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ARTICLE

Hyperglycaemic clamp test for diabetes risk assessment in IA-2-antibody-positive relatives of type 1 diabetic patients

E. Vandemeulebroucke · B. Keymeulen · K. Decochez · I. Weets · C. De Block · F. Féry · U. Van de Velde · I. Vermeulen · P. De Pauw · C. Mathieu · D. G. Pipeleers · F. K. Gorus · The Belgian Diabetes Registry

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Abstract
Aims/hypothesis The aim of the study was to investigate the use of hyperglycaemic clamp tests to identify individuals who will develop diabetes among insulinoma-associated protein-2 antibody (IA-2A)-positive first-degree relatives (IA-2A$^+$ FDRs) of type 1 diabetic patients.
Methods Hyperglycaemic clamps were performed in 17 non-diabetic IA-2A$^+$ FDRs aged 14 to 33 years and in 21 matched healthy volunteers (HVs). Insulin and C-peptide responses were measured during the first (5–10 min) and second (120–150 min) release phase, and after glucagon injection (150–160 min). Clamp-induced C-peptide release was compared with C-peptide release during OGTT.
Results Seven (41%) FDRs developed diabetes 3–63 months after their initial clamp test. In all phases they had lower C-peptide responses than non-progressors ($p<0.05$) and HVs ($p<0.002$). All five FDRs with low first-phase release also had low second-phase release and developed diabetes 3–21 months later. Two of seven FDRs with normal first-phase but low second-phase release developed diabetes after 34 and 63 months, respectively. None of the five FDRs with normal C-peptide responses in all test phases has developed diabetes so far (follow-up 56 to 99 months). OGTT-induced C-peptide release also tended to be lower in progressors than in non-progressors or HVs, but there was less overlap in results between progressors and the other groups using the clamp.
Conclusions/interpretation Clamp-derived functional variables stratify risk of diabetes in IA-2A$^+$ FDRs and may more consistently identify progressors than OGTT-derived variables. A low first-phase C-peptide response specifically predicts impending diabetes while a low second-phase response may reflect an earlier disease stage.

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Keywords Beta cell function · C-peptide · Hyperglycaemic clamp test · IA-2 antibodies · Insulin · Prediction · Prevention · Type 1 diabetes

Abbreviations

ΔCP_{30}	Increase in C-peptide between 0 and 30 min during OGTT

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E. Vandemeulebroucke · B. Keymeulen · K. Decochez · I. Weets · U. Van de Velde · I. Vermeulen · P. De Pauw · D. G. Pipeleers · F. K. Gorus (✉)
Diabetes Research Center, Brussels Free University-VUB,
Laarbeeklaan 103,
1090 Brussels, Belgium
e-mail: Frans.Gorus@uzbrussel.be

C. De Block
Department of Endocrinology, University of Antwerp,
Antwerp, Belgium

F. Féry
Department of Endocrinology,
Université Libre de Bruxelles-ULB,
Brussels, Belgium

C. Mathieu
Department of Endocrinology, Catholic University of Leuven,
Leuven, Belgium

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$\Delta Glucose_{30}$	Increase in glucose between 0 and 30 min during OGTT
$\Delta CP_{30}/\Delta glucose_{30}$	Incremental response for C-peptide during OGTT
DI	Disposition index
FDR	First-degree relative
HV	Healthy volunteer
GADA	Glutamate decarboxylase antibodies
IA-2A	Insulinoma-associated protein-2 antibodies
IAA	Insulin autoantibodies
ICA	Islet cell cytoplasmic antibodies
IGT	Impaired glucose tolerance
ISI	Insulin sensitivity index
MMTT	Mixed-meal tolerance test
P10	10th percentile

