diabetesresearchandclinicalpractice 205 (2023) 110974

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.journals.elsevier.com/diabetes-research-and-clinical-practice

Baseline plasma proinsulin response to glucose for predicting therapeutic response to otelixizumab in recent-onset type 1 diabetes

Aster K. Desouter [a,b,*], Bart Keymeulen [a,b], Simke Demeester [a,c], Ursule Van de Velde [a,b], Pieter De Pauw [a], Annelien Van Dalem [a,c], Bruno Lapauw [d], Christophe De Block [e], Pieter Gillard [f], Daniel G. Pipeleers [a], Frans K. Gorus [a,b]

[a] Diabetes Research Center, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Brussels, Belgium
[b] Department of Diabetes and Endocrinology, Universitair Ziekenhuis Brussel (UZ Brussel), Laarbeeklaan 101, 1090 Brussels, Belgium
[c] Department of Clinical Biology, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium
[d] Department of Endocrinology, University Hospital Ghent–UGent, Corneel Heymanslaan 10, 9000 Ghent, Belgium
[e] Department of Endocrinology, Diabetology and Metabolism, University of Antwerp–Antwerp University Hospital, Drie Eikestraat 655, 2650 Edegem, Belgium
[f] Department of Endocrinology, University Hospital Leuven–KU Leuven, Herestraat 49, 3000 Leuven, Belgium

ARTICLE INFO

Keywords:
anti-CD3 treatment
beta cell function
C-peptide
proinsulin
proinsulin/C-peptide ratio
type 1 diabetes

ABSTRACT

Aims: In recent-onset type 1 diabetes, clamp-derived C-peptide predicts good response to anti-CD3. Elevated proinsulin and proinsulin/C-peptide ratio (PI/CP) suggest increased metabolic/inflammatory beta cell burden. We reanalyzed trial data to compare the ability of baseline acutely glucose-stimulated proinsulin, C-peptide and PI/CP to predict functional outcome.

Methods: Eighty recent-onset type 1 diabetes patients participated in the placebo-controlled otelixizumab (GSK; NCT00627146) trial. Hyperglycemic clamps were performed at baseline, 6, 12 and 18 months, involving 3 h of induced euglycemia, followed by acutely raising and maintaining glycemia to $\geq$ 10 mmol/l for 140 min. Plasma proinsulin, C-peptide and PI/CP were determined after acute (minute 0 at 10 mmol/l; PI_0, CP_0, PI/CP_0) and sustained glucose stimulation (AUC between minutes 60–140). Outcome was assessed as change in $AUC_{60\text{-}140}$ C-peptide from baseline.

Results: In multiple linear regression, higher baseline ($\geq$median [P50]) PI_0 independently predicted preservation of beta cell function in response to anti-CD3 and interacted significantly with IAA. During follow-up, anti-CD3 tempered a further increase in PI/CP_0, but not in PI_0. CP_0 outperformed PI_0 and PI/CP_0 for post-treatment monitoring.

Conclusions: In recent-onset type 1 diabetes, elevated acutely glucose-stimulated proinsulin may complement or replace acutely or sustainedly stimulated C-peptide release for identifying good responders to anti-CD3, but not as outcome measure.

1. Introduction

Preserving (residual) beta cell function is an important goal in (a)symptomatic type 1 diabetes [1]. Several immune interventions could achieve this transiently in subgroups of patients, with Fc receptor non-binding monoclonal antibodies to CD3 as most successful approach, both at clinical onset and in asymptomatic disease [2,3]. At diagnosis, ChAglyCD3 (otelixizumab, GSK) or hOKT3gamma1(Ala-Ala) (teplizumab, Provention Bio-Sanofi) were able to preserve beta cell function up to 4 years with associated positive effects on metabolic control, but only in patients at high risk of rapidly losing residual beta cell function, i.e. relatively young individuals with short duration of clinical symptoms, and only moderately reduced beta cell function at baseline [4–8].

In a randomized placebo-controlled phase 2 trial (NCT00627146),

* Corresponding author at: Diabetes Research Center, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Jette, Belgium.
E-mail addresses: aster.desouter@uzbrussel.be (A.K. Desouter), bart.keymeulen@uzbrussel.be (B. Keymeulen), simke.demeester@uzbrussel.be (S. Demeester), ursule.vandevelde@uzbrussel.be (U. Van de Velde), pieter.de.pauw@vub.be (P. De Pauw), annelien.vandalem@uzbrussel.be (A. Van Dalem), bruno.lapauw@uzgent.be (B. Lapauw), christophe.deblock@uza.be (C. De Block), pieter.gillard@uzleuven.be (P. Gillard), daniel.pipeleers@vub.be (D.G. Pipeleers), frans.gorus@uzbrussel.be (F.K. Gorus).

https://doi.org/10.1016/j.diabres.2023.110974
Received 10 May 2023; Received in revised form 12 October 2023; Accepted 23 October 2023
Available online 25 October 2023

the effectiveness of otelixizumab was limited to new-onset patients with baseline residual function – as assessed by hyperglycemic clamp test (HCT) [9] – exceeding 25 % of matched healthy controls, and presenting with insulin autoantibody (IAA) positivity [7,8,10]. The HCT is considered the gold standard for measuring beta cell function [1,9,11]. In type 1 diabetes patients, C-peptide release is measured under prolonged, constant intravenous glucose stimulation (target glycemia 10.0–13.9 mmol/l during 140 min), after 3 h of insulin-induced euglycemia [7,9]. Teplizumab was recently shown to delay clinical onset in stage 2 asymptomatic type 1 diabetes and was subsequently granted approval by the USA Food and Drug Administration for prescription use [12,13]. Hence, it becomes increasingly important to identify individuals who are most likely to respond well to immunomodulation [14]. This should avoid needlessly exposing non-responders – especially when still symptom-free – to the potentially harmful side effects of intravenous anti-CD3 administration, including signs of cytokine release syndrome and transient Epstein-Barr virus reactivation [6,7]. Treatment at lower doses is better tolerated but may not suffice to replicate the beneficial effects of immunomodulation [15,16]. Therefore, future trials may benefit from replacing cumbersome or lengthy beta cell stimulation tests, which are difficult to perform in young children, by less tedious or time-consuming tests for the selection of participants of choice.

