CLINICAL STUDY

Subtle hyperproinsulinaemia characterises the defective insulin secretory capacity in offspring of glutamic acid decarboxylase antibody-positive patients with latent autoimmune diabetes mellitus in adults

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Abstract

Objective: We set out to assess whether hyperproinsulinaemia is an early finding in latent autoimmune diabetes in adults (LADA).

Research design and methods: We measured plasma proinsulin and C-peptide responses during a 2-h oral glucose tolerance test (OGTT) and in the hyperglycaemic clamp in 21 normoglycaemic offspring of LADA patients testing positive for glutamic acid decarboxylase antibodies (GADA) or islet cell antibodies (ICA), and in 17 healthy control subjects without a family history of diabetes.

Results: The study groups had comparable areas under the curves of blood glucose, plasma proinsulin, C-peptide and proinsulin/C-peptide in the OGTT. However, the offspring of LADA patients had higher proinsulin/C-peptide in the hyperglycaemic clamp ($P < 0.01$ versus the control group). The offspring of GADA-positive LADA patients ($n = 9$) had higher proinsulin and proinsulin/C-peptide than did the control group in the OGTT ($P < 0.05$ for both comparisons) and in the hyperglycaemic clamp ($P < 0.001$ and $P < 0.05$ respectively). They also had higher proinsulin than the offspring of ICA-positive LADA patients ($n = 12$) ($P < 0.001$) in the hyperglycaemic clamp. The offspring of ICA-positive LADA patients did not clearly show hyperproinsulinaemia during the tests, but they had lower maximal glucose-stimulated insulin secretory capacity than the control group ($P < 0.05$) and the offspring of GADA-positive LADA patients ($P < 0.05$) in the hyperglycaemic clamp.

Conclusions: These results suggested that insulin secretion in the offspring of GADA-positive LADA patients is characterised by subtle defects in the processing of insulin precursors. Furthermore, various proinsulin responses among the offspring of LADA patients with different autoimmune markers provided further evidence that LADA is a heterogeneous disorder.

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Introduction

Latent autoimmune diabetes mellitus in adults (LADA) is a common disorder accounting for 10–15% of diabetes diagnosed in adulthood (1–6). LADA has genetic and metabolic features characteristic of type 1 diabetes (7). A recent study showed that LADA comprises a heterogeneous population of diabetic patients ranging from an aggressive form of β -cell autoimmunity to a more slowly progressing form with features of type 1 diabetes (8). We have previously shown in the offspring of LADA patients that impaired insulin secretion in LADA is familial (9). Interestingly, in that study

impaired insulin secretory capacity was associated with parental history of islet cell antibodies (ICA) but not with glutamic acid decarboxylase antibodies (GADA). These findings support the concept of LADA being ultimately a heterogeneous disorder (8, 10).

High proinsulin concentrations and/or disproportionately increased proinsulin levels relative to insulin and/or C-peptide concentrations are commonly present at the diagnosis of type 1 diabetes (11–14) and are even seen in normoglycaemic first-degree relatives of affected patients (13–19). The significance of hyperproinsulinaemia in the pathogenesis of type 1 diabetes is, however, unclear but earlier studies have suggested

that it may reflect an immunological β -cell insult as a secondary phenomenon (15, 19, 20).

Although increased proinsulin concentrations are a characteristic feature of common type 2 diabetes (21), there is convincing evidence based on studies in first-degree relatives of patients with this disease that hyperproinsulinaemia is not present before the deterioration of the glucose tolerance (22). Consequently, hyperproinsulinaemia in 'common' type 2 diabetes has been considered as a secondary phenomenon due to hyperglycaemia-induced β -cell stress (23).

No studies are available that have investigated whether hyperproinsulinaemia is an early finding in LADA. We therefore measured proinsulin concentrations in normoglycaemic offspring of patients with LADA. We also compared proinsulin responses in offspring of LADA patients in relation to GADA and ICA positivity to further assess the heterogeneity of LADA.

