Endocrine
https://doi.org/10.1007/s12020-020-02287-4

ORIGINAL ARTICLE

Diagnosis of Flier's syndrome in a patient with nondiabetic hypoglycemia: a case report and critical appraisal of the literature

Ilaria Cimmino[1] · Antongiulio Faggiano[2] · Giuseppe Perruolo[1] · Roberta Modica[3] · Filomena Bottiglieri[3] · Bianca Covelli[1] · Annamaria Colao[3] · Francesco Beguinot[1] · Pietro Formisano[1] · Francesco Oriente[1]

Received: 12 December 2019 / Accepted: 25 March 2020

Abstract

Purpose Autoimmune hypoglycemia includes rare syndromes characterized by the presence of either anti-insulin antibodies (IAA) (Hirata's disease) or anti-insulin receptor (anti-ISR) antibodies (Flier's syndrome). Diagnosis is usually based on identification of the specific antibodies, in presence of the Whipple triad. However, most of these cases are classified as idiopathic diseases due to the difficulty to define the pathogenic culprit.

Methods Basic research methodologies, including Western Blot and ELISA tests, have been used in this study.

Results We describe a 21-year-old young woman (PT), non-obese and non-diabetic, with a positive history of autoimmune diseases, admitted to the hospital for recurrent episodes of severe symptomatic hypoglycemia. Counterregulatory response to hypoglycemia was normal as well as the fasting test, so excluding both hormone deficiencies and insulinoma. Since an autoimmune hypoglycemic syndrome was suspected, the hyperactivation of the insulin pathway was experimentally evaluated. At this purpose, human hepatocarcinoma (HepG2) cells were incubated with serum obtained from the patient (PT) and from control individuals. Interestingly, a significant increase of phosphorylation of insulin receptor, Akt, and ERK1/2 was observed in the HepG2 cells incubated with PT serum compared with the controls. ELISA tests revealed significantly increased levels of anti-ISR antibodies in PT serum, while IAA were similar both in PT and in control sera, supporting diagnosis of Flier's syndrome.

Conclusions This study emphasizes the importance to identify new strategies for the differential diagnosis of hypoglycemia, not always possible with the routinely used diagnostic tests.

Keywords Hypoglycemia · Flier's syndrome · Hirata's disease · Autoimmune disease · Insulin signaling

Introduction

Hypoglycemia is a metabolic disorder characterized by reduced plasma glucose levels, which may induce several symptoms and clinical signs, including anxiety, irritability, hunger, confusion, visual disturbances, loss of consciousness, and other cognitive dysfunction due to the poor intake of glucose by brain. The diagnostic investigations for the identification of causes of hypoglycemia are recommended in patients with the Whipple's triad: symptoms of hypoglycemia, evidence of low plasma glucose (<55 mg/dL) concentration, and resolution of symptoms after glucose administration [1, 2]. A precise clinical evaluation is essential to find the correct treatment. The most frequent causes of low blood sugar levels are related to hypoglycemic drugs. Other causes are represented by: insulinoma, nesidioblastosis, post gastric bypass hypoglycemia, autoimmune disorders, and other less common diseases.

These authors contributed equally: Ilaria Cimmino, Antongiulio Faggiano,Giuseppe Perruolo

✉ Pietro Formisano
fpietro@unina.it

[1] Department of Translational Medicine, Federico II University of Naples and URT "Genomic of Diabetes" of Institute of Experimental Endocrinology and Oncology, National Council of Research (CNR), Naples, Italy

[2] Department of Experimental Medicine, Sapienza University, Rome, Italy

[3] Department of Clinical Medicine and Surgery, Federico II University of Naples, Naples, Italy

Autoimmune forms of hypoglycemia are rare syndromes usually misdiagnosed as idiopathic due to the lack of validated diagnostic methods. The main two forms are the Hirata's disease, which is more common and is characterized by the presence of autoantibodies directed against endogenous insulin (IAA) [3], and the Flier's syndrome, characterized by the presence of autoantibodies directed against the cell surface insulin receptor (anti-ISR) [4]. Both conditions can be associated with normal or high insulin levels and postprandial and fasting hypoglycemia.