In this context we wondered whether – and to what extent – the discharge of various beta cell secretory peptides during a shorter glucose stimulus may also reliably predict functional outcome after anti-CD3 therapy. Rises in plasma proinsulin levels and the proinsulin/C-peptide ratio (PI/CP) precede and accompany the clinical course of type 1 diabetes and reflect impaired processing of proinsulin to insulin, allegedly due to increased metabolic and/or inflammatory burden upon the beta cells [17–33]. As anti-CD3 treatment is believed to be most effective in the peri-onset period when immune aggression against the beta cells is allegedly at its highest [4–8,12,34], we wanted to investigate whether these markers of beta cell stress may complement or replace C-peptide release for predicting a good therapeutic response to anti-CD3.

Taking advantage of stored data from the above-mentioned otelixizumab trial (NCT00627146) [7], we investigated (i) how acutely glucose-stimulated proinsulin (PI_0), C-peptide (CP_0) and PI/CP (PI/CP_0) relate to each other, to sustained glucose-stimulated area under the curve C-peptide release during minutes 60–140 of a HCT ($AUC_{60\text{-}140}$), and to other patient characteristics at baseline, (ii) whether PI_0 and PI/CP_0 may help predict the effectiveness of anti-CD3 treatment for preserving residual beta cell function in new-onset disease, and (iii) how their evolution during follow-up relates to the therapeutic response.

2. Subjects, materials and methods

2.1. Study design

Eighty recent-onset type 1 diabetes patients were enrolled in a multicentric randomized phase 2 placebo-controlled trial after written informed consent (NCT00627146) according to the following inclusion criteria: 12–39 years of age, treated with insulin $\leq$ 4 weeks before enrolment, polyuria for $<$ 6 months, $\leq$10 % weight loss during the previous 6 months, random C-peptide $\geq$ 200 pmol/l at a glycemia of 10.0–13.9 mmol/l, ICA^+ and/or $GADA^+$, and Epstein-Barr virus IgG^+ [7]. Treatment was assigned by a third-party member after randomization according to trial center, age, and the presence or absence of ICA [7]. Patients received an infusion of ChAglyCD3 (otelixizumab, n = 40) or placebo (n = 40) via intravenous infusion over 2–4 h on six consecutive days (64 mg cumulative dose in the first 4 patients; 48 mg in the following 36 patients) [7]. The primary endpoint was residual beta cell function, determined as AUC between minutes 60–140 ($AUC_{60\text{-}140}$) C-peptide release during a HCT [1,7,9,10] and expressed per minute. Efficacy and safety data were previously reported [7]. Originally, follow-up was planned for 18 months [7], but later extended to evaluate the 48-month outcome [8]. During this extension, the number of participants who underwent HCTs became insufficient (n = 36) to use HCT-derived C-peptide release as endpoint; therefore we opted to limit the current analyses to 18 months of follow-up [8,10].

2.2. Patient follow-up

All patients received intensive insulin therapy during the entire trial. Insulin doses were adjusted at least once every three months with the aim of maintaining blood glucose levels (home capillary measurements) between 4.4 and 7.8 mmol/l and HbA_{1c} levels below 7.0 % (53 mmol/mol) [7]. Type and dose of insulin, body weight, home capillary glucose measurements, concomitant medication and adverse events were recorded during three-monthly follow-up visits [7].

HCTs were performed at baseline (at start of treatment, after a median [IQR] of 20 [15-25] days of insulin treatment) and every 6 months thereafter, if the patient agreed. After 180 min of euglycemia (glycemia between 3.3 and 5.0 mmol/l), maintained by intravenous insulin infusion, glycemia was acutely raised by intravenous administration of a concentrated glucose solution aiming to increase blood glucose levels $\geq$ 10.0 mmol/l in 30 min [7]. Glycemia was measured bedside every 15 min, and the intravenous glucose load adapted according to Supplemental Table S1. Glycemia $\geq$ 10.0 mmol/l was reached after on average 30 min (cumulative glucose load: 3.75 g), with some interindividual variability, and defined the start (minute 0) of the hyperglycemic phase (Supplemental Fig. S1). During sustained stimulation, glycemia was maintained at 10.0–13.9 mmol/l. Proinsulin and C-peptide were measured at minutes 0, 60, 90, 120 and 140 of the hyperglycemic plateau [7].

During the euglycemic phase of the HCT, C-peptide values often fell below the lower limit of quantification (30 pmol/l) of the assay [21] used in the original study [7]. Therefore, values of proinsulin, C-peptide and PI/CP are reported here after a standardized acute glucose stimulation at the beginning of the hyperglycemic plateau phase of the HCT (minute 0 at $\geq$ 10 mmol/l glucose) as PI_0, CP_0 and PI/CP_0, respectively. C-peptide release under sustained glycemic stimulation during HCT was expressed as AUC between minutes 60–140 of the hyperglycemic plateau at $\geq$ 10 mmol/l ($AUC_{60\text{-}140}$). Data availability for PI_0, CP_0 and PI/CP_0 compared to $AUC_{60\text{-}140}$ C-peptide, was similar at baseline and during follow-up (Supplemental Table S2).

