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The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: May 03, 2018
Accepted: July 26, 2018
First Online: July 31, 2018

Insulin-Binding Antibodies: Assessment & Management

Assessment and Management of Anti-insulin Autoantibodies in Varying Presentations of Insulin Autoimmune Syndrome

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Received 03 May 2018. Accepted 26 July 2018.

Context: Insulin autoimmune syndrome (IAS), spontaneous hyperinsulinemic hypoglycemia due to insulin-binding autoantibodies, may be difficult to distinguish from tumoral or other forms of hyperinsulinemic hypoglycemia including surreptitious insulin administration. No standardized treatment regimen exists.
Objectives: To evaluate an analytic approach to IAS and responses to different treatments.
Design and Setting: Observational study in the UK Severe Insulin Resistance Service.
Patients: 6 patients with hyperinsulinemic hypoglycemia and detectable circulating anti-insulin antibody (IA).
Main outcome measures: Glycemia, plasma insulin and C-peptide concentrations by immunoassay or mass spectrometry (MS). Immunoreactive insulin was determined in the context of polyethylene glycol (PEG) precipitation and gel filtration chromatography (GFC). IA quantification using enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), and IA were further characterized using radioligand binding studies.
Results: All patients were diagnosed with IAS (5 IgG, 1 IgA) based on high insulin:C-peptide ratio, low insulin recovery after PEG precipitation, and GFC evidence of antibody-bound insulin. Neither ELISA nor RIA result proved diagnostic for every case. MS provided

a more robust quantification of insulin in the context of IA. 1 patient was managed conservatively, 4 were treated with diazoxide without sustained benefit, and 4 were treated with immunosuppression with highly variable responses. IA affinity did not appear to influence presentation or prognosis.
Conclusions: IAS should be considered in patients with hyperinsulinemic hypoglycemia and a high insulin:C-peptide ratio. Low insulin recovery on PEG precipitation supports the presence of insulin-binding antibodies, with GFC providing definitive confirmation. Immunomodulatory therapy should be customized according to individual needs and clinical response.

A case series of insulin autoimmune syndrome highlighting a range of dysglycemic presentations, diagnostic challenges and variable responses to therapy.

Introduction

Insulin autoimmune syndrome (IAS) features hyperinsulinemic hypoglycemia due to insulin autoantibodies in exogenous insulin-naïve individuals (1,2). IAS presents with recurrent postabsorptive or fasting hypoglycemia, alternating with postprandial hyperglycemia, due to "buffering" by autoantibodies, which sequester insulin in immune complexes during the acute phase of insulin secretion, only to release it slowly later, at physiologically inappropriate times.

IAS cannot easily be distinguished on clinical grounds from tumoral or other forms of hyperinsulinemic hypoglycemia, which includes hypoglycemia caused by surreptitious insulin administration (3). Altered kinetics of insulin clearance in the presence of antibody-binding also commonly skews insulin:C-peptide molar ratios upwards, sometimes dramatically so, as insulin clearance is delayed whilst C-peptide clearance is unaffected. As insulin:C-peptide molar ratios are often used to discriminate exogenous from endogenous hyperinsulinemic hypoglycemia (4), this raises the risk that maleficent insulin use may be erroneously diagnosed, with potentially decisive implications for criminal and child custody proceedings.

Anti-insulin antibody (IA) assays are not standardized, and yield variable, qualitative or semi-quantitative results (5) and moreover detection of IA does not prove the presence of circulating insulin-antibody complexes (6). Methods currently used to confirm hormone-antibody complexes include precipitation with polyethylene glycol (PEG), which is not specific (7), and gel filtration chromatography (GFC), which may be used in conjunction with *ex vivo* addition of insulin to enhance sensitivity (6). Mass spectrometry (MS) methods now offer quantification of insulin (8) that is more robust in the face of anti-hormone antibody interference than immunoassay (9).

Effective use of different immunosuppressive regimens in IAS has been described, including prednisolone (10), hydrocortisone (11), azathioprine (12), cyclophosphamide (13), mycophenolate mofetil (MMF) (14,15), rituximab (16), and plasmapheresis (17,18), but no consensus exists about optimal therapy. We now extend experience by presenting clinical and biochemical characteristics of six patients with varying presentations of IAS and responses to immunosuppression.

Materials and Methods

Patients and blood sampling

Studies were performed in accordance with the Declaration of Helsinki (2000). Six exogenous insulin-naïve patients presenting with hyperinsulinemic hypoglycemia and high insulin:C-peptide ratio were evaluated by the UK Severe Insulin Resistance Supraregional Assay Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge.

Immunoassays and insulin immunocomplex detection

Blood was collected on ice and plasma/serum rapidly separated and frozen at -80°C. Plasma insulin and C-peptide were measured using immunoassay platforms approved for clinical use. PEG precipitation studies were performed as previously published (6), with analyte recovery taken to be the PEG supernatant insulin concentration expressed as a percentage of insulin measured in matched saline-diluted samples. GFC was performed as previously described (6).

Anti-insulin IgG was determined using an in-house human insulin-specific ImmunoCAP® enzyme-linked immunosorbent assay (ELISA). IA were also determined using a competitive IA radioimmunoassay (RIA) (19). In brief, 5µL of serum, neat or diluted with IA negative serum, was incubated with A14-^{125}I-labeled human insulin ± unlabeled synthetic human insulin at 40 µmol/L. ^{125}I-IA complexes were precipitated using glycine-blocked Protein A Sepharose (PAS), ethanolamine-blocked Protein G Sepharose (PGS) (20), and/or IgA agarose.