Diagnosis of autoimmune hypoglycemia is complex because methods to allow identification of specific autoantibodies are not widely available. Therefore, besides Whipple's triad, it is necessary to evaluate the presence of anti-insulin or anti-insulin receptor (anti-ISR) antibodies.

We report a clinical case of a young woman with frequent symptomatic hypoglycemic episodes with normal insulin levels due to anti-ISR antibodies (Flier's syndrome), detected with methodologies generally used in basic research.

Materials and methods

Materials

Media, sera, and antibiotics for cell culture were all from Invitrogen (Grand Island, NY, USA). IR and pIR antibodies were from Cell Signaling Technology (Beverly, MA, USA). pAkt, Akt, ERK1/2, and 14.3.3θ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pERK1/2 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA, USA). Western blotting and ECL reagents were purchased from Amersham Biosciences (Arlington Heights, IL, USA).

Collection of serum samples and hormone tests

Blood samples from patient (PT) and two control individuals (Ctrl1 and Ctrl2) were collected in serum tubes with separator gel and centrifuged at 2500 rpm for 10 min. Insulin and C-peptide were measured by chemiluminescent immunoassay (CLIA) on the ADVIA Centaur analyzers (Siemens). ACTH was evaluated by CLIA on the IMMULITE 2000 analyzers (Siemens). GH was measured by CLIA on the LIAISON analyzers (DiaSorin).

Oral glucose tolerance test (OGTT)

After oral intake of a 75 g glucose solution blood samples are drawn at 0, 30, 60, 90, and 120 min to measure serum glucose (normal value 70–110 mg/dL), insulin (normal value 1–20 µU/mL), and C-peptide (normal value 0.5–3 ng/mL).

ACTH test

The corticotropin test is performed measuring cortisol levels before, 30 and 60 min after intravenous (iv) administration of 250 µg corticotropin as bolus injection to establish the diagnosis of adrenal insufficiency. Peak cortisol levels below 500 nmol/L indicate adrenal insufficiency. Q5

GHRH+arginine test

After administration of GHRH analog (1 µg/kg) as an iv bolus, and arginine hydrochloride 30 g simultaneously as an iv infusion in normal saline from 0–30 min. Serum GH levels are measured at 30 and 60 min. Peak serum GH levels <11.0 µg/L at every time point during testing in patients with BMI <25 kg/m^2 is diagnostic of adult GH deficiency.

72 h fasting test

During a fast of up to 72 h, detection of symptoms, signs of hypoglycemia or both with plasma glucose <55 mg/dL, insulin of at least 3.0 µU/mL, C-peptide of at least 0.6 ng/mL, and proinsulin of at least 5.0 pmol/liter document endogenous hyperinsulinism.

Cell culture

Human hepatocellular carcinoma (HepG2) cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 2% L-glutamine, 10,000 u/mL penicillin, and 10,000 µg/mL streptomycin in humidified 95% air and 5% CO_2 atmosphere (all v/v).

Western blot analysis and ELISA assay

Total cell lysates were obtained and separated by SDS-PAGE. Briefly, cells were solubilized with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, pH 7.4, and 1% (v/v) Triton X-100. Lysates were clarified by centrifugation at 12,000 g for 20 min at 4 °C. The protein concentrations in the cell lysates were measured using a Bio-Rad DC (detergent compatible) assay. Western blot analysis was performed as previously described [5].

Anti-insulin antibodies (IAA) or anti-ISR antibodies were determined by ELISA (Enzyme-Linked ImmunoSorbent Assay) methods (Cloud-Clone Corp, Katy, TX, USA), according to the manufacturer's instructions.

