

Antibody Response to Inhaled Insulin in Patients with Type 1 or Type 2 Diabetes. An Analysis of Initial Phase II and III Inhaled Insulin (Exubera) Trials and a Two-Year Extension Trial

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Objective: To compare antibody responses to inhaled human insulin *vs.* sc human insulin and to determine whether insulin antibody binding is associated with adverse clinical consequences.

Research Design and Methods: Insulin antibody data from initial phase II/III trials were analyzed comparing the efficacy and safety of inhaled insulin with various agents, including sc insulin. Additionally, data from a 24-month extension of the phase III studies were examined. Data were pooled into the following three groups based on insulin treatment status at baseline: patients with type 1 diabetes, and patients with type 2 diabetes using insulin and not using insulin at baseline. Ig class analysis was also performed on randomly selected sera from type 1 patients at the end of the initial trials.

Results: In the initial trials, greater insulin antibody binding was observed in patients receiving inhaled insulin *vs.* sc insulin. The greatest antibody responses to inhaled insulin were observed in patients with type 1 diabetes [nonparametric comparison of medians at the end of the study, 22.0% binding (unadjusted 95%

confidence interval: 19.5, 24.5)], and the lowest responses were observed in non-insulin-using patients with type 2 diabetes in which there was no difference in median values at the end of the study. There were no correlations between antibody binding and glycemic control (measured using glycosylated hemoglobin), insulin dose requirements, hypoglycemic events, or pulmonary function (measured by changes in forced expiratory volume in 1 sec and diffusion capacity of carbon monoxide). Antibody responses were IgG in type. Differences in antibody levels observed in patients with type 1 *vs.* type 2 diabetes were maintained over the 24-month extension trials. Peak antibody levels across all groups were generally observed after 6–12 months of insulin therapy. Inhaled insulin therapy was not associated with a greater incidence of allergy or other hypersensitivity reactions.

Conclusion: Inhaled insulin was observed to produce a larger antibody response than sc insulin. Insulin antibody binding has not been associated with adverse clinical consequences in trials to date. (*J Clin Endocrinol Metab* 90: 3287–3294, 2005)

INHALED INSULIN DELIVERY systems have been developed to overcome patient resistance to insulin therapy. The use of this alternative to injected insulin may result in improved patient compliance, enable intensified therapies, and better approximate physiologic hormone replacement. The pulmonary alveoli have a surface area of 75–100 m² and provide a favorable environment for the delivery of small polypeptides, such as insulin (~6000 Da). Absorption through the alveolar surface is inversely related to molecular mass, and small peptides having a molecular mass of less than 30,000 Da are readily absorbed (1–3).

Parenteral human insulins can elicit various types of local and systemic immunologic responses, although allergic adverse events are rare (4). Previous studies have suggested that the delivery of peptide antigens to the pulmonary mucosa may induce immunologic tolerance (3, 5, 6). The addi-

tional possibilities of developing humoral and/or cellular immune responses resulting in allergic reactions or the formation of neutralizing antibodies that could diminish insulin's action are important assessments to be made in inhaled insulin development programs.

Inhaled insulin (Exubera) (Pfizer, Inc., New York, NY/Aventis Pharmaceuticals, Inc., Bridgewater, NJ in collaboration with Nektar Therapeutics, San Carlos, CA) is a novel aerosol delivery system that enables delivery of inhaled rapid-acting, dry-powder human insulin.

In the present study, serum samples were collected during trials with inhaled insulin and analyzed to determine: 1) whether human insulin delivered via the inhalation route would be more or less immunogenic than sc human insulin comparators; 2) whether patterns of immunologic responses differed from those previously observed with sc insulin; 3) whether antibody development affected glycemic control, dose requirements, or pulmonary function; and 4) whether there was evidence of allergy or other hypersensitivity reactions to inhaled insulin.

Subjects and Methods

The studies included in this manuscript consisted of phase II/III studies and a phase III extension study. These studies were designed to

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Abbreviations: AAV-Ova, Adeno-associated virus encoded for ovalbumin; CI, confidence interval; DL_{CO}, diffusion capacity of carbon monoxide; FEV₁, forced expiratory volume in 1 sec; HbA_{1c}, glycosylated hemoglobin.

