

Simultaneous Measurement of Plasma Concentrations of Proinsulin and C-Peptide and Their Ratio with a Trefoil-Type Time-Resolved Fluorescence Immunoassay

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BACKGROUND: When the concentrations of 2 or more substances are measured separately, their molar ratios are subject to the additive imprecisions of the different assays. We hypothesized that the cumulative error for concentration ratios of peptides containing a common sequence might be minimized by measuring the peptides simultaneously with a “trefoil-type” immunoassay.

METHODS: As a model of this approach, we developed a dual-label time-resolved fluorescence immunoassay (TRFIA) to simultaneously measure proinsulin, C-peptide, and the proinsulin–C-peptide ratio (PI/C). A monoclonal antibody captures all C-peptide-containing molecules, and 2 differently labeled antibodies distinguish between proinsulin-like molecules and true C-peptide.

RESULTS: The trefoil-type TRFIA was capable of measuring plasma C-peptide and proinsulin simultaneously without mutual interference at limits of quantification of 48 and 8125 pmol/L, and 2.1 and 197 pmol/L, respectively. Within-laboratory imprecision values for the trefoil-type TRFIA ranged between 8.4% and 12% for the hormone concentrations. Unlike the hormone results obtained with separate assays, imprecision did not increase when PI/C was calculated from trefoil assay results ($P < 0.05$). Peptide concentrations were highly correlated with results obtained in individual comparison assays ($r^2 \geq 0.965$; $P < 0.0001$). The total error for PI/C obtained with the trefoil-type TRFIA remained $\leq 25\%$ over a broader C-peptide range than with separate hormone assays (79–7200 pmol/L vs 590–4300 pmol/L C-peptide). Preliminary

data indicate little or no interference by heterophile antibodies.

CONCLUSIONS: The developed trefoil-type TRFIA is a reliable method for simultaneous measurement of proinsulin, C-peptide, and PI/C and provides proof of principle for the development of other trefoil-type multiple-label immunoassays.

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The present report describes a new type of immunoassay based on the trefoil-type principle that can reliably quantify 2 or more related peptides simultaneously and measure their ratios more precisely than separate assays. We have investigated the simultaneous measurement of proinsulin, C-peptide, and their ratio as a test case.

Pancreatic β cells secrete insulin and C-peptide in equimolar concentrations into the circulation after enzymatic removal of C-peptide from the precursor proinsulin molecule (1, 2). Proinsulin cleavage is largely achieved before secretion, but proinsulin and its partially processed forms—predominantly the des-31,32 derivative—are still found in the circulation (3). Increased proinsulin concentrations and an increased proinsulin–insulin ratio or proinsulin–C-peptide ratio (PI/C)² are indicative of incomplete precursor conversion and have been noted prior to and at diagnosis of both type 1 (4) and type 2 diabetes (5), as well as in insulinoma (6). In prediabetes, such an increase may reflect sustained β cell activation in the face of increasing demands and/or phenotypic changes in (some) β