Statistical procedures

Data were analyzed with the GraphPad Prism 7.0 (GraphPad Inc., San Diego, CA, USA) by unpaired two-tailed Student's *t* test. *P* values equal or less than 0.05 were considered statistically significant.

Results

A 21-year-old woman was admitted to the Endocrinology Unit of the University-Hospital of Naples Federico II because of frequent episodes of symptomatic, mostly fasting hypoglycemia. The onset of symptoms, diplopia, confusion, sweating, tachycardia, and tremor occurred in the previous 3 months. Her family history was positive for autoimmune disease and she was affected by Hashimoto thyroiditis since the age of 18. Physical examination revealed a well-appearing woman with normal vital signs (arterial pressure 120–70 mmHg, heart rate 80 bpm), rhythmic cardiac activity, and body mass index (BMI) 22.12 kg/m^2. She did not take any medication. During hospitalization hypoglycemia was biochemically confirmed with normal basal insulin levels; other routine biochemical tests were within the normal range, as well as thyroid function tests. OGTT revealed high insulin levels with normal C-peptide. The ACTH test for cortisol and GHRH + arginine test for GH excluded a deficiency of counter-regulatory hormones but they did not clarify the cause of hypoglycemia. Then, the prolonged fasting test was performed and stopped after 24 h, due to severe clinical and biochemical hypoglycemia (37 mg/dL), which was associated with normally suppressed insulin and C-peptide levels, so excluding the diagnosis of insulinoma (Table 1). These results, together with the personal and familial history of autoimmune diseases, lead to suspect an autoimmune cause of hypoglycemia. Therefore, we evaluated "in vitro" the key insulin-regulated signals. To this aim, HepG2 cells were incubated for 10 min with serum obtained by the patient (PT) and two control individuals (Ctrl1 and Ctrl2) having similar insulin levels. Experiments were performed with 2.5 and 10% serum.

Total and phosphorylation levels of insulin receptor (IR), Akt, and ERK1/2 were analyzed by Western blot with specific antibodies. As shown in Fig. 1, the amount of phosphorylated IR, Akt, and ERK1/2 was 25%, 50%, and 42% higher after incubation of HepG2 cells with 2.5% PT serum than the Ctrl1 and Ctrl2 sera at same concentration. In presence of 10% PT serum, we observed a 92%, 75%, and 40% increase of IR, Akt, and ERK1/2 phosphorylation, respectively, compared with the control sera. In parallel, time-course experiments performed at 10, 30, and 60 min upon incubation with 10% PT serum revealed a statistically significant increase of pIR at 10 and 30 min, while ERK1/2 phosphorylation was induced by serum at 10 min compared with the controls. pAkt also showed a tendency to increase, albeit difference did not reach statistical significance (Fig. 2). These effects were comparable with that of 100 nM insulin-stimulated cells for 10 min. Both in dose-response and time-course experiments, the total protein levels of IR, Akt, and ERK1/2 did not change. Next, titles of IAA and anti-ISR antibodies, typical of Hirata's disease and Flier's syndrome, respectively, were measured in different controls ($n = 3$) and in the patient sera, by using the ELISA method. As shown in Fig. 3, no difference in the titles of IAA was observed between the control and PT sera. On the contrary, the titles of anti-ISR antibodies in the PT serum were significantly higher than the controls.

Therapy with prednisone 60 mg daily was started with remission of hypoglycemic episodes.

Discussion

Autoimmune hypoglycemia is a rare disease usually misdiagnosed as idiopathic due to the lack of validated diagnostic methods. The major two forms are the Hirata's disease and Flier's syndrome described in 1994 and 1975, respectively [3, 4, 6].

Hirata's disease is characterized by hyperinsulinemic hypoglycemia, high titles of IAA, absence of previous exposure to exogenous insulin, and absence of pathological changes of pancreatic islets. This disease can occur at any age, without no difference between genders. The mechanism of hypoglycemia in Hirata's disease is not fully known and probably two mechanisms are involved in the onset of this disease. Initially, in the immediate postprandial period, the autoantibody binds immediately to insulin, and, subsequently, insulin dissociates from the complex with IAA, causing hypoglycemia [7].