Subjects and methods

Subjects

The subjects for the present study were offspring of the patients who participated in a follow-up study with newly diagnosed type 2 diabetes (24, 25). The formation and representativeness of the study population have been described in detail earlier (24, 25). The probands for the present study were patients with type 2 diabetes who tested positive for ICA (3) or GADA (4) during the follow-up. Exclusion criteria for the selection of the offspring were: (a) diabetes mellitus in both parents or in the offspring, (b) drug treatment or any disease that could potentially modify carbohydrate metabolism, (c) pregnancy, (d) overt psychiatric disease and (e) age under 30 or over 55 years.

Offspring of the probands with GADA or ICA positivity (offspring of LADA patients)

The formation of the study population for the present study has previously been described in detail (9). Briefly, there were 12 probands who tested positive for GADA at the baseline examination (4) and 15 probands who tested positive for ICA at the baseline and/or at the 5-year follow-up examination (3). Three probands were positive for both GADA and ICA and were excluded from this study. Originally, the offspring of LADA patients comprised 36 subjects (9). Proinsulin samples were available from 32 subjects. Only the normoglycaemic offspring of probands with GADA and the normoglycaemic offspring of probands with ICA positivity were included in the present study. Thus, the study groups for the present study comprised 21 normoglycaemic offspring of LADA patients. Nine of these subjects were offspring of five probands with GADA (offspring of GADA-positive LADA patients) and 12 subjects were offspring of four probands with ICA (offspring of ICA-positive LADA patients).

Control group The formation of the control group for the present study has been described in detail earlier (9). Briefly, the control subjects were offspring of individuals with repeatedly normal glucose tolerance according to the World Health Organisation criteria and GADA and ICA negativity (26). Furthermore, the control subjects had to fulfill the following inclusion criteria: (a) age from 30 to 55 years, (b) no diabetes, (c) first-degree relatives without a history of diabetes, (d) no drug treatment or any disease that could potentially modify carbohydrate metabolism and (e) no history of hypertension. Originally, the control group comprised 19 offspring (ten men and nine women) of 12 probands (9). Since plasma proinsulin samples were available from 17 subjects, the control group for the present study comprised 17 subjects.

Study protocol

The subjects were admitted for 2 days to the metabolic ward of the Department of Medicine, Kuopio University Hospital. On the first day a 2-h oral glucose tolerance test (OGTT) was performed after fasting for 12 h followed by a hyperglycaemic clamp. On the second day the hyperinsulinaemic euglycaemic glucose clamp was performed.

The protocol was approved by the Ethics Committee of the University of Kuopio. All subjects gave their informed consent.

OGTT

In a 2-h OGTT (75 g glucose) samples for blood glucose, plasma proinsulin and plasma C-peptide were drawn at 0, 30, 60, 90 and 120 min to evaluate the degree of glucose tolerance and the β -cell response to an oral glucose load.

Hyperglycaemic clamp

At 120 min immediately after the 2-h OGTT the hyperglycaemic clamp was performed (27). Blood glucose was acutely increased to 20 mmol/l by a constant 20% glucose infusion and clamped at 20 mmol/l until 180 min by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals. At 150, 165 and 180 min samples were taken for the measurement of plasma C-peptide. In addition, at 180 min samples for the determination of plasma proinsulin were obtained.

Intravenous glucose tolerance test (IVGTT)

An IVGTT was performed to determine the first phase insulin secretory capacity. At 0800 h after a 12 h overnight fast, an intravenous catheter was placed in the antecubital vein for the infusion of glucose. Another canula for blood sampling was inserted into a wrist vein surrounded by a heated box (40°C). After baseline

blood collection, bolus of glucose (300 mg/kg in a 50% solution) was given (within 30 s) into the antecubital vein to acutely increase the blood glucose level. Samples for the measurement of blood glucose and plasma insulin were drawn at -5, 0, 2, 4, 6, 8 and 10 min.

Euglycaemic clamp

The degree of insulin resistance was evaluated with the euglycaemic hyperinsulinaemic clamp technique (27). A priming dose of insulin infusion (Actrapid (100 IU/ml); Novo Nordisk, Gentofte, Denmark) was administered during the initial 10 min to acutely raise plasma insulin to the desired level, where it was maintained by a continuous insulin infusion at a rate of 80 mU/m² body surface area per min. Blood glucose was clamped at 5.0 mmol/l for the next 180 min by infusing 20% glucose at varying rates according to blood glucose measurements performed at 5-min intervals (mean coefficient of variation of blood glucose was <4% in both study groups and the control group). The data were calculated for each 20-min interval; the mean value for the period from 120 to 180 min was used to calculate the rates of whole body glucose uptake (WBGU).