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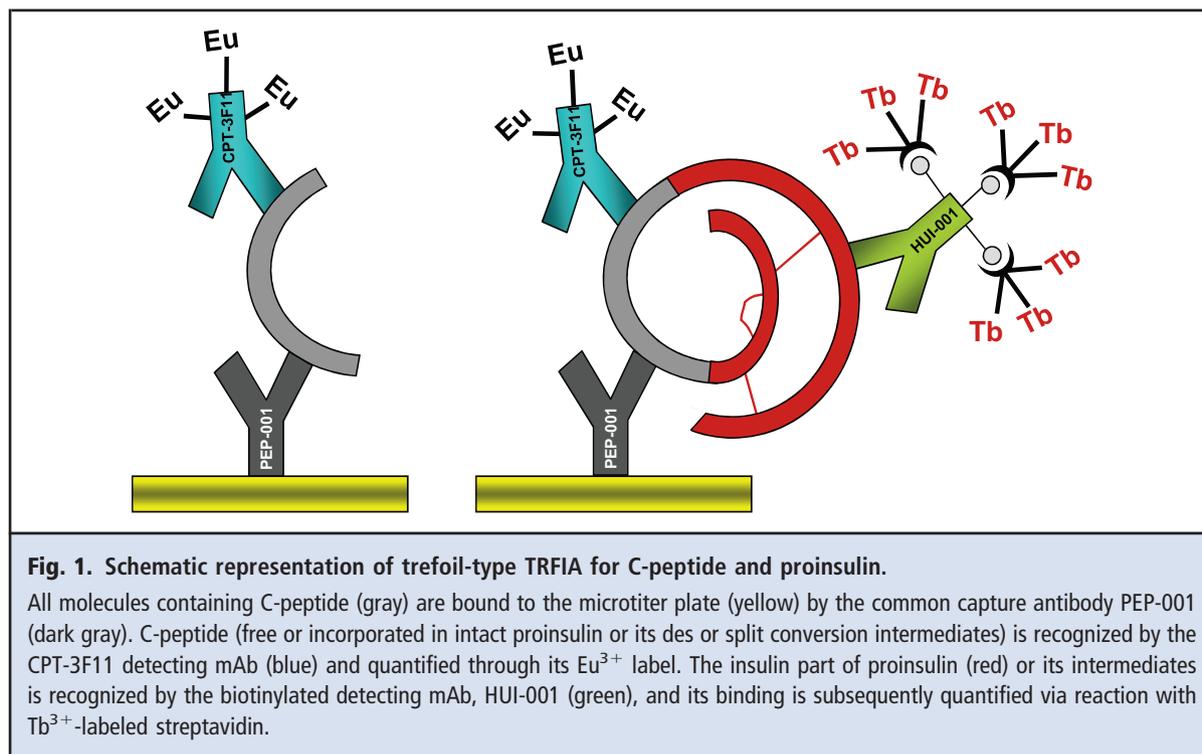
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² Nonstandard abbreviations: PI/C, proinsulin–C-peptide ratio; TRFIA, time-resolved fluorescence immunoassay; mAb, monoclonal antibody; NIBSC, National Institute for Biological Standards and Control; IRR, international reference reagent; LOQ, limit of quantification.



cells due to local inflammation (7, 8). An increased PI/C in baseline or random blood samples has been identified as an independent predictor of β cell dysfunction in patient groups at risk for type 1 diabetes (4, 9, 10). The simplicity of PI/C measurement compared with the standardized but more laborious oral or intravenous glucose-tolerance tests facilitates its large-scale application for predicting diabetes in the general population or in risk groups, especially because there is no strict need to sample the seemingly healthy and active population being screened in the fasted state (10).

Subtle changes in PI/C are predictive of diabetes (10); consequently, high precision in its measurement is necessary. Proinsulin and C-peptide are currently measured with separate assays (11, 12), however, and the precision of PI/C determination is affected by the additive analytical errors of the individual assays. To avoid this problem, we proposed to apply the trefoil-type assay principle. In this approach, one antibody pair captures and detects 2 or more analytes that share a common sequence to measure the sum of their concentrations. The analytes are then distinguished with detecting antibodies against the analytes' distinctive moieties. Three antibodies form a complex with each analyte, except when one of the analytes does not contain a specific amino acid sub-sequence. The concentration of this analyte can be quantified by subtracting the concentration of the other analytes from the total concentration. The intra- and interassay variations in

pipetting volume and reaction conditions during the capturing step are identical for all of the analytes; hence, the related error is mathematically neutralized when analyte ratios are determined.

Materials and Methods

OVERVIEW

The trefoil-type time-resolved fluorescence immunoassay (TRFIA) we describe (Fig. 1) is a combination of an individual proinsulin ELISA (13) and the AutoDELFI A C-peptide Kit from PerkinElmer. We used the same monoclonal antibodies (mAbs), each binding to a different proinsulin epitope. The common capture antibody has an epitope specificity for the C terminus of the C-peptide (mAb PEP-001, $K_a = 10^{10}$ L/mol), and the mAb that detects all captured molecules containing the intact C-peptide (CPT-3F11, $K_a = 10^{10}$ L/mol) recognizes the N-terminal part of C-peptide (Dako). A third antibody (HUI-001, $K_a = 10^9$ L/mol) serves to detect among the captured molecules those that also contain an insulin moiety, i.e., proinsulin, including its partially converted des and split forms (13). mAb CPT-3F11 is directly labeled with Eu^{3+} (14), whereas mAb HUI-001 is biotinylated and detected with Tb^{3+} -labeled streptavidin. This labeling strategy allows differentiation between the detecting antibodies within the same reaction compartment

(15, 16). The difference between the 2 measurements yields the amount of C-peptide unbound to insulin.