Introduction

In type 1 diabetic patients preservation of residual beta cell function reduces glycaemic excursions and hence the risk of severe hypoglycaemia and chronic complications of hyperglycaemia [1]. A short treatment with humanised monoclonal anti-CD3 antibodies preserves beta cell function in recent-onset type 1 diabetes [2, 3], particularly in individuals with residual beta cell function ≥25% of controls [3]. This opens perspectives for immunomodulatory interventions in the late preclinical phase where beta cell function is likely to be even better preserved [4] and where anti-CD3 antibodies have also shown efficacy in animal models [5]. A trial in humans requires the identification of individuals with a beta cell mass that is sufficiently intact to expect efficacy, yet sufficiently reduced to warrant exposure to a possibly harmful medication. We wanted to investigate whether hyperglycaemic clamps [6, 7] can be used in this respect. An advantage of the clamp is that insulin release is not limited to the first phase, but is also quantified after prolonged glycaemic stimulation followed by a glucagon bolus. It was already instrumental in monitoring residual beta cell function in recent-onset type 1 diabetic patients and in recipients of a beta cell graft [3, 8].

In a secondary prevention trial with prophylactic insulin injections [9] we performed hyperglycaemic clamp tests and OGTTs at baseline in first-degree relatives (FDRs) of type 1 diabetic patients at high risk for developing diabetes within 5 years on the basis of insulinoma-associated protein-2 antibody (IA-2A) positivity [10–13] and in a group of matched healthy volunteers (HVs). The specific aims were to investigate whether: (1) during the various test phases insulin and C-peptide responses differed between IA-2A-positive FDRs and HVs; (2) hormone release during glucose clamps could stratify diabetes risk in antibody-positive FDRs and provide more information on clinical outcome than release after acute glycaemic stimulation alone; (3) clamp-derived hormonal variables may better discriminate between rapid and slow progressors to diabetes than OGTT-derived variables.

Methods

Participants

High-risk first-degree relatives Hyperglycaemic clamps were performed in non-diabetic FDRs (nine siblings and eight offspring) of type 1 diabetic patients recruited by the Belgian Diabetes Registry (BDR). They did not differ in age, BMI and sex ratio from the HVs (Table 1) and were at high risk of the disease (about 50% within 5 years), based on positivity for IA-2A in absence of protective HLA *DQA1**-*DQB1**genotypes [9–11, 14]. All but one tested positive for two or three other diabetes-associated autoantibodies. All FDRs were enrolled in an intervention study aiming at prevention of type 1 diabetes by prophylactic subcutaneous administration of low dose (0.05 U/kg twice a day) human regular insulin (Actrapid, Novo Nordisk, Brussels, Belgium) [9] (Fig. 1). The relatives and—in the case of minors—also

Table 1 General characteristics of IA-2A-positive relatives and healthy volunteers

Characteristics	High-risk relatives	Healthy volunteers
n	17	21
Age, years	21 (17–26)	23 (20–26)
Family history of diabetes	Yes	No
Male-to-female ratio (*n*/*n*)	10/7	9/12
BMI (kg/m^2)	23 (20–26)	22 (20–24)
Basal glycaemia (mmol/l)	4.7 (4.3–5.0)	4.5 (4.4–4.8)
Prevalence of diabetes antibodies, *n*/*n* (%)		
IA-2A	17/17 (100)†	0/21 (0)
ICA	16/17 (94)†	0/21 (0)
GADA	16/17 (94)†	0/21 (0)
IAA	6/17 (35)‡	0/21 (0)
GADA and/or IAA	16/17 (94)†	0/21 (0)
HLA- DQ2/DQ8 genotype, *n*/*n* (%)	3/17 (17)	0/17 (0)
Normal basal glycaemia at entry, *n*/*n* (%)	17/17 (100)	21/21 (100)
Normal OGTT at entry, *n*/*n* (%)	14/17 (82)	15/15 (100)
Development of diabetes, *n*/*n* (%)	7/17 (41)§	0/21 (0)

Data are median (interquartile range), *n*/*n* or *n*/*n* (%)