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assess the efficacy and safety of inhaled human insulin. The efficacy data have been published elsewhere (7, 8) (also see Refs. 10–13). Informed consent was obtained from all patients before participation. Protocols for these multicenter studies were reviewed and approved by the respective institutional review boards, and all studies were conducted according to the ethical principles of the Declaration of Helsinki and guidelines for Good Clinical Practice.

Initial studies

The insulin-using patients included those with type 1 diabetes, protocols 106, 107, and 1009 (7, 8), who were on a stable insulin regimen involving at least two daily injections of insulin for at least 2 months before screening, and patients with type 2 diabetes who had been receiving insulin for at least 2 months before screening, protocol 108, a phase III study (9). Non-insulin-using type 2 diabetes patients were included in two phase III studies, protocols 109 and 110 (10, 11), and one phase II study, protocol 104 (12), which enrolled patients with type 2 diabetes who had never received insulin or had not received insulin for at least 1 month before screening. Patients in each study were randomized to receive either an inhaled insulin-containing regimen or a comparator regimen consisting of oral agents or sc insulins. Specific treatment regimens for each study are presented in Table 1.

Extension trial

Patients who completed any of the arms of the initial phase III studies were asked to participate in an open-label 24-month extension trial. Percentage participation, by protocol group, ranged from 72% for protocol 110 to 95% for protocol 1009 (11).

Participants in the extension study received inhaled insulin as their only short-acting insulin. All other antidiabetic therapies, including oral agents, basal insulins, or combinations thereof, were prescribed at the treating physician's discretion.

Laboratory methods

Insulin antibodies were measured by a semiquantitative method at Mayo Medical Laboratories, using 100 μ l patient sera mixed with 300 μ l diluted radiolabeled recombinant human insulin. Antibody-bound insulin was precipitated with goat antihuman γ -globulin and separated after the addition of 600 μ l of 3% polyethylene glycol solution. Percent binding was determined by subtracting counts in a parallel incubation with normal human serum. Antibody binding below the lower limit of quantitation of 3% was imputed to a value of 1.5% for statistical analyses. Levels higher than the maximal reported level (>90%) were imputed to 91% binding. In the initial trials, antibody binding was determined at baseline and at the end of each study. In the extension studies, antibody binding was determined at baseline and after 6, 12, 18, and 24 months of inhaled insulin therapy.

Radioligand binding assays were also used to measure specific classes

of antibodies (IgE, IgA, IgG, and IgM) in random samples that reflected a wide range of antibody levels from patients with type 1 diabetes (13, 14). These assays used anti-IgE, anti-IgA, and anti-IgM antibodies conjugated to Sepharose beads to measure insulin-specific IgE, IgA, and IgM, respectively. The assay for insulin-specific IgG used protein-G-Sepharose beads. The binding capacity and specificity of the antibody-conjugated beads were validated by using radiolabeled IgG, IgE, IgA, and IgM (14).

Glycosylated hemoglobin (HbA_{1c}) was determined using the Bio-Rad VARIANT II analyzer (Bio-Rad Laboratories, Inc., Hercules, CA) in a central laboratory compliant with the National Glycohemoglobin Standardization Program (15). Pulmonary function tests were measured in local pulmonary function laboratories, compliant with American Thoracic Society standards.

Data analysis

Data from all initial studies and the extension trial were pooled into the following three groups: patients with type 1 diabetes, patients with type 2 diabetes (insulin-using at baseline), and patients with type 2 diabetes (non-insulin using at baseline). Scatter plot figures and Spearman correlation coefficient were calculated to explore the correlation between insulin antibody levels and clinical parameters.

Insulin antibody data were distributed asymmetrically and were summarized using descriptive statistics, including mean and median values with sd levels and 25th and 75th percentile ranges. Hypoglycemic events rates, calculated as total hypoglycemic events experienced per subject-month of treatment, were plotted against increasing levels of end-of-study insulin antibody responses. A similar analysis was performed for the change from baseline in forced expiratory volume in 1 sec (FEV₁) at the end of the study. Incidences for allergic or hypersensitivity reactions were also summarized. Because of the skewed distribution of insulin antibody results, the Hodges-Lehmann nonparametric approach, comparing medians, was performed for each of the three patient groups as a *post hoc* analysis.