CHEMICALS AND BUFFERS

All reagents were of analytical grade, and all solutions were brought to 20 °C before use. Buffer A contained 40 mmol/L sodium phosphate, 30 g/L purified BSA (Probumin; Millipore), 1 g/L bovine gamma globulin (Sigma–Aldrich), 60 g/L NaCl, 0.5 g/L NaN₃, and 1 mL/L Tween 20 (Merck), pH 7.4 at 20 °C. Before use, fetal bovine serum (Invitrogen) was added to a concentration of 100 mL/L. Buffer B was 50 mmol/L Tris-HCl, pH 7.75 at 20 °C, containing 9 g/L NaCl, 0.5 g/L NaN₃, 0.5 g/L bovine gamma globulin, 5 g/L purified BSA, 7.44 mg/L disodium EDTA, and 0.1 mL/L Tween 20. Wash Concentrate, Enhancement Solution, Enhancer, Diluent II, and Eu³⁺- or Tb³⁺-labeled streptavidin were from PerkinElmer. Diluent II was used as a zero calibrator in all assays developed in house, and all washings were performed with a DELFIA 1296 Plate-washer (PerkinElmer) filled with a 40-mL/L aqueous dilution of the Wash Concentrate.

REFERENCE PEPTIDE MATERIAL

Recombinant human proinsulin (gift of Prof. R. B. Mackin) (17) was calibrated against WHO international reference reagent (IRR) 84/611 [National Institute for Biological Standards and Control (NIBSC)], and synthetic C-peptide (Bachem) was calibrated against WHO IRR 84/510 for human C-peptide (NIBSC). For the trefoil-type TRFIA, these 2 calibrators were combined in Diluent II to produce a PI/C of 2.44%. For separate analysis of C-peptide with RIA and TRFIA, we included the calibrators from the commercial kits. The IRR 66/304 reference for insulin (NIBSC) (18) was used as such in the interference and recovery experiments.

ANTIBODIES

The mAbs directed against C-peptide (PEP-001 and CPT-3F11) and proinsulin (HUI-001) were obtained from Dako. HUI-001 was biotinylated as described by Bayer and Wilchek (19), starting from the EZ-Link™ NHS-Biotin (Pierce/Perbio Science). The mAb CPT-3F11 was labeled with Eu³⁺, starting from isothiocyanate-activated europium chelate (DELFLIA Eu-N1 ITC chelate; PerkinElmer). Labeled antibodies were stored at –20 °C in 500 mL/L glycerol.

PLASMA SAMPLES

The present study has complied with the principles of the Helsinki Declaration (2000) and has been conducted in accordance with the Council for International Organizations of Medical Sciences [CIOMS (1991)] International Guidelines for Ethical Review of

Epidemiological Studies. Analyses were performed on anonymous surplus plasma samples without obtaining informed consent from the individuals concerned, but the analyses were conducted with full respect for the individuals' right to confidentiality and according to procedures supervised by local authorities responsible for ethical research. Whole blood (10 mL) was collected in S5-Monovettes® (Sarstedt) containing 600 kallikrein inactivator units/mL aprotinin (Trasyol; Bayer HealthCare) and 1.6 g/L disodium EDTA. After centrifugation at 1600g for 15 min, the plasma was aliquoted and stored at –80 °C. For the method comparisons, no attention was given to the glycemic state of the patients tested. The results for nondiabetic first-degree relatives of type 1 diabetes patients were for randomly obtained plasma samples (10).

ASSAY METHODS

Individual assays. C-peptide was measured by RIA (¹²⁵I–human C-peptide, guinea pig antihuman C-peptide serum; Linco) (20), and proinsulin was measured by ELISA (13). The proinsulin TRFIA was based on this ELISA, but Eu³⁺-labeled streptavidin and Enhancement Solution were used for detection. For separate measurement of C-peptide with TRFIA, we used an adaptation of the AutoDELFLIA C-peptide Kit (PerkinElmer). We eliminated disodium EDTA interference by washing 4 additional times after the capture step and before the addition of Eu³⁺-labeled antibody.

Trefoil-type TRFIA of proinsulin and C-peptide. Buffer A (100 μL) and sample or standard (25 μL) were pipetted in duplicate into PEP-001–coated microtiter plate wells (for the coating procedure, see Supplemental Data Part 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol54/issue12>). The plate was covered and incubated for 2 h at room temperature with continuous horizontal shaking (50% of maximum speed on a DELFLIA 1296 Plateshaker). After 4 washings, each well was filled with 135 μL buffer B containing 35 μg/L biotinylated HUI-001 and 750 μg/L Eu³⁺-labeled CPT-3F11. The film-protected plate was incubated for 16 h at room temperature with shaking. After another 4 washings, 135 μL of Tb³⁺-labeled streptavidin (0.12 μg/L) in buffer B was added and shaken for 1 h at 20 °C. After washing, Enhancement Solution (140 μL/well) was added, the plate was shaken for 5 min, and the Eu³⁺ time-resolved fluorescence was measured with a VICTOR² fluorometer (PerkinElmer). Enhancer Solution was then added (35 μL/well), the plate was shaken for another 5 min, and the Tb³⁺ time-resolved fluorescence was measured. We then used the smoothed spline algorithm of the