$^{\dagger}p<0.005$, $^{\ddagger}p<0.004$, $^{\S}p=0.002$ vs healthy volunteers

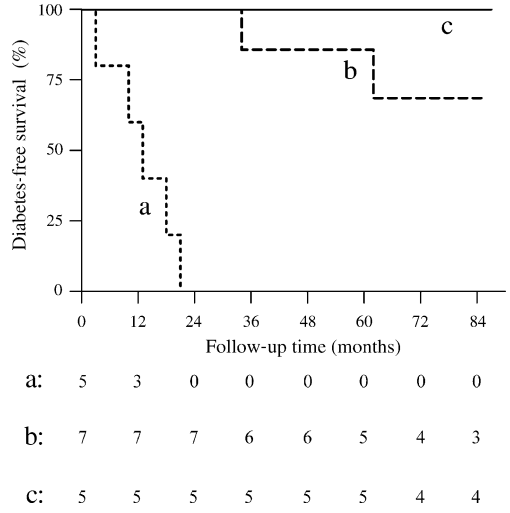

Fig. 1 Diabetes-free survival (%) in IA-2A-positive relatives stratified according to C-peptide response (AUC) during various phases of the hyperglycaemic clamp test at entry. **a** First-phase C-peptide response <P10 (n=5, dotted line). **b** First-phase C-peptide response >P10 and second-phase C-peptide response <P10 (n=7, dashed line). **c** First-phase C-peptide response >P10 and second-phase C-peptide response >P10 (n=5, solid line). The number of participants still under follow-up in each arm is indicated under the respective time point. Overall logrank $p<0.001$

their parents signed informed consent forms approved by the Ethics Committees of the BDR and of the four participating universities. The study was conducted in accordance with the guidelines in the Declaration of Helsinki as revised in 2000 (www/wma.net/e/policy/b3.htm, accessed 6 June 2008).

At entry, glucose tolerance was assessed by a 75 g oral glucose tolerance test (OGTT) [15]. One week later a hyperglycaemic clamp was performed in the FDRs and low-dose prophylactic insulin treatment was initiated for 36 months [9]. Relatives were prospectively followed (median [range]: 64[3–99] months) for glucose tolerance and diabetes onset (OGTT after 3, 9, 15, 18, 21, 27, 30, 33, 36, 42 and 48 months; glucose self-monitoring) and for beta cell function (hyperglycaemic clamp after 6, 12, 24, 36 and 48 months). At diagnosis, patients were shifted to intensified insulin treatment and excluded from the trial.

Healthy volunteers Hyperglycaemic clamps were performed three times with 1–3 week intervals in 21 HVs aged 16–34 years. None of them took medications or had a family history of type 1 diabetes, or an FDR with type 2 diabetes. All had normal BMI, tested negative for diabetes-associated antibodies (against islet cell cytoplasm [ICA], glutamate decarboxylase [GADA], IA-2 antigen [IA-2A], insulin [IAA]) and had normal fasting glycaemia. Of the 21 HVs, 15 underwent an OGTT that proved normal 1 to 3 weeks prior to the first hyperglycaemic clamp. Seventeen participants were genetically tested; they all lacked the high-risk *HLA DQA1*0301–DQB1*0302/DQA1*0501-DQB1*0201(DQ2/DQ8)* genotype (Table 1).

Hyperglycaemic clamps

All participants maintained their normal diet and physical activities during the days prior to the clamps. After an overnight fast a catheter was inserted into a right antecubital vein for blood sampling and into a left antecubital vein to infuse a concentrated glucose solution (1.1 mol/l; Baxter, Brussels, Belgium). At time 0 the plasma glucose concentration was acutely raised to reach the desired hyperglycaemic plateau of 10 mmol/l with a priming dose of glucose during 14 min. The latter was calculated as a function of the body surface area and amounted to 48.3 mmol/m^2 (time 0–5 min), 19.4 mmol/m^2 (5–10 min), 11.7 mmol/m^2 (10–14 min), and 10 mmol/m^2 (after 14 min). The hyperglycaemic plateau was maintained by adjusting the infusion rate according to bedside blood glucose levels measured every 5 min (HemoCue, Angelholm, Sweden). At time 150 min, 1 mg glucagon (Glucagen, Novo Nordisk, Bagsvaerd, Denmark) was injected via the left vein. The glucose infusion was stopped after 170 min. Blood samples for C-peptide, insulin and glucose were taken at time points −30, −15, 0, 2.5, 5, 7.5, 10, 60, 120, 135, 150, 155, 160, 170, 190 and 210 min. The normal patterns of glycaemia and hormone secretion during the clamp are shown in the Electronic supplementary material [ESM] Fig. 1.