Flier's syndrome, also known as type B insulin resistance, is a disease, more common in women, characterized

Table 1 Patient's fasting test

	0	6 h	12 h	18 h	24 h
Serum glucose	69 mg/dL	72 mg/dL	65 mg/dL	43 mg/dL	37 mg/dL
Insulin	8.6 µU/mL	3.3 µU/mL	<2 µU/mL	<2 µU/mL	<2 µU/mL
C-Peptide	1.8 ng/mL	1.2 ng/mL	0.5 ng/mL	0.3 ng/mL	0.3 ng/mL

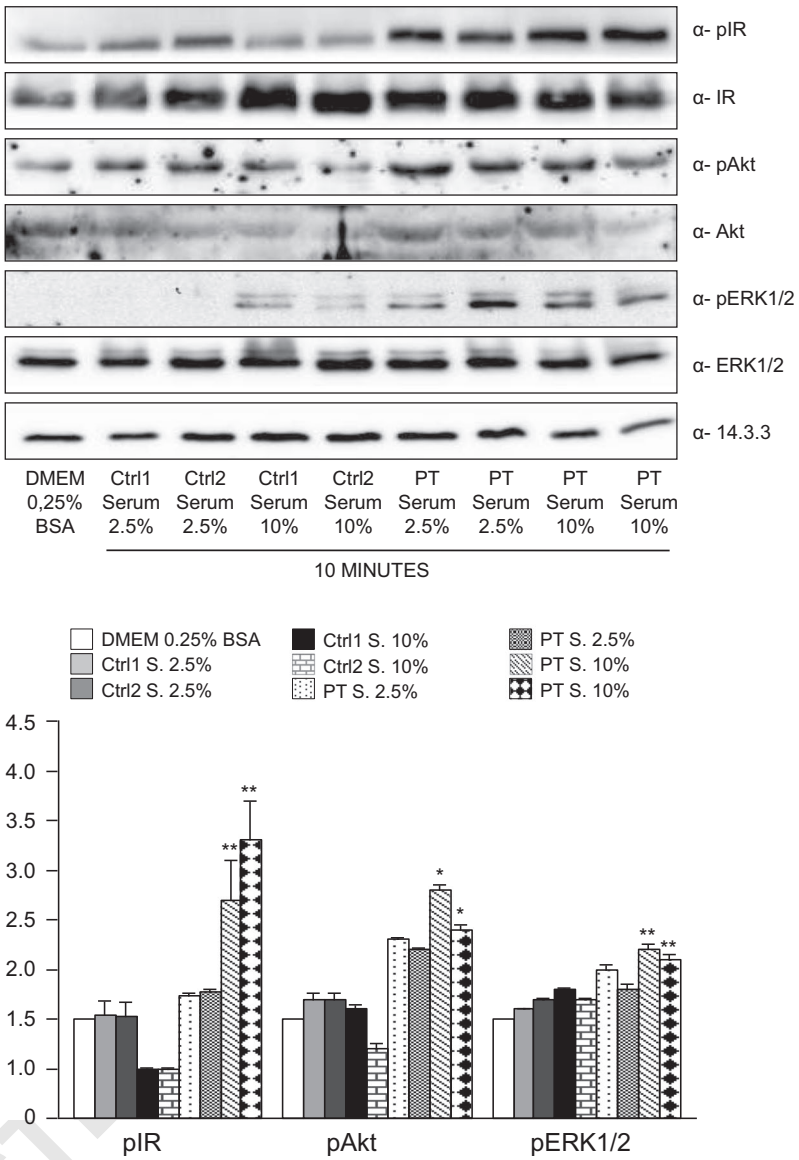

Fig. 1 Protein levels of the main insulin signaling mediators in HepG2 cells stimulated at different serum concentrations for 10 min. HepG2 cells were stimulated with the control and patient sera at 2.5 or 10% concentration. Protein levels were analyzed by Western blot using antibodies for pIR, pAkt, Akt, pERK1/2, ERK1/2, and 14.3.3, which was used for normalization. Blots were detected by ECL and the autoradiographs shown are representative of three different experiments and subjected to densitometric analysis. Asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$)