IA affinity was assessed in neat and diluted serum (21,22), with immune complexes precipitated using a 50:50 mixture of PAS and PGS to include all possible IA-reactive IgG antibodies. IC50, Kd (mol/L) were calculated by non-linear regression analysis using a one-site model (22) (R^2 values 0.88-0.99), assuming equal antibody binding by labeled and unlabeled insulin.

Immunosubtraction using anti-human immunoglobulin-agarose

Synthetic human insulin, diluted in 5% BSA, was added to plasma before 24-hour incubation at 24°C. Agarose-conjugated anti-immunoglobulin (anti-human IgA; anti-human IgM; anti-human IgG) was washed thrice with 0.9% saline and stored at 4°C. Agarose conjugates were added to plasma at ratios based on in-house data (volume ratios of agarose-antibody:plasma were 5:1 for anti-IgA, 29:20 for anti-IgM, and 32:3 for anti-IgG). IgA antibody−agarose experiments for Patient 6 were performed in triplicate. Samples were mixed for 60 minutes prior to centrifugation at 13,200*g* for 15 minutes. To overcome sampling error due to increased sample viscosity, agarose supernatant was diluted in saline prior to analysis. Insulin recovery was calculated as percentage insulin recovery in agarose supernatant of dilution-matched plasma.

Quantitative mass spectrometric analysis of insulin and C-peptide

Pooled human plasma was fortified with insulin lispro and C-peptide to generate concentrations of 8,610 pmol/L to 17 pmol/L and 16,548 pmol/L to 33 pmol/L, respectively. 250µL of each sample of known peptide concentration, of available patient plasma, and of unfortified pooled plasma were transferred to different wells of a 2mL 96-well plate. 5 patient and 34 control samples were extracted using a combination of acetonitrile precipitation and solid phase extraction-liquid chromatography (23) along with quality control (QC) samples and analyzed with two separately extracted sets of calibration samples. MS data were acquired from *m/z* 700-1600, with a resolution of 70,000 and an automatic gain control target of 3e6 ions. Insulin and C-peptide calibration curves were generated using *m/z* values for the $[M+5H]^{5+}$ charge states relating to the monoisotopic (1161.7362) and multiple ^{13}C isotopes of human insulin and for the $[M+3H]^{3+}$ charge state of C-peptide (1007.1783). Calibration curves for insulin and C-peptide gave a linear fit with R^2 values of 0.995 and 0.994, respectively, after correcting for endogenous analyte, and calibration standards and QC samples were all within ±25% of expected values. Regression between immunoassay and MS control plasma values were linear for insulin (0.8727x-27.025; R^2=0.974), and C-peptide (1.317x-56.86; R^2 = 0.997).

Results

A summary of the clinical characteristics of patients studied and the investigations undertaken on initial presentation is given in **Table 1**. Case histories follow:

Patient 1 presented after 20 months of shakiness, sweating, pallor, and confusion, generally 1-2 hours postprandially, and alleviated by carbohydrate ingestion. She had concurrently gained 7kg in weight. On emergency admission plasma glucose concentration was 30 mg/dL (1.7 mmol/L) (normal range (NR) 72-110 mg/dL), with concomitantly inappropriate plasma immunoassay insulin and C-peptide concentrations of 267 pmol/L (NR <60) and 899 pmol/L (NR 174-960) respectively, and a molar ratio of insulin:C-peptide of 0.30 (NR 0.03-0.25) (24,25). 72-hour fast and mixed meal tolerance test (MMTT) failed to solicit hypoglycemia, however a 75g oral glucose tolerance test (OGTT) produced a glucose nadir of 39 mg/dL (2.2 mmol/L) (Fig. 1a) at 240 minutes. Continuous glucose monitoring system (CGMS) demonstrated labile glycemia including late post-prandial hypoglycemia (Fig. 1b). IA were grossly elevated at 722.4 U/mL (NR <0.4) (RiARSR® IAA, Cardiff, UK).

Gross hyperinsulinemia was confirmed using MS (Table 2). Low insulin recovery following PEG precipitation using an immunoassay suggested IA. GFC with and without addition of exogenous human insulin showed predominantly high molecular weight (HMW) insulin immunoreactivity, confirming IAS (6). IA were positive by ELISA and RIA, the latter indicating a high insulin-binding capacity. Competitive insulin-binding studies (Fig. 2) suggested a sub-nanomolar dissociation constant (analyzed at ten-fold serum dilution, with a two-site model offering the best fit, both sites binding with high affinity).

Two 1g intravenous methylprednisolone doses were given one day apart monthly for 4 months, however symptoms continued over the ensuing 2 years with hypoglycemia remaining demonstrable on OGTT and CGMS. Rituximab (750 mg/m^2 X2) was administered, reducing glycemic lability (Fig. 1c) with only two capillary blood glucose (CBG) readings <55mg/dL (<3.1 mmol/L) recorded over 9 months following rituximab. At this stage, IA concentration had decreased to 153 U/mL (NR<0.4, RiARSR® IAA), and fasting plasma insulin and C-peptide concentrations by immunoassay were 173 pmol/L (NR <60) and 500 pmol/L (NR 174-960) respectively.