Assays and calculations

Blood glucose concentrations in the fasting state, during the OGTT and during clamp studies were measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus; Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA). For the determination of plasma immunoreactive insulin, plasma proinsulin and plasma C-peptide, blood was collected into EDTA tubes. After centrifugation, the plasma for the determination of immunoreactive insulin and C-peptide was stored at -20 °C until the analysis. Plasma immunoreactive insulin and C-peptide were determined by RIA (Phadeseeph Insulin RIA 100; Pharmacia Diagnostics AB, Uppsala, Sweden and a ¹²⁵I RIA kit from Incstar Co., Stillwater, MN, USA respectively). Proinsulin concentrations were measured by a non-equilibrium RIA method (Human Proinsulin RIA kit; Linco, St Louis, MO, USA) (28). The polyclonal antibody used in this assay recognises a proinsulin-specific epitope formed by the intact A-chain-C-peptide junction. In this assay, the potency of human insulin and C-peptide is <0.1% of that of proinsulin. Cross-reactivity with des 31,32 proinsulin is 95%. The intra-assay coefficient of variation ranged from 6 to 21% using controls prepared at 5, 50 and 250 pmol/l (29).

The immunoreactive insulin area under the curve (AUC) in the AUC_(IVGTT) and the AUCs of blood glucose, plasma proinsulin, plasma C-peptide and the plasma proinsulin to plasma C-peptide ratio during the OGTT

were calculated by the trapezoidal method. The maximal glucose-stimulated C-peptide secretion during the hyperglycaemic clamp was calculated as mean C-peptide values measured at 150, 165 and 180 min.

Autoantibody tests

The ICA analyses of the probands were performed in the Research Laboratory, Department of Pediatrics, University of Oulu, Oulu, Finland in 1990, while the ICA testing of the offspring was done in the same laboratory in 1997. The ICA assay was performed as previously described in detail (30). The results are expressed in Juvenile Diabetes Foundation units (JDF units) based on a standard curve. The cut-off value for ICA was 5 JDF units. The substrate used in 1997 gave a standard curve identical to that obtained with the substrate used in 1990. The laboratory has participated in the international workshops on the standardisation of the ICA assay with a sensitivity of 100% and a specificity of 98% in the most recent round.

GADA antibody tests for the probands were performed with a radio-immunoprecipitation assay (31) on serum taken at the baseline examination between 1979 and 1981. This method has been described previously in more detail (4). GADA antibody tests for the offspring in the present study were done by a radio-immunoprecipitation method employing ³⁵S-labelled recombinant human GADA₆₅ produced by *in vitro* transcription/translation (32, 33). The *E. coli* clone with full-length human GADA₆₅ cDNA was a kind gift from Dr Allan E Karlsen and Catherine E Grubin, University of Washington (Seattle, WA, USA). The results are expressed as relative units (RU), RU = (sample c.p.m. - mean c.p.m. of three negative controls)/(c.p.m. of a positive internal reference serum - mean c.p.m. of three negative controls) × 100. Antibody levels exceeding 5 RU which represent the mean + 3 s.d. of 296 Finnish healthy control subjects were considered positive. In the Combined Autoantibody Workshop (Orvieto, Italy 1995), the sensitivity of the assay was 75% and the specificity 99%.

Statistical analysis

All calculations were performed with the SPSS for Windows program (SPSS Inc., Chicago, IL, USA). Data are shown as means ± s.e.m. The differences between two groups were analysed by the Mann-Whitney non-parametric test or by the χ^2 test when appropriate. In the subgroup analyses, the differences between the groups were tested by ANOVA for continuous variables and by Mantel-Haenszel's test for dichotomised variables. Only when the *P* value was <0.05 were the two groups compared by Mann-Whitney non-parametric test or by the χ^2 test. The analysis of covariance (ANCOVA) was used to adjust for confounding variables.

Results

Clinical and biochemical characteristics of the study groups

The groups were comparable with respect to age, gender, body mass index and the rates of WBGU (Table 1). The offspring of LADA patients tended to have a lower AUC_(IVGTT) than the control group ($P = 0.056$). Otherwise, there were no differences in fasting blood glucose, fasting plasma proinsulin and C-peptide concentrations between the study groups.