2.3. Analytical methods

For peptide measurements, venous whole blood was collected in K-EDTA monovettes (Sarstedt) containing aprotinin (Trasylol; Bayer; final concentration 600 kallikrein inactivator units/ml) and aliquoted plasma samples were stored at −80 °C before analysis. The present report is based on peptide concentrations obtained with the immunoassays used in the original study [7] because exhaustion of plasma samples precluded reanalysis with more recent methods. Plasma proinsulin and C-peptide were measured by enzyme-linked immunosorbent assay (ELISA) [35] and time-resolved immunofluorescence assay (TRFIA; AutoDelfia-C-peptide kit, Perkin Elmer, with in-house modifications), respectively [21]. Up to a 500-fold excess of C-peptide did not interfere in the proinsulin assay. Because of high cross-reactivity with conversion intermediates (74 % for split[32-33] –, 65 % for des[31-32] –, 78 % for split[65–66] – and 99 % for des[64–65] proinsulin) is considered to measure total proinsulin immunoreactive material [35]. This is not considered a disadvantage as conversion intermediates are also reported to increase in pre-diabetes [36]. Given the 100 % cross-reactivity of proinsulin immunoreactive material in the C-peptide assay used, the concentration of true, intact C-peptide (pmol/l) was calculated by subtracting the proinsulin concentration (pmol/l) from the measured concentration of total C-peptide (pmol/l) [37,38]. For the calculation of PI/CP (as percentage), proinsulin values were used as numerator and intact C-peptide values as denominator.

IAA levels were determined at screening (i.e. after a median [IQR] of 6 [3-14] days of insulin treatment) as previously described [10]. The cut-off value for IAA-positivity was determined as the 99th percentile (P99) of IAA levels in a healthy control population and amounted to 0.6 % tracer binding [10]. HbA_{1c} levels were measured using high-performance liquid chromatography [7].

2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, version 29.0. Figures were generated using Graphpad Prism, version 9.4.1. All tests were performed two-tailed with *P*-values below 0.05 considered significant. Statistical differences between groups for unpaired continuous and discontinuous data were assessed by Mann-Whitney U and Chi-Square tests with test-by-test exclusion of missing data, respectively, and correlations between variables by Spearman rank-order correlation coefficient (r_s) with pairwise exclusion of missing data. No adjustments were made for multiple testing, except where specified. C-peptide, proinsulin and PI/CP levels were used as continuous variables in regression and correlation analysis, or as categorical variables to define subgroups of participants with baseline values above or below the median (P50) for the graphical analysis.

The predictive value of baseline PI_0, CP_0 and PI/CP_0 for preserving residual beta cell function with anti-CD3 therapy was tested in multiple linear regression analysis against variables other than C-peptide that were found to be significant in the original anti-CD3 trial and its follow-up studies, i.e. age at start of treatment, and IAA levels at screening [7,8,10]. Functional decline expressed as change in clamp-derived $AUC_{60\text{-}140}$ C-peptide release at 18 months of follow-up versus baseline, was used as dependent variable. IAA values below the limit of detection (0.3 % tracer binding) were assigned a value of 0.2 % [10]. The parameters that were significant ($P < 0.05$) in univariable analysis were included in the forward stepwise multiple linear regression analysis, and eventual interactions investigated. Missing data were excluded list-wise.

In graphical analyses, $AUC_{60\text{-}140}$ C-peptide, PI_0, CP_0, and PI/CP_0 at month 0 (baseline, at start of treatment), 6, 12 and 18, were plotted as means and standard error of the mean (SEM) for each subgroup. Changes in these variables from baseline were compared statistically between subgroups to assess their potential as markers of therapeutic response.

3. Results

3.1. Baseline characteristics

At baseline, the anti-CD3-treated group and the placebo-treated group did not differ significantly in proinsulin (PI_0), C-peptide (CP_0) and PI/CP (PI/CP_0) levels after acute glucose stimulation following a period of induced euglycemia (i.e. at minute 0, the start of the hyperglycemic plateau during baseline HCT). Neither did they differ in age, sex, duration and dosage of insulin treatment, HbA_{1c}, $AUC_{60\text{-}140}$ C-peptide release during HCT, BMI and autoantibody profile (prevalence, number and levels of the 4 main molecularly defined specificities) (Supplemental Table S3) [7,10]. Plasma glucose values at minute 0 were $\geq$ 10 mmol/l and not significantly correlated with CP_0 ($P = 0.900$; $r_s = -0.014$), PI_0 ($P = 0.479$; $r_s = 0.081$), nor with PI/CP_0 ($P = 0.474$; $r_s = 0.082$). $AUC_{60\text{-}140}$ C-peptide was significantly correlated with CP_0 and PI_0 (both $P < 0.001$; $r_s = 0.851$ and $r_s = 0.648$, respectively), but not with PI/CP_0 (Supplemental Fig. S2). PI_0 and CP_0 were mutually correlated ($P < 0.001$; $r_s = 0.748$), as were PI_0 and PI/CP_0 ($P < 0.001$; $r_s = 0.674$), but not PI/CP_0 and CP_0. Two-by-two scatterplots for the various peptide markers are shown in Supplemental Fig. S2.

At baseline, PI_0 was inversely correlated with HbA_{1c} ($P < 0.001$; $r_s = -0.441$) and insulin dose ($P = 0.017$; $r_s = -0.270$), particularly after adjustment for body weight ($P = 0.001$; $r_s = -0.378$). Similar correlations were found when substituting PI_0 with CP_0, but not with PI/CP_0 (not shown). BMI tended to be more closely correlated with baseline values of PI_0 ($P = 0.003$; $r_s = 0.332$) than those of PI/CP_0 ($P = 0.038$; $r_s = 0.235$) or CP_0 ($P = 0.039$; $r_s = 0.234$).