Patient 2 presented with fasting symptoms of hypoglycemia including syncope. She became hypoglycemic after 10 hours of fasting with a venous plasma glucose of 34 mg/dL (1.9 mmol/L), and concomitant plasma insulin immunoassay concentration of 68,123 pmol/L, C-peptide 3690 pmol/L, and insulin:C-peptide molar ratio of 18 (NR 0.03-0.25). Gross hyperinsulinemia was confirmed by immunoassay (Table 2) and low insulin recovery following PEG precipitation suggested IA. GFC of plasma showed HMW insulin immunoreactivity consistent with insulin-binding antibodies, confirming IAS (Fig. 3a). IAs were positive by ELISA and RIA, the latter result consistent with GFC findings of a very high insulin-binding capacity. Competitive insulin-binding studies (Fig. 2) suggested a nanomolar dissociation constant (analyzed at ten- and fifty-fold dilution).

Initial diazoxide treatment was ineffective and caused neutropenia, leading to discontinuation. Prednisolone 30mg daily was begun with addition of MMF 1.5g daily after IAS confirmation. Hypoglycemia resolved over the subsequent 4 weeks, anti-insulin IgG falling to 5 mg/L, plasma insulin to 322 pmol/L, and C-peptide to 1,210 pmol/L, although insulin recovery after PEG precipitation increased only modestly to 17%. Following treatment GFC demonstrated reduction of HMW insulin (Fig. 3b). The patient remained euglycemic on maintenance MMF for 12 months before discontinuing immunosuppressive therapy with no evidence of recurrence during the twelve months of follow-up to date.

Patient 3 presented with 2 years of recurrent anxiety, confusion, perioral paraesthesiae and generalized diaphoresis on fasting. Typically, she would wake during the night with feelings of terror and agitation. These symptoms would swiftly resolve following carbohydrate ingestion. Emergency medical attendants had recorded CBG readings of 36 and

43 mg/dL (2.0 and 2.4 mmol/L). During inpatient supervised fasting symptomatic hypoglycemia was recorded at 4 hours with a venous plasma glucose of 39 mg/dL (2.2 mmol/L) and paired immunoassay plasma insulin and C-peptide concentrations of 17,800 and 409 pmol/L respectively, with an insulin to C-peptide molar ratio of 44 (NR 0.03-0.25).

Hyperinsulinemia was confirmed using MS (Table 2). Insulin measurement by immunoassay underestimated total insulin in neat plasma, and was non-linear to dilution, with low insulin recovery following PEG precipitation, all suggesting IA. GFC showed predominantly HMW insulin immunoreactivity, confirming the diagnosis of IAS (Fig. 3c). IA were positive by ELISA and RIA, the latter result consistent with GFC findings of a very high insulin-binding capacity. Competitive insulin-binding studies (Fig. 2) suggested a sub-nanomolar dissociation constant (analyzed at hundred-fold serum dilution).

Initial diazoxide treatment was ineffective and was discontinued. Prednisolone 60mg daily, later changed to dexamethasone 8mg twice daily, was commenced after IAS confirmation, with MMF twice daily later added. CGM demonstrated both hyper- and hypoglycemia (Fig. 1d). Following nausea and raised serum transaminases, MMF was replaced by azathioprine 50 mg twice daily. High-dose steroid treatment for hypoglycemia produced Cushing's syndrome, including agitated depression, and avascular necrosis of the hip. Rituximab (1g X2) was administered and dexamethasone weaned to 1mg daily, but no evidence of depletion of the pathogenic antibody (Fig. 4a) nor glycemic improvement were seen. Plasma exchange (thrice weekly X8), in contrast, led to resolution of hypoglycemia, disappearance of serum IA, improvement in insulin immunoassay linearity (Fig. 4b) and an increase in insulin recovery after PEG precipitation. Although transient, this proved the efficacy of immunodepletion and plasma exchange followed by a course of rituximab (750 mg/m^2 X4) was administered. Despite amelioration of hypoglycemia, euglycemia was not achieved, leading to further plasma exchange and administration of rituximab (750 mg/m^2 X4), for recrudescent hypoglycemia six months later. After a further six months, the patient was taking azathioprine but no glucocorticoid. She no longer suffered with fasting hypoglycemia, but had persistent reactive hypoglycemia, managed with dietetic support in combination with acarbose (alpha-glucosidase inhibitor) to limit postprandial insulin secretion.

Patient 4 presented with 9 months of episodic diaphoresis, headache, hunger and confusion, attributed to spontaneous hypoglycemia. Three days after initial consultation he had a myocardial infarction and coronary artery bypass surgery. Initial plasma immunoassay insulin concentration was 1,732 pmol/L, and C-peptide 794 pmol/L during spontaneous hypoglycemia. Over two 72-hour fasts a blood glucose nadir of 45 mg/dL (2.5 mmol/L) was recorded. MMTT revealed early post challenge hyperglycemia, with a peak concentration of 232 mg/dL (12.9 mmol/L), and a late glucose nadir of 29 mg/dL (1.6 mmol/L) (Fig. 1e). Plasma immunoassay insulin was concomitantly >6,945 pmol/L (C-peptide not measured). Glycemic lability was confirmed by CGM (Fig. 1f).