Oral glucose tolerance tests

Blood samples were collected before and 30, 60, 90 and 120 min after a 75 g glucose load and analysed for glucose and C-peptide. In three relatives only the fasting and the 120 min samples were available.

Laboratory analyses

Metabolic and hormonal variables Plasma glucose was measured on Vitros 950 IC (Ortho Clinical Diagnostics, Rochester, NY, USA), C-peptide by time-resolved fluoroimmunoassay [16], proinsulin by ELISA [16] and insulin by a radioimmunometric assay (BI-INS-IRMA, Cisbio International, Gif-sur-Yvette, France). The proinsulin assay was considered to measure total proinsulin immunoreactive material [17]. As there is 100% cross-reactivity of proinsulin in the C-peptide assay, free C-peptide concentrations were obtained by subtracting the proinsulin concentration from the measured total C-peptide result. The interassay CVs at, respectively, low, median and high levels were 6.7% (mean 147 pmol/l), 5.2% (mean 530 pmol/l) and 4.9% (mean 1773 pmol/l) for C-peptide, 11% (mean

4.4 pmol/l), 7.2% (mean 28.7 pmol/l) and 12.5% (mean 111.4 pmol/l) for proinsulin, and 10.4% (mean 98.3 pmol/l), 6.0% (mean 353.5 pmol/l) and 4.8% (mean 901 pmol/l) for insulin. The cross-reactivity of the proinsulin-like molecules in the insulin assay is negligible (manufacturer's specifications).

Antibody assays and HLA-DQ genotyping ICA were determined by indirect immunofluorescence. IA-2A, GADA and IAA by liquid-phase radiobinding assays [18] and *HLA-DQA1-DQB1* haplotypes by allele-specific oligotyping [14]. Cut-off values for antibody positivity represented the 99th percentile of antibody levels in 790 non-diabetic controls [18]. In the 2007 Diagnostic Autoantibodies Standardization Program, diagnostic sensitivities adjusted for 95% specificity were, respectively, 63% for IAA, 94% for GADA, and 74% for IA-2A. cDNAs for the preparation of radiolabelled GAD and the intracellular domain of IA-2 were kindly donated by Å. Lernmark (University of Washington, Seattle, WA, USA) and M. Christie (King's College, London, UK), respectively.

Data analysis and statistical tests

In the hyperglycaemic clamp tests basal hormone levels were calculated as the mean of measurements obtained at −30, −15 and 0 min. The release of C-peptide and insulin was calculated as the AUC using y=0 as baseline (first phase: between 5 and 10 min, $AUC_{5–10\ min}$; second phase: between 120 and 150 min, $AUC_{120–150\ min}$; after glucagon: between 150 and 160 min, $AUC_{150–160\ min}$) and expressed per min. A response below the 10th percentile (P10) of the control group was considered abnormal. Intra- and inter-individual CVs were determined according to Smith et al. [19] Insulin sensitivity was expressed as insulin sensitivity index (ISI) by dividing the amount of glucose metabolised between 120 and 150 min (M expressed as mmol $kg^{-1} min^{-1}$) the average insulin concentration during the same period (I expressed as pmol/l), multiplied by 100. To correct for minor blood glucose fluctuations, M was calculated as the average glucose infusion rate between 120 and 150 min minus a 'space correction' [6].

For OGTTs the $AUC_{0–120}$ was calculated using y=0 as baseline and values were expressed per min. The increase in concentration between time 0 and 30 min was determined for glucose ($\Delta glucose_{30}$) and C-peptide (ΔCP_{30}). The incremental response was defined as $\Delta CP_{30}/\Delta glucose_{30}$ as previously described [20]. The disposition index (DI) [21] was computed by multiplying ISI from the clamp with the OGTT-derived $AUC_{0–120}$ for C-peptide.

Statistical tests were performed two-tailed using SPSS version 15.0 (SPSS, Chicago, IL, USA) and considered significant whenever $p<0.05$. Differences between groups were assessed by Mann–Whitney U or Kruskall–Wallis tests for continuous variables and by χ^2 test, using Yates' correction or Fisher's exact test for categorical variables. Correlations between variables were assessed using the Spearman Rank test and differences between diabetes-free survival by Kaplan–Meier analysis and the logrank test.