Multicalc™ 120 software (version 2.6; PerkinElmer) for regression analysis and to calculate the results for proinsulin and total C-peptide. The concentration of true C-peptide was calculated by subtracting the proinsulin concentration (including the partially converted des and split forms) from the measured concentration of total C-peptide; the concentration of true C-peptide was then used to calculate PI/C as a percentage: $100 \times [\text{proinsulin}]/[\text{C-peptide}]$.

VALIDATION EXPERIMENTS

Standard curves and linearity experiments are described in the online Supplemental Data Part 2. Within-run, between-run, and within-laboratory imprecision values were estimated according to the EP5-A2 CLSI protocol. Pooled human plasma samples with low (A), medium (B), and high (C) concentrations of C-peptide and proinsulin were used. The trefoil-type TRFIAs were performed on 20 separate days with 4 duplicates per run for each concentration (i.e., $n = 80$ duplicates/concentration). Statistical analysis of the data was carried out as described in the online Supplemental Data Part 3. Deming regression analysis was used to compare methods (21). The effects of hemolysis, lipemia, and hyperbilirubinemia were tested according to procedures described in CLSI document EP7-A2, and a bias of $\geq 10\%$ was considered to indicate interference. For the heterophile antibody interference experiments and the recovery experiments, see the online Supplemental Data Parts 4 and 5, respectively.

We calculated “related” PI/C values with proinsulin and C-peptide data obtained from the same microtiter plate well ($n = 80$) and calculated “unrelated” PI/C values from proinsulin and C-peptide results obtained in different runs. As outlined in the online Supplemental Data Part 3, we used *F* statistics to compare imprecision values for related and unrelated PI/C measurements. The lower and upper limits of quantification (LOQs) were evaluated as described by Findlay et al. (22). We spiked C-peptide–negative plasma (< 30 pmol/L) with IRR 84/510 and IRR 84/611 to obtain 8 preparations with final concentrations of 30–8484 pmol/L for C-peptide and 0.7–197 pmol/L for proinsulin. Each preparation was aliquoted ($n = 20$) and stored at -80°C . An aliquot of each preparation was thawed each day, randomly pipetted into 8 wells of a microtiter plate, and analyzed. This procedure was repeated in 20 independent assays on different days. The bias was defined as the difference between the overall mean of the measurements (\bar{z}_i) and the nominal (expected) value (μ_T). The intermediate precision (S_{IP}) was determined by ANOVA (EP5-A2 CLSI) estimation of the within-laboratory error (S_T), and the Satterthwaite approximation of degrees of freedom was used to calculate the 90% confidence interval (23, 24). Total

error (%RE) including both bias and imprecision was estimated with the formula: $\%RE = (100/\mu_T)[(\bar{z}_i - \mu_T) \pm t_{v,0.95}(S_{IP})]$, and the LOQs were defined as the concentrations where $|\%RE|$ is $\geq 25\%$ (22).

Results

CALIBRATION CURVES AND LINEARITY

Calibration curves and the results of linearity experiments are shown in the online Supplemental Data Part 2. In both the individual assays as well as the trefoil-type TRFIA, the measured fluorescence counts were proportional to the concentrations of the calibrators over a large measurement range, up to 8024 pmol/L for total C-peptide and 168 pmol/L for proinsulin. With the trefoil-type TRFIA, results for plasma proinsulin and C-peptide were linear to at least 160 pmol/L and 7038 pmol/L, respectively (method linearity), and a 4-fold dilution produced recoveries of 95%–107% and 99%–103%, respectively (dilution linearity).

IMPRECISION

Total imprecision for the trefoil-type TRFIA assay ranged from 8.7% to 12% for C-peptide and from 8.4% to 12% for proinsulin (Table 1). As expected, the overall imprecision of PI/C is significantly lower ($P \leq 0.05$ at all levels) when proinsulin and C-peptide data are obtained from the same microtiter plate well (CV range, 6.4%–11%) than when the data are obtained from 2 different wells (CV range, 13%–19%).