Compared to participants with baseline $PI_0 <$ P50, patients with $PI_0 \geq$ P50 tended to have higher levels of IA-2A, a higher BMI, and lower insulin needs and HbA_{1c}; unsurprisingly PI/CP_0 and C-peptide (both under acute and chronic glycemic stimulation) were significantly higher in the group with $PI_0 \geq$ P50, given their significant correlation with PI_0 (Supplemental Table S4).

3.2. Response to anti-CD3 treatment according to baseline proinsulin and PI/CP

3.2.1. Multiple linear regression analysis

We investigated the relation of PI_0, CP_0, and PI/CP_0 values after acute glucose stimulation at baseline with the change in beta cell function – expressed as $AUC_{60\text{-}140}$ C-peptide under chronic glucose stimulation – during follow-up in both treatment arms separately, taking previously identified relevant baseline variables (age, IAA levels) [7,8,10] into consideration (Table 1). Both uni- and multivariable linear regression analysis confirmed that IAA levels were significantly associated with a more pronounced drop in $AUC_{60\text{-}140}$ C-peptide after 18 months in the placebo-treated group (Table 1, Model 1), as previously reported [7,10]. Higher PI_0 was the only variable at baseline that was significantly associated with better preserved $AUC_{60\text{-}140}$ C-peptide in the anti-CD3-treated group (hence the negative standardized beta coefficient) after 18 months of follow-up, both in uni- and multivariable analysis (Table 1, Model 1). When interactions between variables were also taken into consideration, PI_0 and IAA interacted significantly in the placebo group, while proinsulin remained the only significant variable in the anti-CD3-treated group (Table 1, Model 2).

3.2.2. Changes in beta cell function during follow-up

Fig. 1 illustrates the change in $AUC_{60\text{-}140}$ C-peptide release during HCT over 18 months from baseline, in placebo- vs. anti-CD3-treated participants, before (panel a) or after stratification according to baseline PI_0 values below (panel b) or above (panel c) P50 of all participants. Throughout follow-up, patients with baseline PI_0 values $\geq$ P50 experienced overall significantly less decline in $AUC_{60\text{-}140}$ C-peptide from baseline when treated with anti-CD3 than after receiving a placebo (Fig. 1c). In the patients with initial $PI_0 <$ P50, only borderline significant differences in $AUC_{60\text{-}140}$ C-peptide changes from baseline were observed between both treatment arms at month 6 (Fig. 1b). Similar patterns were obtained for participants stratified according to initial levels of PI/CP_0 or CP_0, albeit with slightly less significant differences in the latter case (not shown).

3.3. Changes in proinsulin and PI/CP during follow-up

During follow-up of the entire patient group, PI_0 levels overall increased further, but changes from baseline did not differ according to treatment arm (Fig. 2a). In contrast, changes in CP_0 (Fig. 2b) remained significantly lower in the anti-CD3 group over the entire follow-up period, with similar confidence as previously reported for changes in $AUC_{60\text{-}140}$ C-peptide [10]. Anti-CD3 treatment tended to temper the increase in PI/CP_0 ratio from baseline (Fig. 2c).

4. Discussion

4.1. Principal findings

Our multivariable analysis of beta cell secretion data from a randomized placebo-controlled anti-CD3 trial (otelixizumab) [7] in recent-onset type 1 diabetes identified relatively high acutely glucose-stimulated levels of proinsulin (PI_0) as an independent predictor of a good therapeutic response in terms of preserved residual beta cell

Table 1
Forward stepwise multiple linear regression analysis in placebo- and anti-CD3-treated subgroups with change in residual beta cell function, expressed as change in $AUC_{60\text{-}140}$ C-peptide release over an 18-month period from start of treatment (start – month 18), as dependent variable. All parameters were included as continuous variables. [a]At screening; [b]At start of treatment (baseline). PI_0, PI/CP_0 and CP_0 (available baseline data in n = 79) represent circulating levels of acutely glucose-stimulated proinsulin, proinsulin/C-peptide ratio and C-peptide, i.e. at the start of the hyperglycemic plateau phase (minute 0). β, standardized coefficient; NM, not selected for entry in the multivariable model. Multivariable model 2 includes interactions between independent variables. Significant *P*-values (<0.050) are indicated with *.

Variables	Univariable analysis		Multivariable model 1				Multivariable model 2			
	Placebo	*Anti-CD3*	*Placebo*		*Anti-CD3*		*Placebo*		*Anti-CD3*	
	P	*P*	*P*	*β*	*P*	*β*	*P*	*β*	*P*	*β*
Age[a]	0.515	0.593	NM		NM		NM		NM	
IAA[a]	**0.030***	0.858	**0.030***	**0.384**	NM		NM		NM	
PI_0^b	0.971	**0.029***	NM		**0.029***	**−0.355**	NM		**0.029***	**−0.355**
CP_0^b	0.425	0.594	NM		NM		NM		NM	
PI/CP_0^b	0.990	0.085	NM		NM		NM		NM	
$PI_0^b \times IAA^a$	**0.022***	0.057	—		—		**0.022***	**0.402**	NM	