Results

Progression to diabetes

Seven of the 17 FDRs developed diabetes after a median (range) of 18 (3–62) months. In five progressors diagnosis was based on 2 h post-glucose-load glycaemia during OGTT, in one on glucose self-monitoring results and in one on clinical signs after his first clamp test; two of the prediabetic relatives had impaired glucose tolerance (IGT) at entry and developed diabetes 3 and 34 months later, respectively. Ten relatives remained non-diabetic after a median (range) follow-up of 94 (55–99) months. Nine of them had a normal OGTT at baseline; one was glucose intolerant at entry but returned to normal later. Three non-progressors had transient IGT during follow-up.

Hyperglycaemic clamp test in high-risk relatives

Hormone release After having assessed the reproducibility of the clamp test by performing it three times in HVs (with 2–3 week intervals between tests) (ESM Table 1), we compared the hormone release of FDR and HV during their initial clamp test (Table 2). In all clamp phases the AUCs for C-peptide and insulin were lower in those who progressed to diabetes than in non-progressors or HVs. The results were similar if delta hormone responses were considered during the different phases (results not shown). HVs and high-risk FDRs did not differ significantly in their insulin-sensitivity index. Less glucose needed to be infused in progressors in order to clamp glycaemia at 10 mmol/l (Table 2). Overall there was a strong correlation between first- and second-phase AUC for C-peptide (r^2=0.838, $p<0.001$) and between first-phase AUC and C-peptide after glucagon (r^2=0.877, $p<0.001$).

Relation with clinical outcome Five relatives with first-phase C-peptide responses below the P10 of HVs developed diabetes 3 to 21 months after their initial clamp test (Fig. 1, group a). They also had low responses in the second and glucagon-stimulated phases and the most rapid progressor had IGT at entry. Seven relatives had a normal first-phase response but a low second-phase response (Fig. 1, group b); four of them had also a low glucagon-induced release. Two relatives of group b developed

Table 2 Hyperglycaemic clamp-derived variables at baseline in healthy volunteers and high-risk relatives according to clinical outcome

Variables	Healthy volunteers (n=21)	High-risk relatives		Overall p value
		Non-progressors (n=10)	Progressors (n=7)	
Basal				
Glucose (mmol/l)	4.6±0.1	4.5±0.1	4.8±0.2	0.211
Insulin (pmol/l)	29±3	40±6	25±2	0.113
C-peptide (pmol/l)	373±23	418±39	308±25	0.090
First phase: $AUC_{5-10\ min}$				
Glucose (mmol/l×min)	8.2±0.2	7.6±0.4	9.1±0.8	0.192
Insulin (pmol/l×min)	134±13	153±35	58±15†	0.014
C-peptide (pmol/l×min)	787±60	843±99	425±57$^{\dagger **}$	0.004
Second phase: $AUC_{120-150\ min}$				
Glucose (mmol/l×min)	9.9±0.2	9.7±0.2	10.4±0.3	0.275
Insulin (pmol/l×min)	338±38	454±145	137±28$^{\dagger *}$	0.010
C-peptide (pmol/l×min)	2341±134	2269±266	1358±162$^{\dagger **}$	0.003
After glucagon: $AUC_{150-160\ min}$				
Glucose (mmol/l×min)	10.8±0.3	11.2±0.3	10.2±0.4	0.258
Insulin (pmol/l×min)	2012±142	2379±434	690±224$^{\dagger ***}$	0.002
C-peptide (pmol/l×min)	4624±259	4618±563	2357±347$^{\dagger ***}$	0.002
Insulin sensitivity index				
$M/I_{120-150\,min}$[a]	0.70±0.11	0.82±0.28	0.78±0.15	0.885
Total amount of glucose infused				
Glucose infused (mmol/kg)	9.8±0.6	10.1±1.0	5.6±0.6$^{\dagger **}$	0.008

Data are expressed as mean ± SE

[a] Ratio of glucose metabolised between 120 and 150 min (expressed as mmol kg^{-1} min^{-1}) and average insulin concentration between 120 and 150 min (expressed as pmol/l)