Results

Population demographics

The majority of patients with type 1 diabetes or type 2 diabetes were white (92 and 77%, respectively). The mean body mass index for patients with type 1 diabetes was 24.0 ± 4.0 kg/m² for men and 23.3 ± 4.1 kg/m² for women. For patients with type 2 diabetes, mean body mass index was 30.3 ± 4.0 kg/m² for men and 31.1 ± 4.9 kg/m² for women. Of the 781 patients with type 1 diabetes in the initial studies, 665 (85.1%) elected to participate in the extension trial by continuing on or switching to inhaled insulin therapy as their

TABLE 1. Treatment regimens in the initial studies included in the analysis

Protocol no.	Treatment regimens
104	Premeal inhaled insulin and oral agent therapy, or Oral agent therapy (13)
106	Premeal inhaled insulin and bedtime human Ultralente, or Twice daily NPH and sc regular human insulin (7)
107	Inhaled insulin prior to each meal and NPH prior to the first meal and at bedtime, or Subcutaneous regular human insulin prior to each meal and NPH prior to the first meal and at bedtime (8)
108	Premeal inhaled insulin and bedtime Ultralente, or NPH and sc regular human insulins twice daily (10)
109	Premeal inhaled insulin, or Premeal inhaled insulin and oral agents, or Oral agents alone (11)
110	Premeal inhaled insulin, or Rosiglitazone 4 mg twice daily (12)
1009 ^a	Premeal inhaled insulin and NPH or Ultralente, or Subcutaneous regular or Lispro insulin twice daily and longer-acting insulins

NPH, Neutral protamine Hagedorn.

^aStudy protocol no. 1009, data on file. Pfizer, Inc., and Aventis Pharmaceuticals.

short-acting insulin. Of the 298 insulin-using patients with type 2 diabetes, 249 (83.6%) participated in the extension trial; and of the 518 initially non-insulin-using patients with type 2 diabetes, 439 (84.7%) participated in the extension study.

Insulin antibodies in patients previously treated with insulin

Figure 1 illustrates the median percentage insulin antibody binding in the inhaled insulin and comparator groups for patients with type 1 and type 2 diabetes from the initial trials.

The median baseline percentage binding in the pooled type 1 diabetes groups was 3.0% (mean, 7.6%; SD, 10.9) for the inhaled insulin group and 4.0% (mean, 7.2%; SD, 9.9) for the sc insulin group. At the end of the initial studies, the median percentage antibody binding in type 1 diabetes patients increased to 29.0% (mean, 31.0%; SD, 20.3) in the inhaled insulin group but did not increase significantly in the sc insulin group. Nonparametric comparison of medians at the end of the study yielded a difference of 22.0% [unadjusted 95% confidence interval (CI): 19.5, 24.5].

Baseline median and mean percentage antibody binding in the insulin-using patients with type 2 diabetes was below the limit of quantitation (<3.0%) for both the inhaled insulin group and the sc insulin group (SD, 4.2 and 8.9, respectively). At study end point, the median percentage insulin antibody binding in type 2 patients increased to 6.0% (mean, 13.1; SD, 18.2) in the inhaled insulin group and remained unchanged in the sc insulin group. Nonparametric comparison of medians at the end of the study yielded a difference of 3.5% binding (unadjusted 95% CI: 1.5, 4.5).

In inhaled insulin-treated patients, there was a clear difference in antibody responses between patients with type 1 diabetes *vs.* insulin-using patients with type 2 diabetes. Greater antibody responses were observed in patients with type 1 diabetes.

Insulin antibodies in non-insulin-using patients with type 2 diabetes

Data for the non-insulin-using patients with type 2 diabetes are also shown in Fig. 1. Baseline median and mean percentage antibody binding in the inhaled insulin and oral agent groups were below the limit of quantitation (<3.0%; SD, 4.5 and 0.3, respectively). At the end of the initial study, the median remained unchanged, and the mean rose to 6.1%

(SD, 8.7) with inhaled insulin. No increases in insulin antibody levels were seen in the oral agent comparator group.

Twenty-four-month cohort data for median percentage antibody binding

Data for subjects with insulin antibody measurements at baseline and at 24 months of exposure in the extension trial are shown in Fig. 2. Patients with type 1 diabetes who completed the 24-month extension trials had a baseline median percentage antibody binding of 3.0% (mean, 7.4%; SD, 10.2). Over the duration of the extension trials, the median values increased to 27, 32, 32, and 29% at 6, 12, 18, and 24 months, respectively (at 12 months: mean, 31.9%; SD, 22.0).