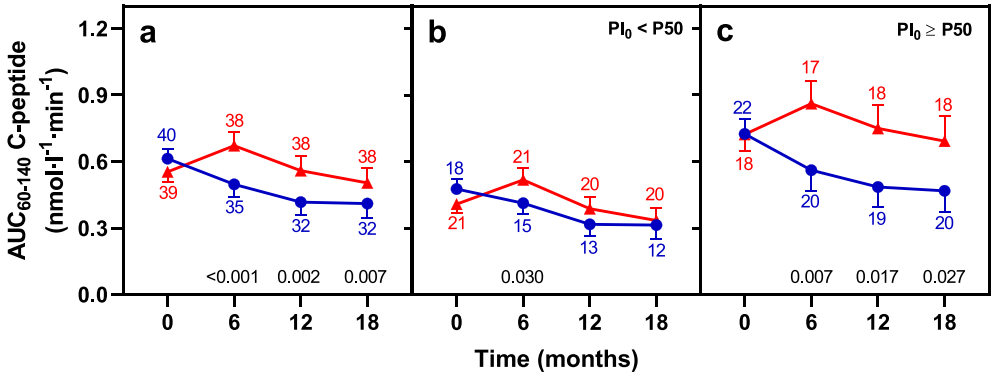

Fig. 1. Evolution of clamp-derived $AUC_{60\text{-}140}$ C-peptide release ($nmol \cdot l^{-1} \cdot min^{-1}$) during 18 months of follow-up in placebo-treated (blue circle) and anti-CD3-treated (red triangle) patients with available baseline data (n = 79) before (panel a) and after stratification according to acutely glucose-stimulated proinsulin (PI_0) at baseline below (panel b) or above/equal to (panel c) the median (PI_0 P50 = 4.49 pmol/l). Values represent means ± SEM. Small numbers next to the whiskers indicate number of observations at the respective time points. Statistical differences in change in clamp-induced $AUC_{60\text{-}140}$ C-peptide release from baseline (month 0, at start of treatment) vs. month 6, 12, 18 of follow-up between the placebo-treated vs. anti-CD3-treated groups were assessed by Mann-Whitney *U* test. Significant *P*-values (<0.050) are indicated above the respective time points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

function as assessed by the change in clamp-derived $AUC_{60\text{-}140}$ C-peptide from baseline, the primary outcome measure of the trial [7,10]. Changes in PI_0 and PI/CP_0 during follow-up could not distinguish between anti-CD3- and placebo-treated patients, and were outperformed by changes in CP_0 as measures of good therapeutic response.

4.2. Strengths and limitations of the study

Strengths of the otelixizumab trial include adherence to stringent patient selection criteria (e.g. short duration of symptoms, positivity for islet autoantibodies at screening, C-peptide levels above a pre-set threshold at study entry), the use of the HCT – the gold standard for assessing residual beta cell function – and validated biomarker assays [7]. The precision of PI/CP values may have been limited by the need to use proinsulin and C-peptide values measured separately in the original study with ELISA and TRFIA, respectively [7], instead of reanalyzing stored samples with more recent immunoassays, such as our trefoil-type TRFIA assay for simultaneous quantification of both secretory peptides and more precise estimation of PI/CP [37,38]. This choice was made to keep missing data to a minimum, as several original plasma samples were no longer available for retesting. This is relevant since the present analysis is limited by the relatively low number of participants, particularly when making subgroups. Measuring both intact and partially converted proinsulin is considered a strength as both increase in pathological situations [36–38]. Calculating instead of measuring true intact C-peptide is a weakness. We acknowledge that the use of subset data may detract from randomization balancing the groups. Another limitation is that the otelixizumab trial did not include children under the age of 12 years, who may benefit most from anti-CD3 treatment in view of an overall more intense immune inflammatory process, allegedly reflected by higher PI and PI/CP levels [20,39–41]. However, the age range of the included patients spans the period of life during which most diagnoses of new-onset type 1 diabetes are made [41]. Finally, during the euglycemic phase of the HCT, C-peptide levels often fell below the assays' lower limit of quantification, prompting the use of proinsulin, C-peptide and PI/CP after a low-dose, acute intravenous glucose stimulation. Albeit less time-consuming and cumbersome than chronic glycemic stimulation during a full HCT, the findings may still need to be replicated using more convenient measurement conditions, e.g. fasting or peak-stimulated proinsulin during a mixed meal tolerance test, prior to implementation, which is considered a limitation. We should also consider that the quality of the studied acute beta cell discharge data is likely to have benefitted from the induced period of euglycemia preceding the glucose challenge [1].

4.3. Interpretation of the findings

Elevated proinsulin and PI/CP levels both reflect incomplete proinsulin processing, allegedly due to an increased metabolic and/or inflammatory burden upon the beta cells. Additionally, the significant correlation at study entry of PI_0 – but not PI/CP_0 – with clamp-induced $AUC_{60\text{-}140}$ C-peptide, CP_0 and lower bodyweight-adjusted insulin needs