*p<0.05, **p<0.01, ***p<0.005 vs non-progressors; †p<0.002 vs healthy volunteers

diabetes after, respectively, 34 and 64 months; the first had IGT at entry and low glucagon-induced release. The five relatives with normal first- and second-phase responses had also normal post-glucagon responses and none of them developed diabetes during follow-up (range 64–99 months; Fig. 1, group c). The different groups in Fig. 1 did not differ in baseline characteristics (age, sex, BMI, *HLA-DQ* genotype, antibody levels or prevalence, and duration of IA-2A positivity). The amount of glucose infused during the clamp tended to be lower in group a than in groups b and c (not shown). Two relatives in group b and one in group c were transiently glucose intolerant.

Comparison with C-peptide response during OGTT Like clamp-derived hormonal variables, OGTT-derived C-peptide responses—in particular the incremental response during OGTT ($\Delta CP_{30}/\Delta glucose_{30}$), peak value, value at 120 min and AUC_{0-120}—tended to be lower in progressors than in non-progressors and HVs, while fasting glycaemia tended to be higher (Table 3). The three groups did not differ in disposition index calculated as the product of the clamp-derived ISI and the OGTT-derived AUC_{0-120} for C-peptide (Table 3). Clamp-derived first-phase AUC for C-peptide was significantly correlated with C-peptide at 30 min (r^2=0.71, p=0.004), AUC_{0-120} C-peptide (r^2=0.64, p=0.014) and peak C-peptide (r^2=0.56, p=0.04) during OGTT, but not with $\Delta CP_{30}/\Delta glucose_{30}$ (r^2=0.5, p=0.06).

Both in the clamp test and OGTT, low C-peptide secretion was associated with later development of diabetes, but—particularly for rapid progressors—there was less overlap in results between progressors and non-progressors or HVs using the clamp (Fig. 2).

Discussion

This study demonstrates for the first time that hyperglycaemic clamps can be used in antibody-positive FDRs of type 1 diabetic patients to consistently identify individuals close to clinical onset at a stage where fasting glycaemia or OGTT are still normal or inconsistent. In all clamp phases C-peptide and insulin responses were lower in individuals who progressed to diabetes than in those who did not and in

Table 3 Oral glucose tolerance test at baseline in healthy volunteers and high-risk relatives according to clinical outcome

Variables	Healthy volunteers (n=15)	High-risk relatives		Overall p value
		Non-progressors (n=10)	Progressors (n=7)	
Basal				
Glucose (mmol/l)	4.5±0.1	4.3±0.1	4.9±0.2*†	0.039
C-peptide (pmol/l)	412±37	444±79	569±148	0.778
Value at 30 min				
Glucose (mmol/l)	7.0±0.3	7.4±0.6	7.1±0.5	0.707
C-peptide (pmol/l)	1,461±131	1,469±206	913±152	0.072
Incremental response				
$\Delta CP_{30}/\Delta glucose_{30}$	430±80	535±216	218±44**	0.037
Peak value				
Glucose (mmol/l)	7.3±0.4	7.4±0.5	7.9±0.7	0.824
C-peptide (pmol/l)	2,309±149	2,000±242	1,515±106**	0.020
Value at 120 min				
Glucose (mmol/l)	5.2±0.2	6.0±0.3	5.8±0.4	0.243
C-peptide (pmol/l)	1,987±183	1,778±210	1,139±154†**	0.022
$AUC_{0–120\ min}$				
Glucose (mmol/l×min)	5.9±0.3	6.3±0.4	6.8±0.4	0.188
C-peptide (pmol/l×min)	1,700±116	1,481±175	1,144±87***	0.019
DI[a]	910±172	1066±314	775±148	0.984

Data are expressed as mean ± SE

[a] Computed as ISI obtained during clamp (see Table 2)×AUC $CP_{0–120\ min}$ obtained during OGTT (expressed as pmol/l×min)

*p<0.05, **p<0.01, ***p<0.005 vs healthy volunteers; †p<0.05 vs non-progressors

HVs. A low first-phase C-peptide release was invariably accompanied by low second-phase and post-glucagon responses, and consistently predicted impending diabetes. Moreover, two FDRs with low second-phase but normal first-phase responses developed hyperglycaemia after more than 2 years. Relatives with normal C-peptide responses in all phases have not progressed to diabetes so far. Clamp-derived C-peptide variables were significantly correlated with OGTT-derived variables, but there was less overlap in results between progressors and non-progressors or HVs using the clamp.