Insulin-using patients with type 2 diabetes who completed the 24-month extension trial had a baseline median percentage antibody binding of less than 3.0% (mean, 3.2%; SD, 6.6). Median values increased to 6.0, 6.5, 7.0, and 6.0% at 6, 12, 18, and 24 months, respectively (at 24 months: mean, 12.4%; SD, 16.3).

Non-insulin-using patients with type 2 diabetes who completed the 24-month extension trials had a baseline median and mean percentage antibody binding of less than 3.0% (SD, 4.8). Median values increased to 3.0, 5.0, 4.0, and 4.0% at 6, 12, 18, and 24 months, respectively (at 24 months: mean, 7.2%; SD, 10.6). Patients with type 2 diabetes who were initially non-insulin-using had lower antibody responses than insulin-treated patients with type 2 diabetes throughout the initial and extension studies, despite the addition of basal sc insulin in 32% of individuals by the end of the extension study (data not shown). Peak antibody levels in patients with type 1 and type 2 diabetes were reached by 6–12 months of inhaled insulin exposure.

Ig class characterization

A wide range of IgG levels was observed (data not shown) in both the inhaled insulin and the sc insulin groups; whereas IgM, IgA, and IgE antibodies were below the lower limits of quantitation for the respective assays.

Relationship of insulin antibody binding to HbA_{1c}, short-acting insulin doses, and hypoglycemic events

There were no correlations between percentage median antibody binding and HbA_{1c} in patients with type 1 or type 2 diabetes in the inhaled insulin group (Fig. 3). No correla-

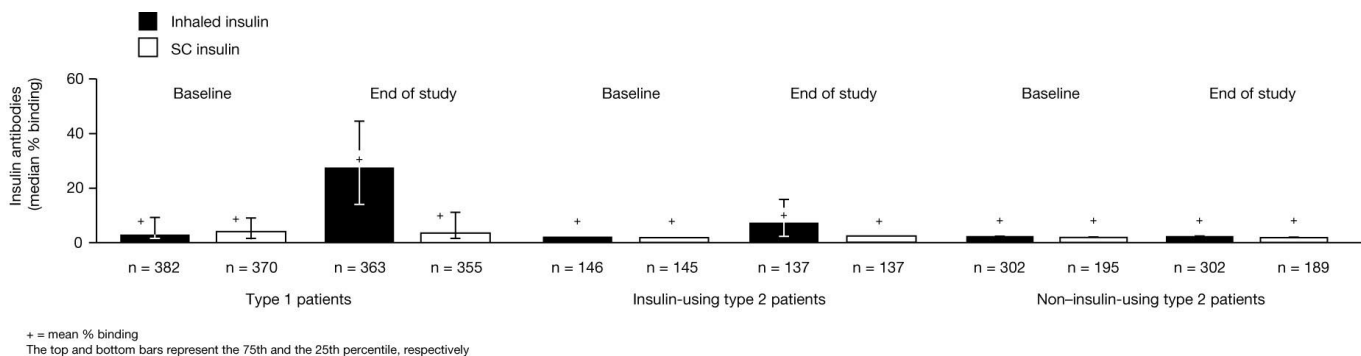
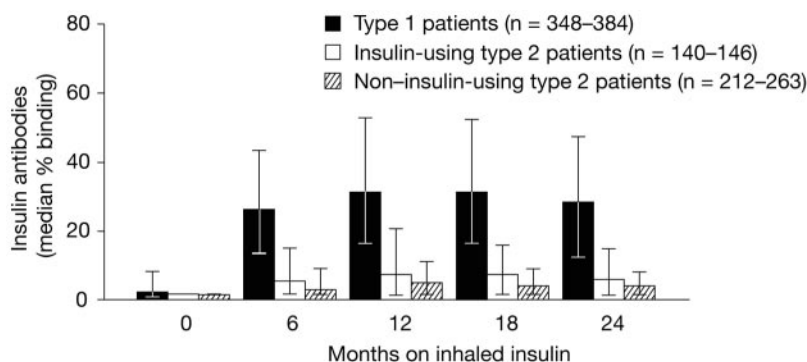


FIG. 1. Insulin antibody binding responses after inhaled insulin *vs.* comparator (sc insulin or oral agents) in the initial studies (7–9).