Gross hyperinsulinemia was confirmed using MS (Table 2). Insulin measurement by immunoassay underestimated total insulin in neat plasma, and was non-linear to dilution, with very low insulin recovery following PEG precipitation suggesting IA. GFC showed predominantly HMW insulin immunoreactivity, confirming IAS (Fig. 3d). IA were strongly positive by ELISA but equivocal by RIA, the former result consistent with GFC findings of a high insulin-binding capacity. Unlike the low levels of RIA binding with protein A immunoprecipitation (Table 2), high levels were demonstrable with protein G that could be explained by insulin-binding due to IgG3. Competitive insulin-binding studies (Fig. 2) (analyzed in neat serum) suggested a micromolar dissociation constant, although interpretation was limited by low baseline binding.

Diazoxide (50mg thrice daily) reduced frequency and severity of hypoglycemia, however after 6 months lanreotide 60mg subcutaneously was added monthly as hypoglycemia continued to compromise quality of life. Lanreotide controlled hypoglycemia but caused gastrointestinal side effects leading to its withdrawal. Acarbose was not tolerated. Diazoxide was continued at increased dose (100mg thrice daily) for three years with concomitant diuretics to manage edema. HbA1c on diazoxide remained around 55 mmol/mol (NR 20-42). Immunomodulatory therapy was declined but remains under consideration.

Patient 5 presented with recurrent falls associated with cognitive decline. Borderline low CBG concentrations at 50 mg/dL (2.8 mmol/L), as well as concentrations as high as 248 mg/dL (13.8 mmol/L) consistent with diabetes mellitus, were noted during admission however no glycopenic symptoms were apparent. Plasma immunoassay insulin concentration, at a time when blood glucose concentration was 37 mg/dL (2.1 mmol/L), was 1,024 pmol/L with concomitant C-peptide of 679 pmol/L and insulin to C-peptide molar ratio of 1.51 (NR 0.03-0.25). Gross hyperinsulinemia was confirmed using MS (Table 2). Insulin measurement by immunoassay underestimated total insulin in neat plasma, and was non-linear to dilution, with very low insulin recovery following PEG precipitation suggesting IA. GFC showed HMW insulin immunoreactivity, confirming IAS. IA were positive by ELISA and RIA, and competitive insulin-binding studies (Fig. 2) (analyzed at ten-fold serum dilution) suggested a sub-micromolar dissociation constant. Further investigation and treatment was declined, and the patient was discharged to residential care with a CBG meter and advice to avoid fasting. Four months later, she was admitted to hospital with reduced consciousness and a CBG reading of 23 mg/dL (1.3 mmol/L). Blood glucose normalized with intravenous glucose. Prednisolone 10mg daily was commenced and the patient was discharged with advice for regular blood glucose monitoring, and glucose gel was provided. She has since died.

Patient 6 presented with two episodes of loss of consciousness due to hypoglycemia. On both occasions low CBG was detected, and he was admitted to hospital for emergency treatment. He had no family history of diabetes or hypoglycemia. Two 72-hour fasts failed to provoke hypoglycemia, with a glucose nadir during the first fast of 72 mg/dL (4.0 mmol/L). In contrast, prolonged 75g OGTT produced a glucose nadir of 26 mg/dL (1.4 mmol/L) (Fig. 1g) with a corresponding immunoassay insulin of 1,285 pmol/L and C-peptide of 1,006 pmol/L and insulin to C-peptide ratio of 1.28 (NR 0.03-0.25) at 180 minutes after the glucose load. This led to loss of consciousness, which was rescued with intravenous glucose. IAS was suspected and prednisolone 60mg with diazoxide 300mg daily was commenced. IA were, however, within reference limits using two RIA.

Gross hyperinsulinemia was confirmed using MS (Table 2). Insulin measurement using immunoassay underestimated total insulin in neat plasma, was non-linear to dilution, with low insulin recovery following PEG precipitation suggesting IA. GFC studies with and without preincubation of plasma with exogenous human insulin showed HMW insulin immunoreactivity consistent with insulin-binding antibodies, confirming the diagnosis of IAS (Fig. 3e). IA were equivocal by ELISA and negative by RIA which was inconsistent with GFC findings of a high insulin-binding capacity. To identify the class of the putative IA, immunosubtraction studies were performed using antibody class-specific antibodies conjugated to agarose. Patient 6 plasma was compared to control plasma with insulin-binding IgG, and three plasma samples with no evidence of insulin autoimmunity, all matched for insulin concentration. To increase the sensitivity of the method to detect IA, plasma was incubated with synthetic human insulin to drive the binding equilibrium in favor of bound insulin. Plasma insulin recovery was close to 100% in all cases except for those with anti-insulin IgG subtracted for IgG, and Patient 6 subtracted for IgA. In both cases recovery fell to around 50-60%, indicating the presence of anti-insulin IgA in Patient 6. In keeping with this,

no increased precipitation of radiolabel was seen using either protein G or protein A, but demonstrably increased precipitation was seen with anti-IgA agarose. The baseline PAS/PGS radioligand binding was too low (analyzed in neat serum) to allow reliable calculation of binding affinity.