A strength of the study is that it has allowed comparison of the abilities of hormone release during the hyperglycaemic clamp and during OGTT to discriminate between progressors and non-progressors to diabetes in a group of well-characterised FDRs with close metabolic follow-up. An advantage of the clamp test is that—unlike acute beta cell stimulation tests—it also allows study of second-phase and glucagon-stimulated hormone release after diagnosis of diabetes and thus it is possible to monitor beta cell function, if necessary, over a longer period [3, 8, 9]. Acute stimulation tests such as IVGTT or mixed-meal tolerance tests (MMTT) measure only hormonal release from the beta cell subpopulation that can be rapidly activated and some (e.g. MMTT) are influenced by gastrointestinal variables [22]. In contrast, prolonged glycaemic stimulation during glucose clamp tests is believed to recruit virtually all beta cells in a glucose-responsive state [23]. A limitation of our study is that all relatives received prophylactic insulin injections after the initial clamp test, but this intervention did not affect clinical outcome [9] nor the ability of clamp variables to predict it. The hyperglycaemic clamp is more laborious to perform than acute stimulation tests that have been widely used in diabetes prediction and prevention studies [20, 24–28], but compliance of relatives or patients was high in our hands [3, 8, 9]. The glucagon injection is the most burdening part of the test and does not seem to provide much additional information: one may therefore consider omitting it. The clamp procedure is too heavy for children under age 12, but performing only the first phase in this group may still allow comparison of C-peptide responses over a large age range. Our results also require confirmation in ongoing studies targeting larger groups of relatives with various risk levels according to their antibody status and followed for longer periods (K. Decochez, unpublished results).

The present study shows for the first time that hormone release during prolonged beta cell stimulation can stratify diabetes risk in FDRs considered already at high risk based on their antibody status [10–13]. It illustrates that positivity

