)	97,5% limit (90% CI)	N
Without diabetes	379 (372–414)	1631 (1466–1713)	590
Without prediabetes (WHO)	376 (369–409)	1479 (1311–1649)	505
Without prediabetes (ADA)	372 (341–390)	1299 (1219–1479)	314

was illustrated with an evaluation of ten commercial insulin assays against an isotope dilution measurement procedure (isotope dilution-liquid chromatography/tandem mass spectrometry (IDMS)) calibrated by using purified recombinant insulin [30]. In only four of ten methods (including the Roche assay, used in our study) ≥ 95% of results were within the 32% total error allowance for desirable method performance. Only the Roche® and Mercodia® assays showed acceptable error occurrence and in general the authors observed increasing tendency towards biases with lower concentration of insulin. We found a 20% difference between our insulin measurements and results at the reference laboratory. This difference is attributed to an incorrect conversion factor for converting in the Cobas e411 assay. As noted previously, the Cobase411 assay uses the factor 6.945 when converting from U/mL to pmol/L. This is in disagreement with the biological activity of insulin which is 166.8×10^6 IU/mol or 6.00 nmol/IU [31]. Both the AutoDELFIA and the Cobas e411 method are calibrated traceable to the WHO IRP 66/304, a lyophilized impure preparation of human pancreatic insulin. One international unit (1 IU) of human insulin is the activity contained in 0.03846 mg of the international standard for human insulin [31]. However, as early reported the reference preparation contains impurities (salt, water, desamido insulin and 'traces' of proinsulin [31,34]. While this only has negligible importance for assigned activity it causes a major error when using the assigned mass to calculate the molar amounts of the IRP. To facilitate and standardize reporting of insulin measurements in pmol/L there is a need for having reference preparations that have been assigned values in concentration. This can be done in several ways; one possibility is simply to assign a substance concentration to the IRP 66/304. Another could be to develop a new IRP which have a substance concentration assigned from the start. One such is the newer Japanese preparation [35]. We therefore urge that actions are taken to quickly make insulin reference preparations with assigned concentration available to the manufacturers so that assays can be correctly calibrated and hence making results portable.

Regarding reference intervals for C-peptide, Little et al. [29] evaluated inter-assay (nine assays) and inter-laboratory (15 laboratories) variation and compared results to mass spectrometry method. Forty serum samples were analyzed, eight at each laboratory. They found some disagreement between C-peptide results, by different laboratories and methods, primarily at high C-peptide concentrations. They recommended calibration of C-peptide measurement to a reference method to increase comparability between laboratories.

Our reference population compares well to the characteristics of the Copenhagen General Population Study and the General Suburban Population Study in which 55.2 and 60.5% were either overweight or obese. Also total blood cholesterol and triglycerides levels match the findings in the two bigger studies [28]. We therefore find that the population matches the average Danish population, making our reference interval applicable to the Danish and similar populations. Reference intervals

Table 6
Insulin reference interval (factor 6.0) (Cobas e411).

Insulin (pmol/L)	2.5% limit (90% CI), pmol/L	97.5% limit (90% CI), pmol/L	n
Without diabetes	16 (14–18)	140 (132–161)	590
Without prediabetes (WHO)	14 (14–17)	126 (117–141)	505
Without prediabetes (ADA)	14 (13–16)	117 (98–132)	304

have also been reported for other ethnic populations. Li et al. [36] generated a reference interval for insulin in non-diabetic Chinese men from 1434 fasting serum insulin measurements. Their criteria for inclusion and exclusion were much broader than the Danish survey from which samples for our study are drawn, e.g. they excluded heavy smokers. Samples were analyzed on the Roche Cobas® 6000 system E601 Elecsys module resulting in a reference interval of 1.57–16.32 mU/L (9.4–98 pmol/L). Larsson et al. [37] reported a reference interval for insulin as 1.74–18.27 mU/L (10.4–110 pmol/L) based on measurements on 75 year old females and males with a median BMI 26.2. This is a little narrower than ours.

A limitation of the current study may be that we classified participants solely based on fasting glucose and HbA1c level and not reviewed medical records. Diabetic patients with well treated disease may have normal fasting glucose and HbA1c level. Such cases were then misclassified. With this in mind we still believe that our reference intervals are valuable information for the clinician.

In summary we have determined reference intervals for insulin and C-peptide not only for a nondiabetic slightly overweight population, but for a population without prediabetes, according to both WHO and ADA definitions. This should make our reference intervals for insulin and C-peptide for Roche assays widely applicable and more specific and may improve clinical practice.

5. Conclusion

This work focused on reference interval for C-peptide and fasting insulin in non-diabetics and prediabetics, the latter according to both the WHO definition of PDM and the definition by ADA. We have generated reliable reference intervals for C-peptide which are ready for implementation, but for fasting insulin a recertification of standards is needed.

Author contributions

- Study concept and design (TH, AL, LFH)
- Acquisition of data (AL, LFH)
- Analysis and interpretation of data (PBL, LFH)
- Drafting of the manuscript (LFH, PBL)
- Critical revision of the manuscript for important intellectual content (PBL, AL, TH, LFH)
- Statistical analysis (LFH, PBL)
- Obtained funding (AL, LFH)
- Administrative, technical, or material support (LFH)
- Study supervision (LFH, TH).

Acknowledgements

Table 5
Insulin reference intervals (factor 6.945) (Cobas e411).

Insulin (pmol/L)	2.5% limit (90% CI), pmol/L	97.5% limit (90% CI), pmol/L	N
Without diabetes	19 (16–21)	161 (152–186)	590
Without prediabetes (WHO)	16 (16–20)	147 (135–174)	505
Without prediabetes (ADA)	16 (15–19)	135 (113–153)	314

Table 7
Insulin reference interval (AutoDELFIA).

Insulin (pmol/L)	2.5% limit (90% CI), pmol/L	97.5% limit (90% CI), pmol/L	n
Without diabetes	≤10 (≤10)	120 (104–142)	590
Without prediabetes (WHO)	≤10 (≤10)	99 (92–117)	505
Without prediabetes (ADA)	≤10 (≤10)	86 (79–96)	314

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