Prednisolone was reduced to 40mg daily and no further symptomatic hypoglycemia was recorded. Four months following diagnosis, during prednisolone treatment, blood tests confirmed the continued presence of insulin-binding antibodies. CGM confirmed labile glycemia, with matutinal hyperglycemia and postprandial hyperglycemia (Fig. 1h) leading to immunodepletion therapy being considered

Quantitative LC-MS insulin and C-peptide results

Individual results are shown in Table 2. There was insufficient plasma from patient 2 for analysis. Molar ratios of IAS insulin:C-peptide ranged from 3.7 to 8.4, and for 34 control plasma samples from 0.2 to 1.5 with one outlier of 0.02.

Discussion

IAS has been reported most widely in Japan (1), and despite numerous but scattered reports elsewhere, and frequent airing of the diagnostic possibility in forensic investigation of suspected insulin poisoning, there is relatively little awareness of the condition in the Western Hemisphere amongst endocrinologists. IAS most often presents with hypoglycemia, which may be postprandial, postabsorptive or fasting. In this series, the presenting symptoms ranged from daytime loss of consciousness to modest symptoms only after overnight fasting. Patients 1, 4 and 6 displayed reactive hypoglycemia on dynamic testing while in patients 2 and 3 hypoglycemia was provoked by fasting. Prolonged fasting of patients 1, 4 and 6 did not result in hypoglycemia using thresholds aimed at excluding insulinoma, as in some published cases of IAS (16,26,27). Four of six patients underwent imaging using modalities including MRI, endoscopic ultrasonography and PET/SPECT before IAS was diagnosed. Suggestive biochemical evidence for IAS existed in each case, and some imaging may have been avoided with earlier access to definitive testing.

In this series, the first clue to IAS came from high insulin concentrations and insulin:C-peptide molar ratios in samples drawn during hypoglycemia. Immunoassay results were shown to be non-linear to dilution at presentation (linearity improving following plasma exchange), and to underestimate MS-detected insulin in neat plasma, consistent with assay interference due to the IA competing with the immunoassay antibodies for insulin-binding sites (6). Consistent with previous observations (28–31) immunoassay C-peptide concentrations, in the five patients in whom it was measured, were reported at hundreds to thousands of picomoles per liter concurrent with hypoglycemia. Immunoassay C-peptide concentrations in patients 1, 3, 4 and 6 conversely overestimated MS C-peptide more than may be expected from assay bias alone (32) possibly due to cross-reacting insulin precursors not detected by the MS method. As MS methods are not susceptible to antibody interference they are more likely to return a correct value for total insulin concentration in IAS and thus increased confidence in the diagnosis.

IA are a *sine qua non* of IAS (33), but assay sensitivity and specificity in the diagnosis of IAS has not been established. Indeed IA were first described in patients receiving exogenous insulin (34,35), with such frequency that in early literature the presence of such antibodies in hypoglycemic ostensibly insulin-naïve patients was regarded as nearly diagnostic of surreptitious insulin administration (36). They are now well established in the repertoire of autoantibodies used to identify type 1 diabetes (37) and to stratify non diabetic people according to risk of autoimmune diabetes (38,39). They may also be detected in healthy blood donors or patients with unrelated autoimmune disorders (40–42). Different diagnostic

laboratories employ different methods; these are non-standardized, and assay concordance remains poor (5,43) despite longstanding attempts at harmonization (44). In all patients, recovery of immunoreactive insulin after PEG precipitation was low and GFC confirmed HMW insulin-containing complexes, however not all patients had elevated IA on initial testing. In this study ELISA and RIA moreover produced different rankings of the magnitude of the results, possibly due to differential effects of high endogenous insulin concentrations. Antibody characteristics will also contribute to assay variability: for Patient 4 the ELISA/RIA discrepancy may be attributable to underrepresentation of IgG3 in immunoglobulins captured by protein A prior to RIA. More strikingly, in Patient 6 equivocal or negative antibody levels were determined using four different IA assays, despite convincing GFC evidence of insulin-antibody complexes. Anti-insulin IgA was ultimately demonstrated by immunosubtraction, explaining the discrepancy. Only around 70% of IgA is removed using PEG precipitation (in-house data), explaining the relatively modest suppression of recovery after PEG precipitation in this case, and raising the possibility that PEG precipitation may offer false reassurance in the presence of IgA IA. The use of alternative immunoprecipitation methods may increase the sensitivity of these tests but offer diminishing returns and increase complexity and cost. For example, further studies using anti- IgA showed Patient 5 also to possess significant IgA IA binding of insulin. Unfortunately, there is no failsafe method for immunosubtraction of immunoglobulin subclasses. It is tempting to speculate that patients 2 and 3 exhibited hypoglycemia principally during fasting due to the high affinity and very high capacity of their IA, however antibody capacity and affinity did not appear to correlate with physiological abnormality across the whole group studied.

Hypoglycemia in IAS has been reported to resolve spontaneously in most patients within three months (1). The severe hypoglycemia seen in this series, sustained over months or years, allied to other reports, demonstrate that this is not always true however. As IAS is antibody-mediated, targeting of pathogenic antibodies is rational. In keeping with this diazoxide, which targets insulin secretion, showed modest or no benefit. Four patients in this series to date were treated with immunomodulatory therapies. Patient 1 was treated with glucocorticoids alone over more than 4 months, however intermittent hypoglycemia persisted and so therapy with rituximab was used. Patient 6 also failed to experience improvement of glycemic lability and immunodepletion therapy is being considered. Patients 2 and 3 were both initially treated with glucocorticoids and MMF, however whilst Patient 2 appears to have gone into remission relatively quickly, Patient 3 continued to experience severe hypoglycemia, despite high-dose glucocorticoids (which caused severe side effects). Ultimately, it was necessary to combine plasma exchange with rituximab therapy. Collectively, this demonstrates that therapeutic responses are variable.