OGTT

There were no significant differences in the AUCs of blood glucose, plasma C-peptide, proinsulin and proinsulin to plasma C-peptide ratio during the OGTT between the control group and the offspring of LADA (Fig. 1).

Hyperglycaemic clamp

There were no significant differences in plasma C-peptide and proinsulin concentrations between the control group and the offspring of LADA patients during the hyperglycaemic clamp (Fig. 2). The offspring of LADA patients had, however, higher proinsulin to plasma C-peptide ratio than did the control group ($P = 0.010$).

Subgroup analyses

Clinical and biochemical characteristics Age, gender, body mass index and the rates of WBGU were comparable in the subgroups of the offspring of LADA patients (Table 1). The offspring of ICA-positive LADA patients had a lower AUC_(IVGTT) than the control group ($P = 0.021$) and the offspring of GADA-positive LADA patients ($P = 0.006$). Fasting blood glucose concentration did not differ significantly among the study groups (ANOVA; $P = 0.11$). The offspring of GADA-positive LADA patients had higher fasting plasma proinsulin and C-peptide concentrations than the control group ($P = 0.034$ and $P = 0.039$ respectively) and the offspring of ICA-positive LADA patients ($P = 0.007$ and $P < 0.001$ respectively), even after adjustment for the rates of WBGU, fasting blood glucose or the family effect (ANCOVA; $P < 0.05$ for all comparisons) (Table 1).

Among the offspring of ICA-positive LADA patients one subject had both ICA and GADA (66 JDF units and 123.5 RU respectively), among the offspring of GADA-positive LADA patients one subject tested positive for GADA (10.0 RU), and among the control subjects one subject tested positive for GADA (13.1 RU).

OGTT There were no significant differences in blood glucose and plasma C-peptide AUCs during the OGTT

Table 1 Characteristics of the study subjects. Values are means $\pm$ S.E.M.

	Controls ($n = 17$)	Offspring of LADA patients ($n = 21$)	P value ^a	Offspring of GADA-positive LADA patients ($n = 9$)	Offspring of ICA-positive LADA patients ($n = 12$)	P value ^b
Age (years)	40.6 $\pm$ 1.4	44.0 $\pm$ 1.1	NS	44.8 $\pm$ 2.0	43.5 $\pm$ 0.9	NS
Gender (M/F)	8/9	11/10	NS	5/4	6/6	NS
Body mass index (kg/m ²)	25.5 $\pm$ 0.9	24.8 $\pm$ 0.6	NS	25.8 $\pm$ 1.2	24.0 $\pm$ 0.5	NS
WBGU (μ mol/kg per min)	60.5 $\pm$ 3.9	63.1 $\pm$ 3.1	NS	58.3 $\pm$ 5.5	66.7 $\pm$ 3.1	NS
AUC _(IVGTT) (pmol min)	3492 $\pm$ 568	2352 $\pm$ 251	0.056	3120 $\pm$ 270	1776 $\pm$ 82†	0.044
Fasting blood glucose (mmol/l)	4.6 $\pm$ 0.1	4.6 $\pm$ 0.1	NS	4.8 $\pm$ 0.1	4.5 $\pm$ 0.1	NS
Fasting plasma proinsulin (pmol)	6.25 $\pm$ 0.48	7.00 $\pm$ 1.04	NS	9.31 $\pm$ 2.05†‡	5.27 $\pm$ 0.69	0.037
Fasting plasma C-peptide (pmol/l)	488 $\pm$ 29	506 $\pm$ 31	NS	608 $\pm$ 48‡§	429 $\pm$ 22	0.004

^aComparisons between the controls and offspring of LADA patients (Mann-Whitney non-parametric test); ^bcomparisons between the controls, offspring of GADA-positive LADA patients and offspring of ICA-positive LADA patients (ANOVA). †The incremental insulin (IRI) AUC during IVGTT.

* $P < 0.05$ offspring of ICA-positive LADA patients versus controls; † $P < 0.01$ offspring of GADA-positive LADA patients versus offspring of ICA-positive LADA patients; ‡ $P < 0.05$ offspring of GADA-positive LADA patients versus controls; § $P < 0.010$ offspring of GADA-positive LADA patients versus offspring of ICA-positive LADA patients. NS, not significant.