RECOVERIES AND INTERFERENCES

The analytical recoveries of proinsulin and C-peptide in their respective parts of the trefoil-type TRFIA approximated 100% (see the online Supplemental Data Part 5). As expected, proinsulin reacted equimolarly in the C-peptide part of the assay, but up to 8000 pmol/L C-peptide (the sum of the endogenous and spiked amounts) did not interfere with the proinsulin part. Insulin was not recognized in either part of the assay.

The addition of an erythrocyte lysate (up to 10 g/L hemoglobin), Intralipid™ (up to 11.29 mmol/L triglycerides), or bilirubin (up to 513 $\mu\text{mol/L}$) to a plasma sample containing 796 pmol/L C-peptide and 19.8 pmol/L proinsulin produced a change in the C-peptide or proinsulin concentration of $< 10\%$, as measured by the trefoil-type TRFIA (data not shown). Interference by heterophilic antibodies could be markedly reduced by adding bovine gamma globulin and fetal bovine serum to the incubation buffer (see the online Supplemental Data Part 4).

METHOD COMPARISONS

Proinsulin and C-peptide results from trefoil-type TRFIAs were highly correlated with and similar to re-

Table 1. Imprecision of trefoil-type TRFIA at different proinsulin and C-peptide concentrations.

Variable	Pool	Mean ^b	CV (95% CI), % ^a		
			Within run	Between run	Within laboratory
C-peptide					
	A	203 pmol/L	6.9 (5.8–8.3)	10.5 (7.8–14.9)	12.0 (9.5–15.6)
	B	1119 pmol/L	4.4 (3.7–5.3)	7.8 (5.8–11.1)	8.7 (6.8–11.5)
	C	3166 pmol/L	5.0 (4.2–6.0)	8.8 (6.5–12.5)	9.8 (7.7–12.9)
Proinsulin					
	A	6.8 pmol/L	7.9 (6.6–9.5)	10.3 (7.6–14.6)	12.3 (9.9–15.7)
	B	26.4 pmol/L	7.3 (6.1–8.8)	5.6 (4.1–8.0)	8.4 (7.1–10.1)
	C	86.6 pmol/L	6.0 (5.0–7.2)	7.5 (5.6–10.7)	9.2 (7.4–11.7)
PI/C					
Related ^c					
	A	3.50%	8.4 (7.1–10.1)	8.1 (6.0–11.5)	10.9 (9.1–13.4)
	B	2.42%	4.4 (3.7–5.3)	5.1 (3.8–7.3)	6.4 (5.2–8.0)
	C	2.82%	4.0 (3.4–4.8)	6.7 (5.0–9.5)	7.5 (5.9–9.9)
Unrelated ^d					
	A	3.50%	15.0 (12.6–18.1)*	15.6 (11.5–22.2)***	18.6 (14.9–23.8)**
	B	2.44%	9.2 (7.7–11.1)*	9.8 (7.3–13.9)***	12.7 (10.4–15.8)**
	C	2.85%	8.1 (6.8–9.8)*	11.8 (8.7–16.8)***	13.7 (11.0–17.6)**

^a CI, confidence interval.

^b Overall mean of variable values in the EP5-A2 CLSI procedure (20 runs with 4 duplicates/run).

^c Related indicates that the PI/C values considered for precision estimation ($n = 80$ for each pool) were calculated from proinsulin and C-peptide concentrations measured within the same well of a microtiter plate.

^d Unrelated indicates that the proinsulin and C-peptide data used for calculating PI/C values originated from different runs. * $P \leq 0.01$, ** $P \leq 0.05$, and *** $P \leq 0.1$; F -test results for comparisons of imprecision data for related and unrelated PI/C ratios (degrees of freedom are for $n = 80$; see methods).

sults obtained with separate TRFIAs, which in turn were strongly correlated with results obtained with established techniques (ELISA and RIA, respectively; Fig. 2). Compared with the TRFIA results, the ELISA results were about 10% lower, and the RIA results were 10% higher. These differences, however, may derive from differences in assay standardization.