In summary, IAS should be considered in cases of spontaneous hypoglycemia with a high insulin:C-peptide molar ratio. Measurement of IA is an appropriate screening step, however although the IA assays used in this series detected antibodies in five patients, they were equivocal or negative in Patient 6, illustrating that IA results are assay-dependent (5). Moreover, detection of IA alone is not specific for actionable antibodies (6) meaning that further measures to confirm plasma insulin-antibody complexes are required for diagnosis. MS-based methods promise to increase diagnostic confidence as they are unaffected by antibody-based assay interference. Immunodepletion is warranted in severely affected patients. Our series demonstrates that therapeutic responses vary, and so a customized, and flexible approach to depleting pathogenic antibodies is required. More standardized approaches to IAS diagnosis will facilitate the systematic therapeutic studies required.

Acknowledgements
Our thanks to Peter Barker and Ashley Clarke (Core Biochemical Assay Laboratory, Cambridge University Hospitals NHS Foundation Trust) for analytical support; to Sally Houghton (Department of Immunology, Cambridge University Hospitals NHS Foundation Trust) for analytical support; to Dircea Rodrigues and Francisco Carrilho (Department of Endocrinology, Diabetes and Metabolism, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal) for the clinical management of Patient 1; to Catriona Goodlad (Department of Nephrology, Royal Free Hospital, London) for the clinical management of Patient 3.

Funding

D.C. is funded by a Diabetes Research and Wellness Fellowship Sutherland-Earl Clinical Fellowship (RG68554) and R.S. by the Wellcome Trust (Grant WT098498). R.G.K was funded by the Wellcome Trust (Grants 106262/Z/14/Z, 106 263/Z/14/Z) and the MS was obtained using the MRC "Enhancing UK clinical research" grant (MR/M009041/1). Funding was also received from the Medical Research Council (MRC_MC_UU_12012/5); the United Kingdom National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre. C.W. is funded by a Diabetes UK PhD studentship.

Wellcome Trust, http://dx.doi.org/10.13039/100004440, WT098498, Robert K Semple; Diabetes Research & Wellness Foundation, http://dx.doi.org/10.13039/501100000273, Sutherland-Earl Clinical Fellowship, David Church; Wellcome Trust, http://dx.doi.org/10.13039/100004440, 106262/Z/14/Z, 106262/Z/14/Z; Wellcome Trust, http://dx.doi.org/10.13039/100004440, 106263/Z/14/Z, Richard G Kay; Medical Research Council, http://dx.doi.org/10.13039/501100000265, MR/M009041/1, Richard G Kay; Medical Research Council, http://dx.doi.org/10.13039/501100000265, MRC_MC_UU_12012/5, Not Applicable; Diabetes UK, http://dx.doi.org/10.13039/501100000361, Claire L Williams; National Institute for Health Research, http://dx.doi.org/10.13039/501100000272, Cambridge Biomedical Research, David Church

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Conflict of interest
The authors have declared that no conflict of interest exists.
Disclosure Statement:
I certify that neither I nor my co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this Work.

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Figure 1: Variable patterns of dysglycemia of patients studied. (a) Venous plasma glucose concentrations during a 75g oral glucose tolerance test (OGTT) at presentation of Patient 1; ○ denotes glucose measurements following glucose rescue. The glucose nadir was 39 mg/dL (2.2 mmol/L). (b) Demonstration of labile glycemia in Patient 1 at presentation by Continuous Glucose Monitoring System (CGMS). (c) Demonstration of normoglycemia in Patient 1 following immunomodulation therapy. (d) Demonstration of labile glycemia in Patient 3 concomitant with glucocorticoid therapy. (e) Demonstration of reactive hypoglycemia in Patient 4 at presentation by mixed meal tolerance test. The peak glucose concentration was 232 mg/dL (12.9 mmol/L) with glucose nadir at 300 minutes of 29 mg/dL (1.6 mmol/L). (f) Demonstration of reactive and nocturnal hypoglycemia in Patient 4 at presentation by CGMS. (g) Demonstration of reactive hypoglycemia in Patient 6 at presentation by 75g OGTT. The glucose nadir was 26 mg/dL (1.4 mmol/L). (h) Demonstration of labile glycemia in Patient 6 at presentation by CGMS.

Figure 2 Displacement curves for samples from the Patient 1-5 serum at various dilutions in antibody-negative serum following competitive displacement with unlabeled human insulin. Although identified as low affinity ($4.1x10^{-7}$ mol/L), Patient 6 plasma was considered unreliable because baseline levels of insulin binding were very low. Serum was diluted as follows, Patient 1: 10-fold; Patient 2: 50-fold; Patient 3: 100-fold; Patient 4: neat; Patient 5: 10-fold.