Figure 1 Individual AUCs of blood glucose, plasma C-peptide, plasma proinsulin concentrations and the proinsulin to C-peptide ratio during the OGTT. The control group (□) and the offspring of LADA patients (●) are shown. The solid line depicts the mean.

(Fig. 3). The offspring of GADA-positive LADA patients had, however, higher AUCs of plasma proinsulin and proinsulin to plasma C-peptide ratio than the control group ($P = 0.025$ and $P = 0.029$, respectively). These differences persisted even after adjustment for WBGU and the family effect (ANCOVA; $P < 0.05$ for all analyses).

Hyperglycaemic clamp The offspring of ICA-positive LADA patients had lower plasma C-peptide concentration than the offspring of GADA-positive LADA patients ($P = 0.034$) and the control group ($P = 0.052$) during the hyperglycaemic clamp (Fig. 4). On the other hand, the offspring of GADA-positive LADA patients had higher plasma proinsulin concentrations compared

Figure 2 Individual maximal glucose-stimulated plasma C-peptide, plasma proinsulin concentrations and the plasma proinsulin to C-peptide ratio in the hyperglycaemic clamp. ** $P = 0.01$ between study groups. The control group (□) and the offspring of LADA patients (●) are shown. The solid line represents the mean.

Figure 3 Individual AUCs of blood glucose, plasma C-peptide, plasma proinsulin concentrations and the proinsulin to C-peptide ratio in the two subgroups of offspring of LADA patients during the OGTT. The control group (□), the offspring of GADA-positive LADA patients (▲) and the offspring of ICA-positive LADA patients (▼) are shown. The control subject testing positive for GADA (○), the offspring of a GADA-positive LADA patient testing positive for GADA (△) and the offspring of an ICA-positive LADA patient testing positive for both GADA and ICA (▽) are shown. * $P < 0.05$, offspring of GADA-positive LADA patients versus the control group. The solid line represents the mean.

Figure 4 Individual maximal glucose stimulated plasma C-peptide, plasma proinsulin concentrations and the proinsulin to C-peptide ratio in the two subgroups of offspring of LADA patients in the hyperglycaemic clamp. The control group (□), the offspring of GADA-positive LADA patients (▲) and the offspring of ICA-positive LADA patients (▼) are shown. The control subject testing positive for GADA (○), the offspring of a GADA-positive LADA patient testing positive for GADA (△) and the offspring of an ICA-positive LADA patient testing positive for both GADA and ICA (▽) are shown. † $P < 0.05$, offspring of ICA-positive LADA patients versus the control group, * $P < 0.05$ and *** $P < 0.001$, offspring of GADA-positive LADA patients versus the control group and † $P < 0.05$ and †† $P < 0.001$, offspring of GADA-positive LADA patients versus offspring of ICA-positive LADA patients. The solid line depicts the mean.

with those of the offspring of ICA-positive LADA patients ($P < 0.001$) or the control group ($P < 0.001$). Furthermore, they had a higher proinsulin to plasma C-peptide ratio than the control group ($P = 0.013$).

Discussion

The novel finding of our study was that the normoglycaemic offspring of LADA patients, although having quite normal plasma proinsulin responses after an oral glucose load, showed disproportionately increased plasma proinsulin concentrations in the hyperglycaemic clamp. These results suggested that the impaired insulin secretory capacity in the offspring of LADA patients (9) is characterised by a subtle defect in the processing of insulin precursors, unlike in the offspring of patients with type 2 diabetes (34). On the other hand, although hyperproinsulinaemia was not so profound as described in first-degree relatives of patients with type 1 diabetes (13–19) it supports the concept that LADA has metabolic features typical of classical type 1 diabetes. Moreover, hyperproinsulinaemia was observed only in the offspring of GADA-positive LADA patients, providing further evidence that LADA is a heterogeneous disorder (8, 10).