by the presence of serum anti-ISR antibodies [6]. Clinical features are variable and may include typical conditions of insulin-resistance such as type 2 diabetes and acanthosis nigricans. Anti-ISR antibodies are able to recognize peripheral insulin receptors, which generally inhibit their signaling. In some cases, however, these antibodies may exert insulin-like activity, sometimes so powerful, to justify deaths from hypoglycemic crises [8, 9]. Furthermore, it has been hypothesized that some of the antibodies directed specifically against insulin receptors may cross-react with epidermal growth factor receptors, which, in part, explain the presence of the acanthosis nigricans in these patients. In addition, one third of the patients with Flier's syndrome can develop other autoimmune disorders such as systemic lupus erythematosus, Sjögren's syndrome, ataxia-telangiectasia, vitiligo, alopecia, and autoimmune hypothyroidism [10]. Our patient did not show acanthosis nigricans but had a personal and familial history of autoimmune diseases.

The crucial role of the interaction between insulin and its receptor in the pathogenesis of rare cases of hypoglycemia was supposed in the first report of insulin receptors disorders in 1976 [11]. Soon after it became evident that in patients with hypoglycemia due to insulin receptors antibodies the clinical course could be fluctuating [12]. Nevertheless, literature data are scarce due to the rarity of the disease. In a recent nationwide survey in Japan, where insulin autoimmune syndromes are the third leading cause of hypoglycemia, after insulinoma and extrapancreatic tumors, 30 cases of Flier's syndrome were identified by questionnaires sent to 1957 academic councilors or responsible individuals at certified facilities [13]. The syndrome was diagnosed most frequently in older individuals

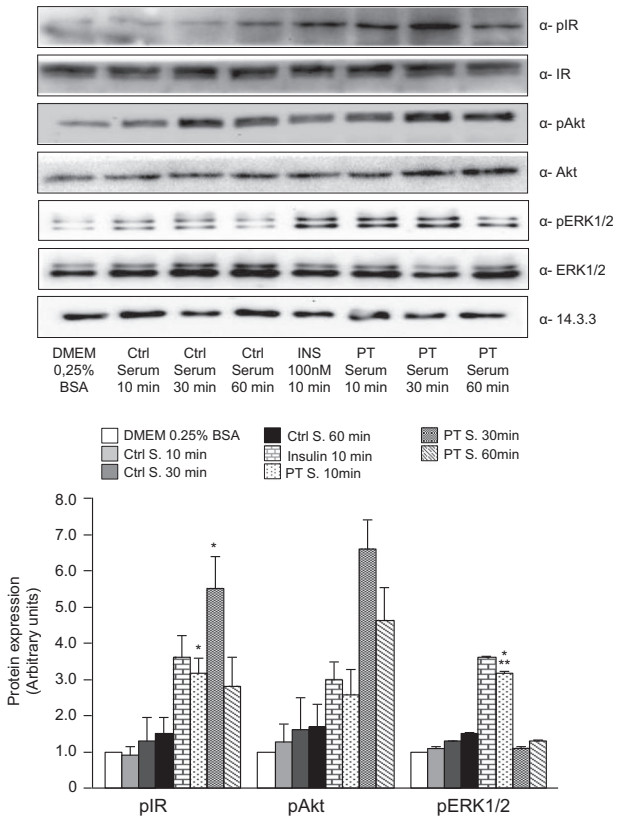

Fig. 2 Protein levels of the main insulin signaling mediators in HepG2 cells stimulated at different serum concentrations for 10 min. HepG2 cells were stimulated with the control and patient sera at 10,30, and 60 min. Protein levels were analyzed by Western blot using antibodies for pIR, pAkt, Akt, pERK1/2, ERK1/2, and 14.3.3, which was used for normalization. Blots were detected by ECL and the autoradiographs shown are representative of three different experiments and subjected to densitometric analysis. Asterisks indicate statistically significant differences (*$p < 0.05$; ***$p < 0.001$)