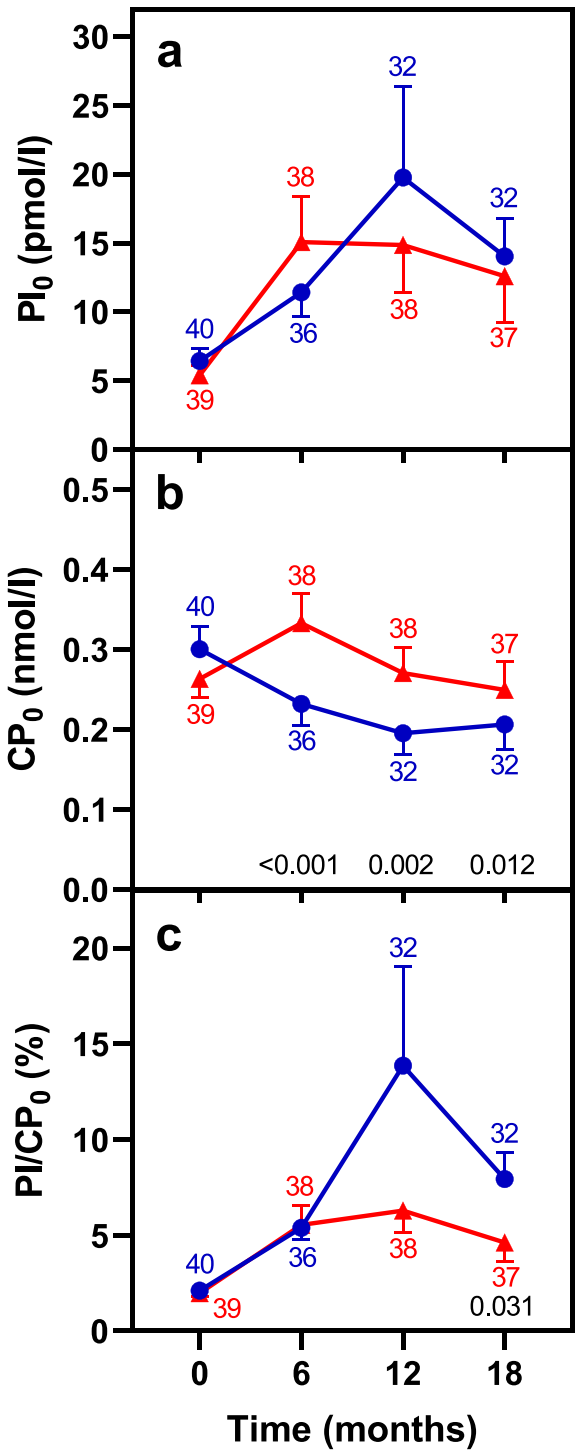

Fig. 2. Evolution of circulating levels after acute glucose stimulation of proinsulin (PI_0; pmol/l; panel a), C-peptide levels (CP_0; nmol/l; panel b) and proinsulin/C-peptide (PI/CP_0; %; panel c) during 18 months of follow-up in placebo-treated (blue circle) and anti-CD3-treated (red triangle) patients with available baseline PI_0 data (n = 79). Values represent means ± SEM. Small numbers next to the whiskers indicate number of observations at the respective time points. Statistical differences in change in proinsulin, C-peptide and PI/CP levels from baseline (month 0, at start of treatment) vs. month 6, 12, 18 of follow-up between the placebo-treated vs. anti-CD3-treated groups were assessed by Mann-Whitney *U* test. Significant *P*-values (<0.050) are indicated above the respective time points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(see §3.1.) supports the notion that proinsulin could serve as an indicator of residual insulin biosynthetic capacity [22]. This extra information conferred by PI_0 could underlie why it is retained as best independent predictor of good functional outcome. Our findings in patients are also in agreement with observations in autoantibody-positive relatives of type 1 diabetes patients in whom proinsulin, but not PI/CP, was significantly correlated with clamp-induced AUC C-peptide release [38].

The association of higher baseline levels of proinsulin with more rapid loss of beta cell function in the placebo group is consistent with a recent study in new-onset type 1 diabetes [24] and our own previous observation of a more rapid functional decline in the placebo group in case of initially relatively preserved clamp-derived $AUC_{60\text{-}140}$ C-peptide [7]. These observations may be ascribed to a higher potential to rapidly lose beta cells in presence of a relatively preserved functional beta cell mass and/or a more aggressive immune-mediated attack on these cells – particularly in individuals positive for IAA – which could preferentially be curbed by anti-CD3 treatment [7,10]. The better preservation of beta cell function in anti-CD3 treated participants with higher proinsulin levels at baseline suggests that a sufficient insulin biosynthetic capacity is needed for an optimal response to this immune intervention [22]. Increased proinsulin and PI/CP levels may also to a variable degree be ascribed to cytokine-induced phenotypical beta cell changes affecting prohormone folding, trafficking and conversion as a consequence of the immune-mediated disease process [22,23,25,27].

In conditions of increased metabolic and/or inflammatory burden on beta cells – allegedly present around the time of clinical onset of type 1 diabetes – proinsulin release may reflect the functional potential of the beta cells better than C-peptide [22,23]. It has become increasingly clear that proinsulin secretion is a persistent feature of human type 1 diabetes, even in long-standing disease and in the absence of detectable C-peptide, indicating the presence of cells that can synthesize – but not timely process – proinsulin [28–30]. Our results in recent-onset type 1 diabetes are compatible with the presence of such partly dysfunctional cells, which may be rescued from a sleeping, degranulated or dedifferentiated state by adequate immune and metabolic intervention [28–30].

In contrast CP_0 was superior to PI_0 or PI/CP_0 for monitoring the response to anti-CD3 treatment during follow-up. Inconsistent results have been reported on the relationship between therapy-induced changes in proinsulin or PI/CP levels and metabolic outcome [31]. Cyclosporin [32] and golimumab [42] reportedly suppressed the rise in PI/CP observed in the placebo group during the first months post-diagnosis. However, PI/CP was a poor predictor for cyclosporin-induced non-insulin requiring remission [43], while the relation between proinsulin and clinical outcomes was not reported in the golimumab study [14,31,42]. In a recent study in stage 2 type 1 diabetes, PI/CP did not differ between anti-CD3- and placebo-treated groups, despite clinical onset being delayed by > 3 years by teplizumab [12,13].

In conclusion, proinsulin discharge after acute glucose stimulation may replace or at least complement C-peptide for identifying putative good responders to anti-CD3 treatment in recent-onset type 1 diabetes. In the present study, proinsulin was measured after a low-dose, acute (on average 3.75 g over 30 min) glucose stimulation during a HCT, as the preceding insulin-induced euglycemic period blunted unstimulated beta cell secretion. We call for confirmation of the present results by retro- and prospective analysis of data and samples (both fasting and stimulated) obtained from other intervention trials – with anti-CD3 or other agents – including younger children and using more convenient and less cumbersome beta cell stimulation tests, to further evaluate the relevance and optimal conditions for assessment of proinsulin discharge as a marker for good therapeutic response to immune interventions in (a) symptomatic type 1 diabetes.