LIMITS OF QUANTIFICATION

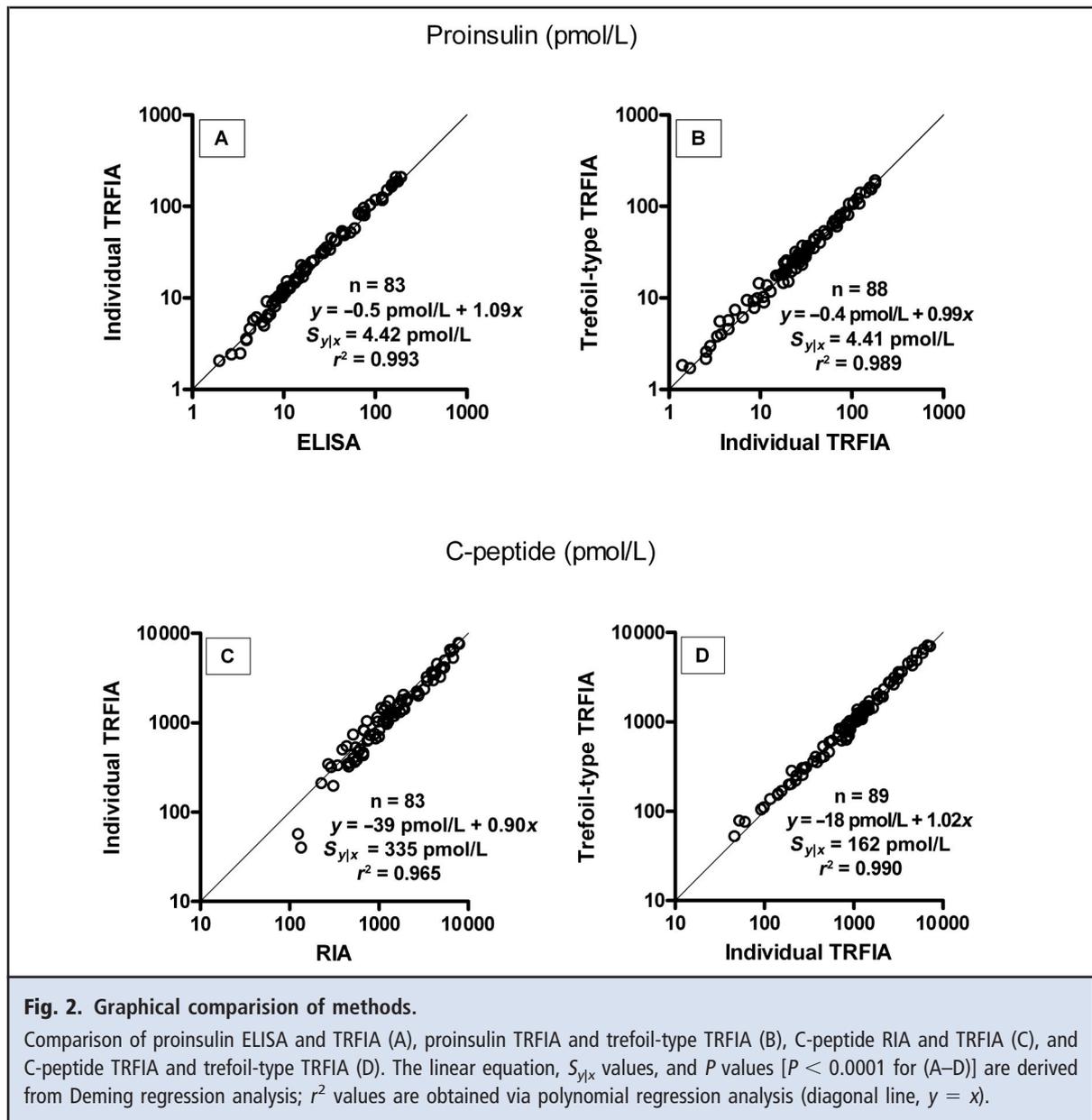
Graphical estimation indicates the lower and upper LOQs for the C-peptide TRFIA to be 48 and 8125 pmol/L (Fig. 3A). For proinsulin, the lower LOQ is 2.1 pmol/L; however, the upper LOQ could not be determined because the limit of >25% total error was not reached at the highest concentration tested (197 pmol/L; Fig. 3B). When this criterion was applied to PI/C, it appeared that this ratio could be reliably measured over a much broader range of C-peptide concentrations when simultaneously measured proinsulin and C-peptide values were used (“related” values, 79–7200 pmol/L C-peptide) than when these data originated from different plates (“unrelated” values, 590–4300 pmol/L C-peptide; Fig. 3, C and D).

RESULTS IN PREDIABETES

With the developed trefoil-type TRFIA, PI/C was significantly higher in randomly sampled normoglycemic prediabetic first-degree relatives ($n = 46$) of a type 1 diabetic proband than in as yet nondiabetic relatives ($n = 292$), with or without diabetes autoantibodies [mean (interquartile range), 3.87% (2.32%–5.82%) vs 2.10% (1.68%–3.81%); $P < 0.001$], as expected (4, 10).