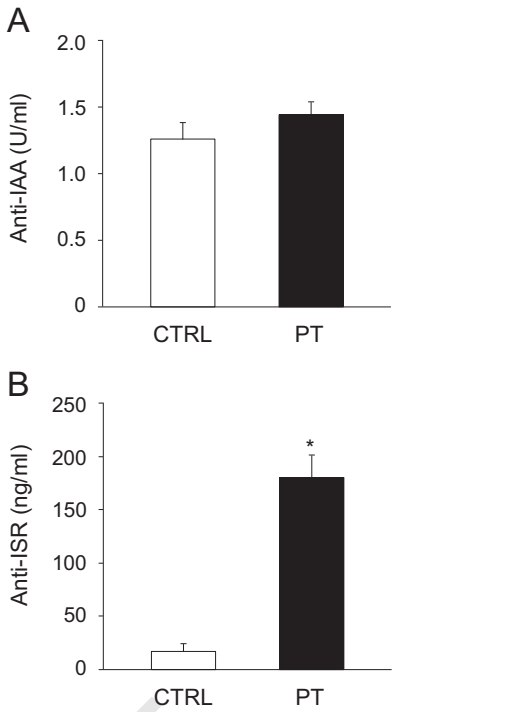

Fig. 3 Anti-insulin antibody (IAA) and anti-insulin receptor (Anti-ISR) assays. Levels of anti-insulin antibody (IAA) (A) and anti-insulin receptor (Anti-ISR) (B) were measured in different control sera ($n = 3$) and in that of the patient being tested, using the ELISA assays. The bars represent the mean ± SD of three independent experiments, in which each is performed in duplicate. Asterisks indicate statistically significant differences (*$p < 0.05$). Detection interval: 3.12–200 ng/mL for Anti-ISR and 0–2.4 U/mL for IAA

and was often associated with other autoimmune diseases. Interestingly, hypoglycemia represented the trigger for diagnosis in 23% of patients and it was found that 60% of these patients experienced hypoglycemia. Hypoglycemia represents a relevant issue not only in diagnosis of this syndrome, but even during its clinical course, as the death of a patient due to hypoglycemia was reported [13]. These findings highlight that Flier's syndrome is frequently not recognized or even misdiagnosed. The lack of standard and reliable diagnostic criteria is cause of late diagnosis and impaired quality of life in these patients. Different treatments are reported including glucocorticoid, immune suppressant, immunoglobulin, and treatment for diabetes too [6, 13]. Low dose of glucocorticoids, as in our patient, have been reported to be effective in a 57-year-old male, whose anti-ISR antibodies became undetectable after 30 months of treatment [14]. Studies in patients with extreme insulin resistance and acanthosis nigricans were performed to obtain new insights in structure and function of the insulin receptor [15], but collecting data about both clinical and molecular aspects of the syndrome is still of utmost importance to improve knowledge and healthcare.

We now describe that insulin levels did not increase during the fasting test and hyperglycemia was never detected in the proband (Table 1). To achieve the correct diagnosis, an "in vitro" study was performed on the serum of the patient to evaluate the presence of some insulin-like factors capable of enhancing insulin signaling. Although laboratory conditions cannot always be directly transferred to normal clinical practice, this basic biomedical approach has been already used in different pathologies to overcome the limitations of clinical observations and to achieve a correct diagnosis [16–19]. Thus, HepG2 cells were stimulated with the patient's serum. Based on the obtained results, it was possible to observe that the cells treated with the patient's serum showed hyperactivation of insulin signaling, despite having the same amount of insulin, compared with the controls. Next, since family's history revealed the presence of autoimmune diseases, ELISA tests were then performed to determine the presence of the IAA and anti-ISR antibodies. Results indicated the presence of anti-ISR antibodies, but not IAA, in the patient, suggesting

that she could be affected by Flier's syndrome. Hence, the patient was effectively treated with low dose glucocorticoids.