Hyperproinsulinaemia in subjects genetically predisposed to type 1 diabetes is considered to be secondary to a previous or ongoing attack on the β -cell (15, 19, 20), although an inherent, non-autoimmune defect in the cleavage process of insulin precursors (16, 35, 36) or decreased metabolic clearance of insulin (37, 38) cannot be excluded. Given that LADA comprises a sub-type of type 1 diabetes, patients with LADA and their first-degree relatives should also have hyperproinsulinaemia. In the present study, the normoglycaemic offspring of LADA patients had disproportionately increased proinsulin concentrations when challenged with high blood glucose in the hyperglycaemic clamp. We have previously shown that the normoglycaemic offspring of autoantibody-negative patients with type 2 diabetes have normal proinsulin responses not only after an oral glucose load but also during the hyperglycaemic clamp, suggesting that hyperproinsulinaemia is not an early feature in common type 2 diabetes (34). Hyperproinsulinaemia in type 2 diabetes has been considered to develop secondary to β -cell stress induced by prolonged hyperglycaemia (23). Therefore, the present study suggested that the mechanisms of impaired insulin secretory capacity in LADA are closer to type 1 than type 2 diabetes.

Although the offspring of LADA patients did not show significant hyperproinsulinaemia at physiological glucose concentrations, proinsulin responses were heterogeneous between these subjects. We have previously reported that defects in insulin secretory capacity in these offspring of LADA patients were associated with

a parental history of ICA positivity but not of GADA positivity (9). These results were somewhat unexpected since autoantibodies to GAD have been considered to be an even better predictor of progression to insulin dependency than are ICA in LADA patients (39, 40). In the present study, the offspring of GADA-positive LADA patients, although having quite normal insulin secretory capacity, showed increased plasma proinsulin levels both absolutely and relatively to plasma C-peptide after an oral glucose load and in the hyperglycaemic clamp. The offspring of ICA-positive LADA patients, on the other hand, had impaired first-phase and maximal glucose-stimulated insulin secretory capacity, but showed quite normal plasma proinsulin responses both in the OGTT and the hyperglycaemic clamp. These findings imply that GADA positivity in LADA reflects β -cell autoimmunity similar to classical type 1 diabetes whereas ICA positivity in LADA may be, at least partly, a secondary phenomenon.

Interestingly, the offspring of GADA-positive LADA patients had increased fasting C-peptide concentrations and somewhat higher fasting blood glucose concentration than the offspring of ICA-positive LADA patients, even after adjustment for their WBGU. This implies that these subjects have increased hepatic glucose output. It is possible that an autoimmune insult in the β -cells leads to disturbed pulsatile/oscillatory insulin secretion and subsequent impaired inhibition of hepatic glucose output. Consequently, the hyperproinsulinaemia in these subjects may be secondary to mild hyperglycaemia. Alternatively, it is possible that an autoimmune insult in the β -cells leads simultaneously to both a defective processing of proinsulin and disturbed pulsatile/oscillatory insulin secretion. Finally, a genetic defect in the cleavage process of insulin precursors without any autoimmune attack on the β -cells cannot be excluded.

Despite clearly reduced first-phase and maximal glucose-stimulated insulin secretory capacity, the normoglycaemic offspring of ICA-positive LADA patients had quite normal plasma C-peptide insulin and blood glucose responses after an oral glucose load. It is therefore possible that their impaired glucose-stimulated insulin secretion is compensated by an increased incretin effect. The impaired glucose-stimulated insulin secretory capacity in these subjects may therefore reflect a reduced quantitative or functional β -cell mass due to a non-autoimmune inherited defect in glucose-stimulated insulin secretory mechanism which could result in slow β -cell destruction and to the formation of antibodies as a secondary event (10). Nonetheless, although the plasma proinsulin response in these subjects was similar to that reported in normoglycaemic first-degree relatives of patients with type 2 diabetes, their insulin secretory capacity differed from that seen in family members of patients affected by type 2 diabetes (41, 42), implying that ICA positivity in LADA patients may represent a specific entity.

We have concluded that the impaired insulin secretory capacity seen in the offspring of LADA patients is characterised by a defect in the processing of proinsulin and could reflect an autoimmune attack on the β -cell in these subjects. However, heterogeneous plasma proinsulin responses among the offspring of LADA patients suggested that LADA is a heterogeneous disorder likely reflecting differences in β -cell function among relatives of LADA patients. These findings have provided additional evidence for the heterogeneity of diabetes mellitus, particularly in relation to phenotypes characterised by impaired insulin secretion.

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