Discussion

This study provides proof of principle for the use of trefoil-type immunoassays for measuring the concentrations of related molecular species simultaneously, with an accompanying improvement in the precision of their ratios. We applied this technique to measure proinsulin and C-peptide in plasma and to obtain reliable PI/C values. The assay, which was calibrated against certified reference materials, displayed a wide dynamic range for both peptides, without any mutual interference and with recoveries that are in line with prior knowledge on the epitope specificities of the mAbs used, as established in individual assays (11, 13). The trefoil-type TRFIA has a large dynamic range and



is unaffected by common interfering substances, such as hemoglobin, bilirubin, and triglycerides, and preliminary data suggest little or no effect of heterophilic antibodies. The measured plasma concentrations differed by $\leq 10\%$ from values obtained with separate assays (PerkinElmer TRFIA or Linco RIA for C-peptide; ELISA or TRFIA for proinsulin) (13, 20). These results are quite acceptable given the large between-method differences that have been reported for C-peptide (25). The trefoil-type TRFIA achieved better precision for PI/C, a variable that expresses the functional state of pancreatic β cells and hence serves as predictor of diabetes (4, 5, 7–10). The total error for this ratio re-

mained $\leq 25\%$ over a much larger range of C-peptide concentrations than obtainable with individual assays. It is anticipated that applying the trefoil-type principle with more precise assay techniques, such as the recently described Tosoh AIA-600II immunoassay (12), will further improve the precision of PI/C determination. This supposition is supported by our preliminary results that adaptation of the trefoil-type assay to the PerkinElmer AutoDELFIA instrument achieves a lower total within-laboratory imprecision for C-peptide, proinsulin, and their ratio (range, 3.4%–5.6%, depending on the concentration; I.V., unpublished results). This imprecision corresponds to a reduction of