Funding.

The anti-CD3 trial was supported by the Juvenile Diabetes Research Foundation (JDRF Grants 4–2001-434 and 4–2005-1327) [7] and biomarker measurements by the Flemish Government Agentschap voor Innovatie door Wetenschap en Technologie (IWT Grant 130138). These

funding sources were not involved in the preparation of the present manuscript. The Strategic Research Program (SRP) of the Diabetes Research Center of the Vrije Universiteit Brussel (VUB) is supported by the Research Council of the VUB (SRP42-Growth and SRP55-Spearhead).

Contribution statement.

A.K.D., B.K., D.G.P. and F.K.G. designed the study; A.K.D., S.D., U.V. d.V. retrieved, statistically analyzed, and interpreted data; B.K., U.V.d. V., P.D.P., A.V.D., B.L., C.D.B. and P.G. contributed to patient recruitment and/or critical discussions; A.K.D. and F.K.G. wrote the first draft of the manuscript; all authors critically reviewed the manuscript for content, and approved the final version. A.K.D. is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the expert technical assistance of coworkers at the central unit and the clinical biology reference laboratory of the Belgian Diabetes Registry, from the Department of Clinical Chemistry and Radio-immunology, Universitair Ziekenhuis Brussel (UZ Brussel) and from the Diabetes Research Center, Vrije Universiteit Brussel (VUB), Brussels, Belgium (list provided in Demeester et al. [10]). The authors sincerely thank all participating patients and all health care workers who contributed to their recruitment and follow-up in the otelixizumab trial (list provided in Keymeulen et al. [7]).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2023.110974.