In conclusion, this report suggests the importance of personalized diagnostic approaches, by taking in account techniques usually used in basic research, to prevent and care diseases for which there are no validated clinical methods.

Acknowledgements This research was supported by grant from Associazione Italiana per la Ricerca sul Cancro - AIRC (IG19001).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ehtical approval This study does not contain any studies with animals performed by any of the authors.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. P.E. Cryer, L. Axelrod, A.B. Grossman, S.R. Heller, V.M. Montori, E.R. Seaquist, F.J. Service, S. Endocrine, Evaluation and management of adult hypoglycemic disorders: an Endocrine Society Clinical Practice Guideline. J. Clin. Endocrinol. Metab. **94** (3), 709–728 (2009). https://doi.org/10.1210/jc.2008-1410
2. M.L. Virally, P.J. Guillausseau, Hypoglycemia in adults. Diabet. Metab. **25**(6), 477–490 (1999)
3. Y. Hirata, Y. Uchigata, Insulin autoimmune syndrome in Japan. Diabet. Res. Clin. Pr. **24**(Suppl), S153–S157 (1994). https://doi.org/10.1016/0168-8227(94)90243-7
4. J.S. Flier, C.R. Kahn, J. Roth, R.S. Bar, Antibodies that impair insulin receptor binding in an unusual diabetic syndrome with severe insulin resistance. Science **190**(4209), 63–65 (1975). https://doi.org/10.1126/science.170678
5. S. Ricci, D. Viggiano, I. Cimmino, G. Perruolo, S. Cabaro, A. Liotti, F. Fiory, R. Spinelli, A. Di Carlo, F. Beguinot, P. Formisano, F. Oriente, Prep1 deficiency affects olfactory perception and feeding behavior by impairing bdnf-trkb mediated neurotrophic signaling. Mol. Neurobiol (2018) https://doi.org/10.1007/s12035-018-0873-7
6. S. Censi, C. Mian, C. Betterle, Insulin autoimmune syndrome: from diagnosis to clinical management. Ann. Transl. Med **6**(17), 335 (2018). https://doi.org/10.21037/atm.2018.07.32
7. M.V. Davi, A. Pia, V. Guarnotta, G. Pizza, A. Colao, A. Faggiano, N. Group, The treatment of hyperinsulinemic hypoglycaemia in adults: an update. J. Endocrinol. Investig. **40**(1), 9–20 (2017). https://doi.org/10.1007/s40618-016-0536-3
8. S.I. Taylor, G. Grunberger, B. Marcus-Samuels, L.H. Underhill, R.F. Dons, J. Ryan, R.F. Roddam, C.E. Rupe, P. Gorden, Hypoglycemia associated with antibodies to the insulin receptor. N. Engl. J. Med. **307**(23), 1422–1426 (1982). https://doi.org/10.1056/NEJM198212023072303
9. D.L. Willard, M. Stevenson, D. Steenkamp, Type B insulin resistance syndrome. Curr. Opin. Endocrinol. Diabet. Obes. **23**(4), 318–323 (2016). https://doi.org/10.1097/MED.0000000000000263
10. S.L. Wong, A. Priestman, D.T. Holmes, Recurrent hypoglycemia from insulin autoimmune syndrome. J. Gen. Intern. Med. **29**(1), 250–254 (2014). https://doi.org/10.1007/s11606-013-2588-9
11. C.R. Kahn, J.S. Flier, R.S. Bar, J.A. Archer, P. Gorden, M.M. Martin, J. Roth, The syndromes of insulin resistance and acanthosis nigricans. Insulin-receptor disorders in man. N. Engl. J. Med. **294**(14), 739–745 (1976). https://doi.org/10.1056/NEJM197604012941401
12. J.S. Flier, R.S. Bar, M. Muggeo, C.R. Kahn, J. Roth, P. Gorden, The evolving clinical course of patients with insulin receptor autoantibodies: spontaneous remission or receptor proliferation with hypoglycemia. J. Clin. Endocrinol. Metab. **47**(5), 985–995 (1978). https://doi.org/10.1210/jcem-47-5-985