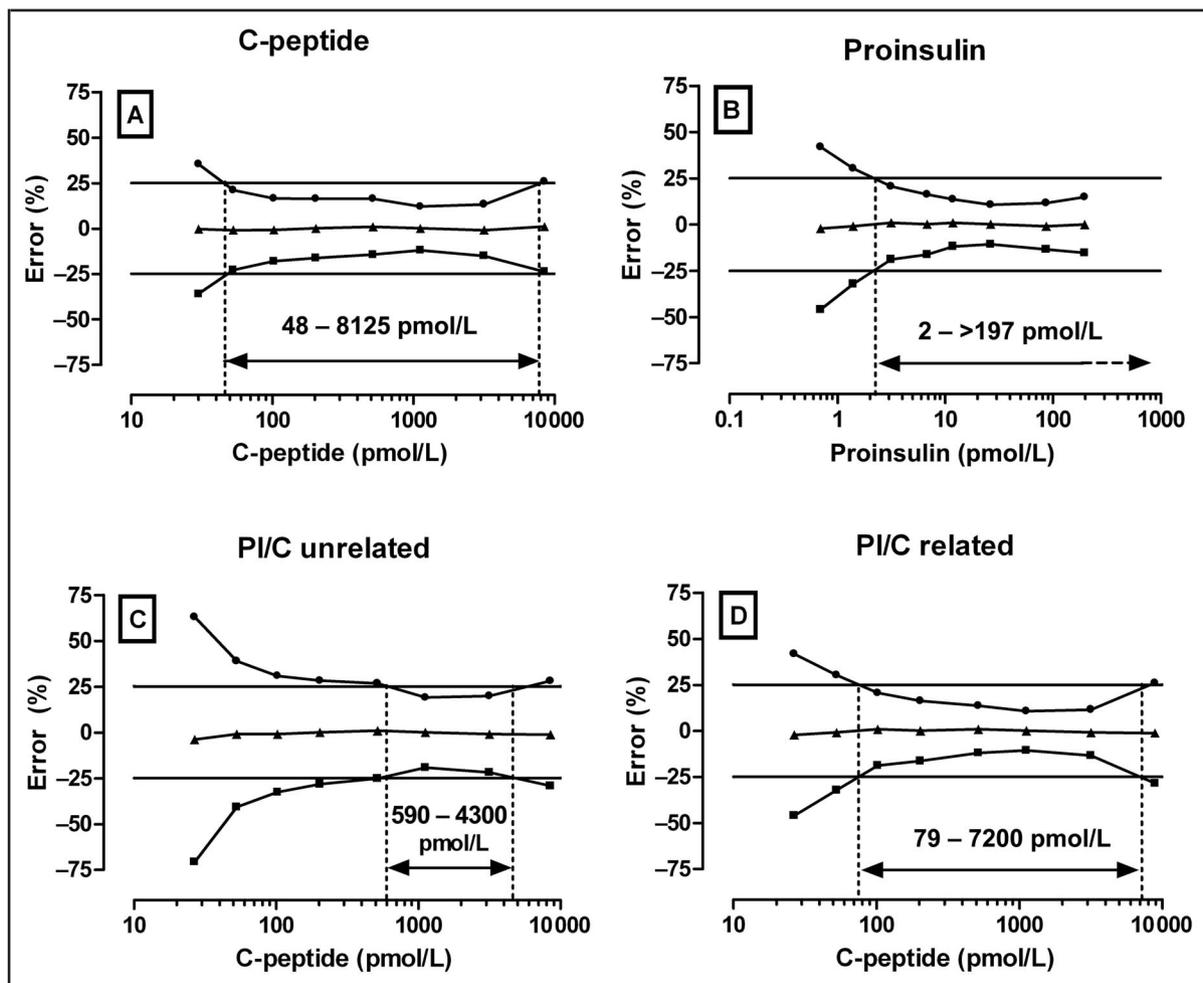


Fig. 3. Graphical determination of LOQs.

Total error was plotted as the bias (\blacktriangle) \pm the 90% confidence limits of imprecision (\bullet , \blacksquare), with the criterion that the total error be $\leq 25\%$. LOQs are shown for C-peptide (A), proinsulin (B), and PI/C derived from different (i.e., unrelated) wells (C) or the same (i.e., related) wells (D). Dashed lines indicate the ranges obtained.

15%–70% compared with similar concentrations in Table 1, as might be expected (26).

With the trefoil-type TRFIA, one mAb is used to capture all of the molecules sharing an epitope; these molecules are then quantified with a detecting antibody raised against another epitope shared by the various molecule species. These molecules are distinguished with differently labeled antibodies that are raised against the molecules' distinctive moieties, leading to an "all in one" assay. A disadvantage of combined assays is that a compromise has to be made between the optimal reaction conditions for each analyte. This disadvantage also applies in the trefoil-type TRFIA, but the problem is minimized thanks to the use of a common capture antibody. Saturation of the microtiter plate well surface with only one kind of anti-

body also avoids a decrease in the analytical range with respect to the individual assays. The trefoil-type TRFIA is amenable to automation (see above) and offers several advantages over individual tests, such as an identical sample history for all analytes, reduced sample handling, and reductions in the plasma volume required, costs for reagents (-30%) and personnel (-49%), and assay time. The assay measures intact proinsulin and most of the partially cleaved proinsulin-like molecules, similar to the parent individual proinsulin assay (13). Although direct assays of intact proinsulin are, strictly speaking, more accurate (27) and may have prognostic value in some rare cases (28), measurement of "total" proinsulin-like material may generate apparently larger increases in most pathologic conditions because the circulating concentrations of conversion interme-

diates also increase in (pre)diabetes (29). The anti-C-peptide mAbs used in the trefoil-type TRFIA are directed against stable epitopes of the peptide (30), a conclusion also supported by the high correlation between results obtained with TRFIA and RIA. A more widespread clinical use of PI/C would benefit from standardization of C-peptide and proinsulin assays, as has been attempted for insulin (26, 31, 32), and would require the use of assays that are not biased by the concentrations of circulating insulin, as is the case for the developed assay.

Apart from the developed example we have described, trefoil-type immunoassays may also apply to other situations in which the measurement of molecules with a common sequence is clinically relevant, e.g., for hormones or isoenzymes with common subunits (33, 34). Increasing the number of analytes for a trefoil-type TRFIA also seems feasible. Detection systems exist that can independently measure more than 2 markers within the same solution (35). Such an improvement may be facilitated by the use of antigen-binding fragments (36) or nanobodies (37), with a low risk of mutual steric hindrance. The trefoil-type immunoassay technique is positioned between individual immunoassays and the multiplex assays that are currently in vogue (38). The number of analytes that can be measured simultaneously with multiplex techniques is virtually unlimited, but whether this capability is always clinically and economically desirable and to what extent compromises in optimal conditions affect the quality of results for the multitude of variables assayed in multiplex approaches remain open questions. Trefoil-type TRFIAs may therefore have a niche in situations in which ratios of a limited number of molecules of the same family have to be measured with the highest possible accuracy and precision.

In conclusion, the trefoil-type TRFIA we have developed is a reliable method for the simultaneous measurement of proinsulin, C-peptide, and PI/C. The present observations provide a proof of principle for the application of trefoil-type multiple-label immunoassays under conditions in which different molecules with shared epitopes need to be quantified simultaneously.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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