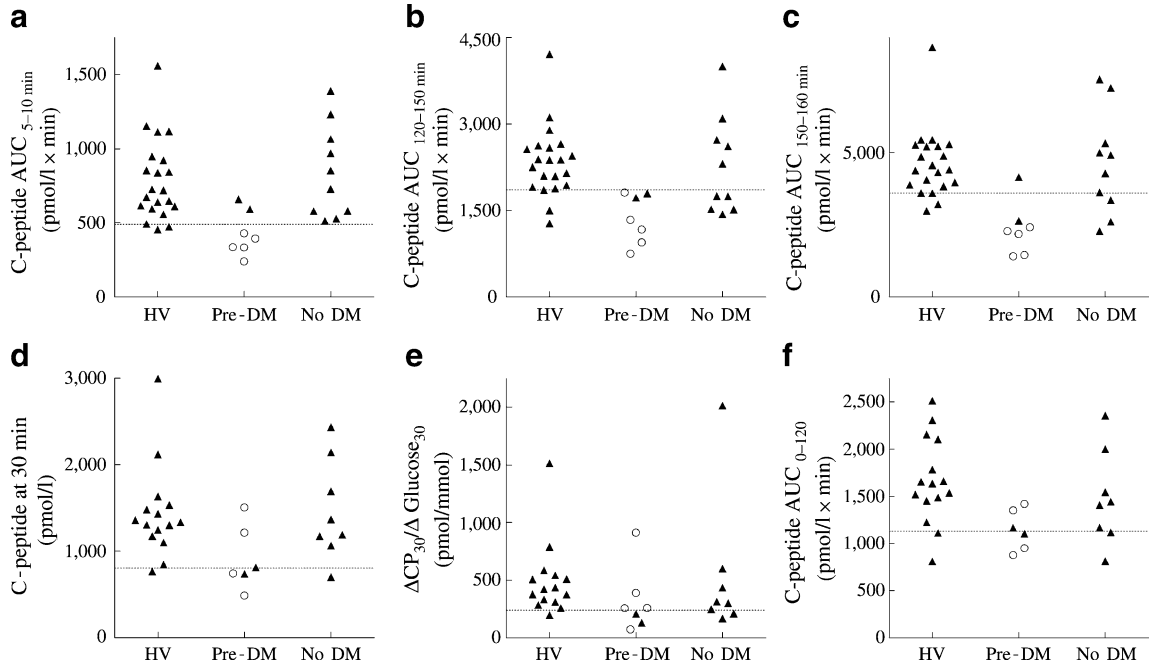

Fig. 2 Clamp-derived (panels **a–c**) and OGTT-derived (panels **d–f**) variables for C-peptide at baseline in healthy volunteers, relatives who progressed to diabetes (Pre-DM) and those who did not (No DM). **a** First-clamp-phase C-peptide $AUC_{5–10\ min}$. **b** Second-clamp-phase C-peptide $AUC_{120–150\ min}$. **c** Post-glucagon clamp phase C-peptide $AUC_{150–160\ min}$. **d** C-peptide at 30 min post-glucose load in OGTT. **e** Incremental C-peptide response during OGTT ($\Delta CP_{30}/\Delta glucose_{30}$). **f** C-peptide $AUC_{0–120\ min}$ during OGTT. Because of some missing data the number of individuals in the various groups is slightly lower for OGTT-derived variables. In each panel the horizontal line represents P10 of healthy volunteers for the variable under study. Rapid progressors (within 2 years), white circles

for IA-2A and multiple autoantibody markers is, in itself, not indicative of a strikingly decreased beta cell function. Autoantibodies reflect an ongoing immune process but cannot be used as markers of residual beta cell function. IA-2A$^+$ FDRs with a low first-phase C-peptide response during hyperglycaemic clamp should be considered for participation in immunointervention trials at the preclinical stage as they are at very high risk for developing diabetes in the short term, but still have better preserved residual function (e.g. overall >50% of control values for second-phase C-peptide release in Table 2 vs 25% at onset of diabetes in [3]). On the other hand, IA-2A$^+$ FDRs with normal C-peptide responses throughout the entire clamp procedure are unlikely to develop hyperglycaemia within 5 years and may not, therefore, be good candidates for immunointerventions.

Our results suggest that at least in some prediabetic FDRs a low second-phase C-peptide release may precede the decrease in first-phase release and may thus reflect an earlier disease stage. Hyperglycaemic clamp tests may thus provide more information on clinical outcome than less cumbersome tests with only acute glycaemic stimulation. However, this requires confirmation in larger ongoing studies. The clamp test also allowed estimation of insulin sensitivity in FDRs and HVs, but in line with previous publications [29, 30] it failed to detect differences in biological measures of insulin sensitivity according to clinical outcome.

Our results suggest that clamp-derived hormonal variables may discriminate better between rapid and slow progressors to diabetes than OGTT-derived variables. The clamp test may in this respect benefit from its good reproducibility (see ESM Table 1). In HVs the intra-individual CV for C-peptide AUC was lower than for insulin and approximated to 12%, which is in general better than values obtained with IVGTT or glucagon tests performed in duplicate in smaller groups [19, 31–34]. In line with an ADA workshop report, C-peptide also proved the most appropriate outcome measure in our hands [35, 36]. By performing clamp tests and OGTTs in parallel we were able to calculate disposition indices for most participants, but this variable could not discriminate between progressors and non-progressors in this study.

In conclusion, clamp-derived hormonal variables stratify risk of diabetes in IA-2A-positive relatives. A low C-peptide response during the first phase was invariably associated with rapid progression to diabetes. A decreased response during the second phase may provide an earlier and more sensitive indication for progression to diabetes, although larger studies are needed to confirm this. C-peptide release during the clamp may better discriminate between progres-

sors and non-progressors to diabetes than OGTT-derived C-peptide values. In the light of previous results in individuals with recent-onset type 1 diabetes, relatives with IA-2A, multiple antibodies and decreased C-peptide release during clamp tests emerge as the most likely candidates to participate in immunointervention trials in the preclinical disease phase.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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