13. T. Takeuchi, Y. Ishigaki, Y. Hirota, Y. Hasegawa, T. Yorifuji, H. Kadowaki, T. Akamizu, W. Ogawa, H. Katagiri, Clinical characteristics of insulin resistance syndromes: a nationwide survey in Japan. J. Diabet. Investig. (2019) https://doi.org/10.1111/jdi.13171
14. M. Kotani, N. Tamura, T. Inoue, I. Tanaka A case of type B insulin resistance syndrome treated with low-dose glucocorticoids. Endocrinol. Diabet. Metab. Case Rep. (2019) https://doi.org/10.1530/EDM-19-0115
15. M. Kasuga, Structure and function of the insulin receptor-a personal perspective. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. **95**(10), 581–589 (2019). https://doi.org/10.2183/pjab.95.039
16. B. Rohrig, J.B. du Prel, D. Wachtlin, M. Blettner, Types of study in medical research: part 3 of a series on evaluation of scientific publications. Dtsch. Arztebl. Int. **106**(15), 262–268 (2009). https://doi.org/10.3238/arztebl.2009.0262
17. M. Takahashi, T. Tanaka, H. Takahashi, Y. Hoshino, S. Nagashima, Jirintai, H. Mizuo, Y. Yazaki, T. Takagi, M. Azuma, E. Kusano, N. Isoda, K. Sugano, H. Okamoto, Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. J. Clin. Microbiol. **48** (4), 1112–1125 (2010). https://doi.org/10.1128/JCM.02002-09
18. A.I. Jaura, G. Flood, H.C. Gallagher, D.J. Buggy, Differential effects of serum from patients administered distinct anaesthetic techniques on apoptosis in breast cancer cells in vitro: a pilot study. Br. J. Anaesth. **113**(Suppl 1), i63–i67 (2014). https://doi.org/10.1093/bja/aet581
19. A. Lernmark, J. Sehlin, I.B. Taljedal, H. Kromann, J. Nerup, Possible toxic effects of normal and diabetic patient serum on pancreatic B-cells. Diabetologia **14**(1), 25–31 (1978). https://doi.org/10.1007/bf00429704

Journal : **12020**
Article : **2287**

Springer
the language of science

Author Query Form

Please ensure you fill out your response to the queries raised below and return this form along with your corrections

Dear Author

During the process of typesetting your article, the following queries have arisen. Please check your typeset proof carefully against the queries listed below and mark the necessary changes either directly on the proof/online grid or in the 'Author's response' area provided below

Queries	Details Required	Author's Response
AQ1	Please check and confirm that the authors names (first and surname)/order of authors/affiliation have been identified correctly.	
AQ2	Author surnames have been highlighted - please check these carefully and indicate if the first name or surname have been marked up incorrectly. Please note that this will affect indexing of your article, such as in PubMed.	
AQ3	Note that the eproof should be amended in only one browser window at any one time; otherwise changes will be overwritten.	
AQ4	Please check your article carefully, coordinate with any co-authors and enter all final edits clearly in the eproof, remembering to save frequently. Once corrections are submitted, we cannot routinely make further changes to the article.	
AQ5	Please check the sentence 'The corticotropin test is performed measuring cortisol levels before...' for clarity.	