Figure 3 Demonstration of insulin-antibody complexes using gel filtration chromatography (GFC). Results of insulin assay after GFC of non-fasting plasma. Elution volumes of immunoglobulin (Ig), albumin (Alb) and monomeric insulin (mIns) are shown. Results are shown for Patient 2 at (a) presentation (pre-therapy), and (b) with and without preincubation of plasma with exogenous insulin post-therapy, and with and without preincubation of plasma with exogenous insulin at presentation for (c) Patient 3, (d) Patient 4, and (e) Patient 6.

Figure 4: Response of biochemical markers to therapy in Patient 3. (a) Cumulative results for Patient 3 over course of treatments, including mycophenolate mofetil (MMF); azathioprine (AZA), prednisolone (Pr), dexamethasone (Dex), rituximab (R), and plasma exchange (PEx), showing anti-insulin IgG concentrations (in-house human insulin specific ImmunoCAP®) and immunoassay insulin recovery following PEG precipitation over time. (b) Effect of plasma exchange on insulin immunoassay linearity to dilution. Calculated insulin concentration plotted against plasma dilution of Patient 3 plasma before plasma exchange and following cycle 1 and cycle 9.

Table 1: Clinical characteristics and initial investigation of patents studied.
*Hypoglycemia with inappropriately elevated plasma insulin were inclusion criteria for this study so are excluded from the table. BMI = body mass index; CGMS = continuous glucose monitoring system; CT = computerized tomography; GAD = glutamic acid decarboxylase; α-INSR = anti-insulin receptor; IA2 = islet antigen-2; MMTT = mixed meal tolerance test; MRI = magnetic resonance imaging; OGTT = oral glucose tolerance test; PET = positron emission tomography; SPECT = single-photon emission computerized tomography; SU = sulfonylurea; US = ultrasound

Patient	Age (years)	Sex	Ethnicity	BMI (kg/m^2)	Pre-existing diagnoses	Medications	Presentation	Investigations with abnormal results*	Investigations with normal results*	Negative imaging
1	56	Female	Caucasian	26.2	Autoimmune hypothyroidism	None	Postprandial hypoglycemia	OGTT nadir 39 mg/dL (2.2 mmol/L)	HbA1c	CT abdomen
					Asthma			CGMS	72-hour fast nadir 59 mg/dL (3.3 mmol/L)	MRI abdomen
					Factor XI deficiency				MMTT	Endoscopic US
									SU screen	
									α-islet, α-GAD65, α-IA2, α-INSR autoantibodies	
2	52	Female	Thai	35.0	None	None	Fasting hypoglycemia		SU screen α-INSR autoantibodies	^{68}Ga-DOTATATE PET/CT
3	28	Fe	Ca	25.1	None	None	Fasting		HbA1c	^{68}Ga-

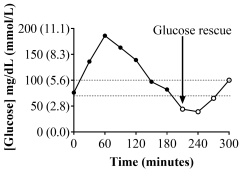
		male	ucasian				hypoglycemia		SU screen	DOTATATE PET/CT
4	76	Male	Caucasian	29.5	Type 2 diabetes Ischemic heart disease Parotid pleomorphic adenoma Glaucoma	Spironolactone Furosemide Losartan Aspirin Bisoprolol Atorvastatin Omeprazole Fluoxetine	Postprandial/ nocturnal hypoglycemia	MMTT nadir 29 mg/dL (1.6 mmol/L) CGMS	72-hour fast nadir 45 mg/dL (2.5 mmol/L)	MRI abdomen Endoscopic US Octreotide SPECT ^{18}F-Deoxyglucose-PET
5	89	Female	Caucasian	19.4	Small B cell lymphoma	Furosemide Fexofenadine Ferrous fumarate	Low capillary blood glucose readings		Short Synacthen® test	nil
6	50	Male	Caucasian	22.3	None	None	Postprandial hypoglycemia	OGTT nadir 26 mg/dL (1.4 mmol/L)	72-hour fast nadir 72 mg/dL (4.0 mmol/L) SU screen	CT abdomen

Table 2: Biochemical evaluation of non-fasting plasma in a single specialized center. GFC = gel filtration chromatography; HMW = high molecular weight; IA = anti-insulin antibody; Kd = dissociation constant; MS = mass spectrometry; PEG = polyethylene glycol. * The reference range used for the anti-insulin IgG assay was provided by a reference laboratory using the same method (Sheffield Protein Reference Unit, Sheffield, UK). Testing 28 of the 34 control samples used in the quantitative mass spectrometric analysis of insulin and C-peptide yielded a 75% percentile insulin antibody concentration of 4.8 mg/L.