References

[1] Pipeleers D, Chintinne M, Denys B, Martens G, Keymeulen B, Gorus F. Restoring a functional beta-cell mass in diabetes. Diabetes Obes Metab 2008;10(Suppl 4):54–62.
[2] Skyler JS. The compelling case for anti-CD3 in type 1 diabetes. Diabetes 2013;62(11):3656–7.
[3] Dayan CM, Korah M, Tatovic D, Bundy BN, Herold KC. Changing the landscape for type 1 diabetes: the first step to prevention. Lancet 2019;394(10205):1286–96.
[4] Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346(22):1692–8.
[5] Herold KC, Gitelman SE, Masharani U, Hagopian W, Bisikirska B, Donaldson D, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. Diabetes 2005;54(6):1763–9.
[6] Herold KC, Gitelman SE, Ehlers MR, Gottlieb PA, Greenbaum CJ, Hagopian W, et al. Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders. Diabetes 2013;62(11):3766–74.
[7] Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, Hale G, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352(25):2598–608.
[8] Keymeulen B, Walter M, Mathieu C, Kaufman L, Gorus F, Hilbrands R, et al. Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. Diabetologia 2010;53(4):614–23.
[9] DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Phys Anthropol 1979;237(3):E214–23.
[10] Demeester S, Keymeulen B, Kaufman L, Van Dalem A, Balti EV, Van de Velde U, et al. Preexisting insulin autoantibodies predict efficacy of otelixizumab in preserving residual beta-cell function in recent-onset type 1 diabetes. Diabetes Care 2015;38(4):644–51.
[11] Elahi D. In praise of the hyperglycemic clamp. A method for assessment of beta-cell sensitivity and insulin resistance. Diabetes Care 1996;19(3):278–86.
[12] Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, et al. An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes. N Engl J Med 2019;381(7):603–13.
[13] Sims EK, Bundy BN, Stier K, Serti E, Lim N, Long SA, et al. Teplizumab improves and stabilizes beta cell function in antibody-positive high-risk individuals. Sci Transl Med 2021;13(583).
[14] Rigby MR, Hayes B, Li Y, Vercruysse F, Hedrick JA, Quattrin T. Two-Year Follow-up From the T1GER Study: Continued Off-Therapy Metabolic Improvements in Children and Young Adults With New-Onset T1D Treated With Golimumab and Characterization of Responders. Diabetes Care 2023;46(3):561–9. https://doi.org/10.2337/dc22-0908.
[15] Ambery P, Donner TW, Biswas N, Donaldson J, Parkin J, Dayan CM. Efficacy and safety of low-dose otelixizumab anti-CD3 monoclonal antibody in preserving C-peptide secretion in adolescent type 1 diabetes: DEFEND-2, a randomized, placebo-controlled, double-blind, multi-centre study. Diabet Med 2014;31(4):399–402.
[16] Keymeulen B, van Maurik A, Inman D, Oliveira J, McLaughlin R, Gittelman RM, et al. A randomised, single-blind, placebo-controlled, dose-finding safety and tolerability study of the anti-CD3 monoclonal antibody otelixizumab in new-onset type 1 diabetes. Diabetologia 2021;64(2):313–24.
[17] Watkins RA, Evans-Molina C, Terrell JK, Day KH, Guindon L, Restrepo IA, et al. Proinsulin and heat shock protein 90 as biomarkers of beta-cell stress in the early period after onset of type 1 diabetes. Transl Res 2016;168:96–106.e1.
[18] Roder ME, Knip M, Hartling SG, Karjalainen J, Akerblom HK, Binder C. Disproportionately elevated proinsulin levels precede the onset of insulin-dependent diabetes mellitus in siblings with low first phase insulin responses. The Childhood Diabetes in Finland Study Group. J Clin Endocrinol Metab 1994;79(6):1570–5.
[19] Ludvigsson J, Heding L. Abnormal proinsulin/C-peptide ratio in juvenile diabetes. Acta Diabetol Lat 1982;19(4):351–8.
[20] Sims EK, Chaudhry Z, Watkins R, Syed F, Blum J, Ouyang F, et al. Elevations in the Fasting Serum Proinsulin-to-C-Peptide Ratio Precede the Onset of Type 1 Diabetes. Diabetes Care 2016;39(9):1519–26.
[21] Truyen I, De Pauw P, Jørgensen PN, Van Schravendijk C, Ubani O, Decochez K, et al. Proinsulin levels and the proinsulin:c-peptide ratio complement autoantibody measurement for predicting type 1 diabetes. Diabetologia 2005;48(11):2322–9.
[22] Hostens K, Ling Z, Van Schravendijk C, Pipeleers D. Prolonged exposure of human beta-cells to high glucose increases their release of proinsulin during acute stimulation with glucose or arginine. J Clin Endocrinol Metab 1999;84(4):1386–90.
[23] Hostens K, Pavlovic D, Zambre Y, Ling Z, Van Schravendijk C, Eizirik DL, et al. Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release. J Clin Invest 1999;104(1):67–72.
[24] Freese J, Al-Rawi R, Choat H, Martin A, Lunsford A, Tse H, et al. Proinsulin to C-Peptide Ratio in the First Year After Diagnosis of Type 1 Diabetes. J Clin Endocrinol Metab 2021;106(11):e4318–26.
[25] Mirmira RG, Sims EK, Syed F, Evans-Molina C. Biomarkers of beta-Cell Stress and Death in Type 1 Diabetes. Curr Diab Rep 2016;16(10):95.
[26] Rodriguez-Calvo T, Zapardiel-Gonzalo J, Amirian N, Castillo E, Lajevardi Y, Krogvold L, et al. Increase in Pancreatic Proinsulin and Preservation of β-Cell Mass in Autoantibody-Positive Donors Prior to Type 1 Diabetes Onset. Diabetes 2017;66(5):1334–45.
[27] Eizirik DL, Miani M, Cardozo AK. Signalling danger: endoplasmic reticulum stress and the unfolded protein response in pancreatic islet inflammation. Diabetologia 2013;56(2):234–41.
[28] Oram RA, Sims EK, Evans-Molina C. Beta cells in type 1 diabetes: mass and function; sleeping or dead? Diabetologia 2019;62(4):567–77.
[29] Kahn SE, Chen Y-C, Esser N, Taylor AJ, van Raalte DH, Zraika S, et al. The β Cell in Diabetes: Integrating Biomarkers With Functional Measures. Endocr Rev 2021;42(5):528–83.
[30] Leslie RD, Vartak T. C-peptide persistence in type 1 diabetes: 'not drowning, but waving'? BMC Med 2019;17(1):179.
[31] Ramzy A, Kieffer TJ. Altered islet prohormone processing: a cause or consequence of diabetes? Physiol Rev 2022;102(1):155–208.
[32] Snorgaard O, Hartling SG, Binder C. Proinsulin and C-peptide at onset and during 12 months cyclosporin treatment of type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1990;33(1):36–42.
[33] Rodríguez-Villar C, Conget I, Casamitjana R, Vidal J, Manzanares JM, Gomis R. High proinsulin levels in late PRE-IDDM stage. Diabetes Res Clin Pract 1997;37(2):145–8.
[34] Bach J-F, Chatenoud L. A historical view from thirty eventful years of immunotherapy in autoimmune diabetes. Semin Immunol 2011;23(3):174–81.
[35] Kjems LL, Roder ME, Dinesen B, Hartling SG, Jorgensen PN, Binder C. Highly sensitive enzyme immunoassay of proinsulin immunoreactivity with use of two monoclonal antibodies. Clin Chem 1993;39(10):2146–50.
[36] Wareham NJ, Byrne CD, Williams R, Day NE, Hales CN. Fasting proinsulin concentrations predict the development of type 2 diabetes. Diabetes Care 1999;22(2):262–70.
[37] De Pauw PEM, Vermeulen I, Ubani OC, Truyen I, Vekens EMF, van Genderen FT, et al. Simultaneous measurement of plasma concentrations of proinsulin and C-peptide and their ratio with a trefoil-type time-resolved fluorescence immunoassay. Clin Chem 2008;54(12):1990–8.

[38] Van Dalem A, Demeester S, Balti EV, Keymeulen B, Gillard P, Lapauw B, et al. Prediction of Impending Type 1 Diabetes through Automated Dual-Label Measurement of Proinsulin:C-Peptide Ratio. PLoS One 2016;11(12):e0166702.
[39] Leete P, Oram RA, McDonald TJ, Shields BM, Ziller C, Hattersley AT, et al. Studies of insulin and proinsulin in pancreas and serum support the existence of aetiopathological endotypes of type 1 diabetes associated with age at diagnosis. Diabetologia 2020;63(6):1258–67.
[40] Battaglia M, Ahmed S, Anderson MS, Atkinson MA, Becker D, Bingley PJ, et al. Introducing the Endotype Concept to Address the Challenge of Disease Heterogeneity in Type 1 Diabetes. Diabetes Care 2020;43(1):5–12.
[41] Gorus FK. Diabetes registries and early biological markers of insulin-dependent diabetes mellitus. Belgian Diabetes Registry Diabetes/Metab 1997;13(4):247–74.
[42] Quattrin T, Haller MJ, Steck AK, Felner EI, Li Y, Xia Y, et al. Golimumab and Beta-Cell Function in Youth with New-Onset Type 1 Diabetes. N Engl J Med 2020;383(21):2007–17.
[43] Brussaard HE, Bravenboer B. Poor predicting values in obtaining non-insulin requiring remission using proinsulin/C-peptide ratios. Diabetologia 1991;34(3):201.