Patient	**MS insulin (pmol/L)**	**Immunoassay insulin pmol/L (<60)**		**Insulin recovery after PEG precipitation % (>102)**	**GFC of insulin**	**Anti-insulin IgG mg/L (0-5)***	**IA cIA units (<0.2)**	**Kd mol/L**	**MS C-peptide pmol/L**	**Immunoassay C-peptide pmol/L (174-960)**	**MS Insulin: C-peptide molar ratio (0.2-1.5)**	**Immunoassay insulin: C-peptide molar ratio (0.03-0.25)**
		Dilution ratio (Plasma:diluent)										
		1:0	**1:4**									
1	5,278	>3,000	7,020	8	HMW insulin present	16	2,408	$3.42x10^{-10}$	1,428	3,750	3.7	1.9
2	-	>3,000	11,585	6	HMW insulin present	38	8,738	$1.16 x10^{-9}$	-	5,580	-	2.1
3	1,583	782	4,601	9	HMW insulin present	11	>10,000	$4.68 x10^{-10}$	215	2,380	7.4	0.3

4	2,912	1,340	3,912	11	HMW insulin present	>200	4.0	6.55×10^{-6}	348	1,190	8.4	1.1
5	6,589	2,781	7,805	3	HMW insulin present	89	300	8.55×10^{-7}	880	3,110	7.5	0.9
6	4,012	2,906	5,630	65	HMW insulin present	5	0.1	-	750	3,280	5.4	0.9

(a)



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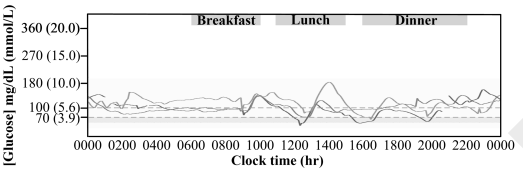

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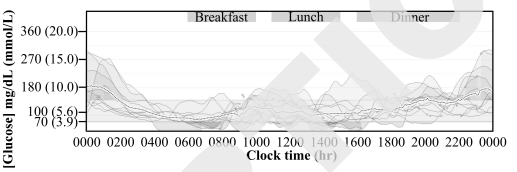

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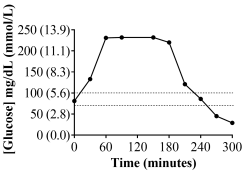

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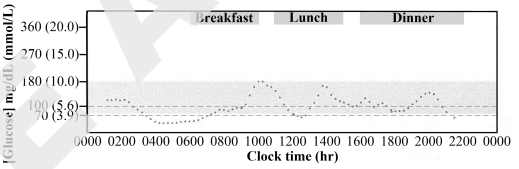

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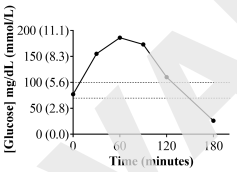

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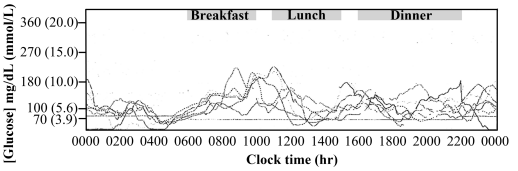

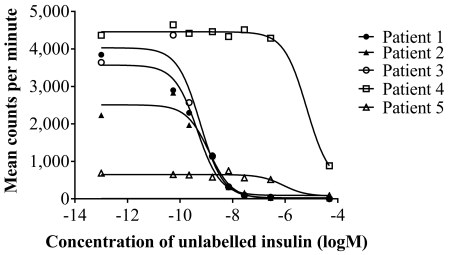

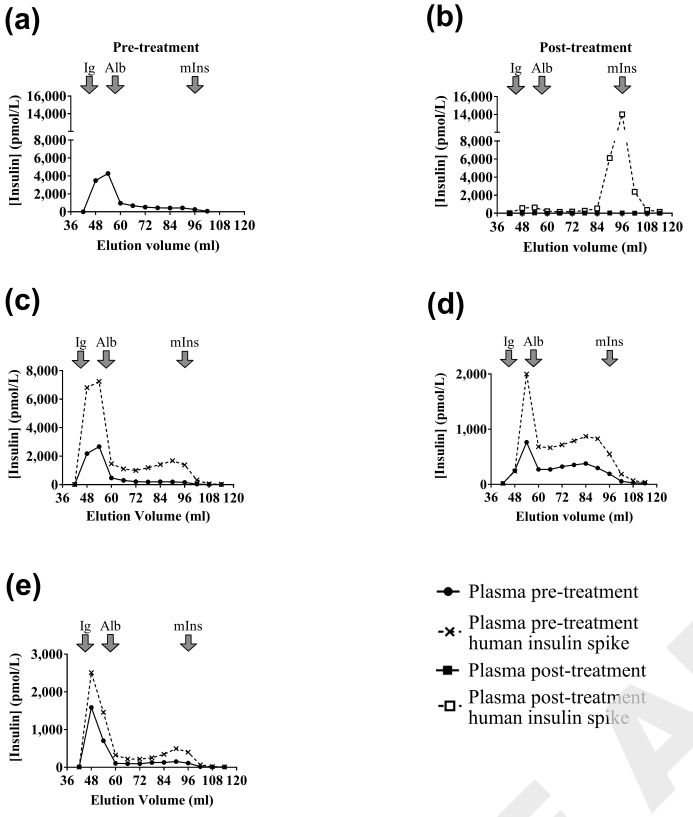

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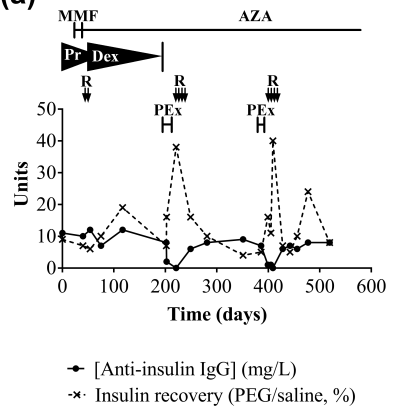

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