FIG. 2. Insulin antibody responses in patients with antibody measurements at baseline and after 24 months of inhaled insulin exposure.



The top and bottom bars represent the 75th and the 25th percentile, respectively

tions were seen between antibody levels and HbA_{1c} in comparator-treated patients with type 1 or type 2 diabetes (data not shown). Similarly, short-acting insulin doses (data not shown) in inhaled insulin and comparator groups for patients with type 1 and type 2 diabetes were unaffected by levels of antibody binding. Furthermore, no correlations were seen when comparing change from baseline in HbA_{1c} or insulin doses with changes in insulin antibody levels.

No correlations between hypoglycemic events and insulin antibody levels were observed at the end of the study, including in study 107, which compared intensive treatment regimens in patients with type 1 diabetes and had the highest hypoglycemic event rate of all inhaled insulin studies. Similar results were observed in patients with type 2 diabetes (data not shown).

Relationship of antibody binding to pulmonary function

The relationship of FEV₁ to antibody binding was assessed to determine whether antibody binding has any effect on pulmonary function. No significant correlations between FEV₁ and antibody binding were observed for the inhaled insulin group with type 1 or type 2 diabetes (Fig. 4) or in comparator-treated subjects. Similar analyses assessing the diffusion capacity of carbon monoxide (DL_{co}) showed no correlation to antibody responses. Change from baseline analyses likewise showed no correlations between FEV₁ or DL_{co} and insulin antibody changes (data not shown).

Allergy and hypersensitivity

Table 2 summarizes all-cause adverse events (using Coding Symbols for Thesaurus of Adverse Reaction Terms (COSTART) dictionary preferred terms) of a potentially allergic nature from the controlled phase II and III studies. There was no significant difference between groups in the incidence of allergy or other hypersensitivity reactions. A slight imbalance in the “skin and appendages” category may reflect the increased exposure in the inhaled insulin compared with the sc insulin groups (3791 *vs.* 2939 subject-months, respectively). A listing of specific investigator text descriptions of adverse events coded to this group was reviewed, and it is clear that this category encompasses a great variety of diagnoses, only a fraction of which are potential manifestations of insulin allergy. No vasculitic syndromes were reported.

Discussion

These studies show that switching short-acting sc human insulin to inhaled human insulin is associated with a larger antibody response than is continuation of sc insulin regimens. In addition, initiation of inhaled insulin is associated with greater insulin antibody levels relative to patients taking only oral agents. Interpretation of the 95% CIs calculated *post hoc* is confounded by the skewed distribution and increased variability found in the inhaled-treated arms relative to comparator arms. However, the statistical analyses are consistent with the qualitative conclusion that larger inhaled insulin associated responses were observed, particularly in patients with type 1 diabetes.

It has been suggested that insulin immunity may cause the formation of neutralizing antibodies, leading to increased insulin dosage, decreased glycemic control, or hypoglycemia (16–20). However, in the inhaled insulin studies reported, there was no relationship between insulin antibody binding and insulin doses or HbA_{1c}. Hypoglycemia has been associated with insulin antibodies in the case of the insulin autoimmune syndrome (21); however, episodes of hypoglycemia in patients receiving sc insulin are rarely attributable to insulin antibodies. We did not observe any relationship between insulin antibodies and hypoglycemic event rates in patients receiving either sc or inhaled insulin, even in protocol 107, which had the highest hypoglycemic event rate among all of the initial inhaled insulin trials (8).

The primary objective of this study was to compare antibody responses to inhaled insulin *vs.* sc insulin and to determine whether insulin antibody binding is associated with adverse clinical consequences. The sensitivity of the assay used in the current analyses does not necessarily allow for optimal determination of antibody prevalence. However, the sensitivity of the assay employed was clearly sufficient to establish a difference in the magnitude of the antibody response between inhaled insulin-treated patients and those treated with comparators. With regard to assessing clinical significance, any potential clinical consequences of insulin antibodies would be expected to correspond to the magnitude of antibody response. Because this assay was sufficient to detect differences in the magnitude of antibody responses between treatment groups, it is reasonable to use data generated from this assay to examine potential clinical consequences associated with this laboratory finding. Although it

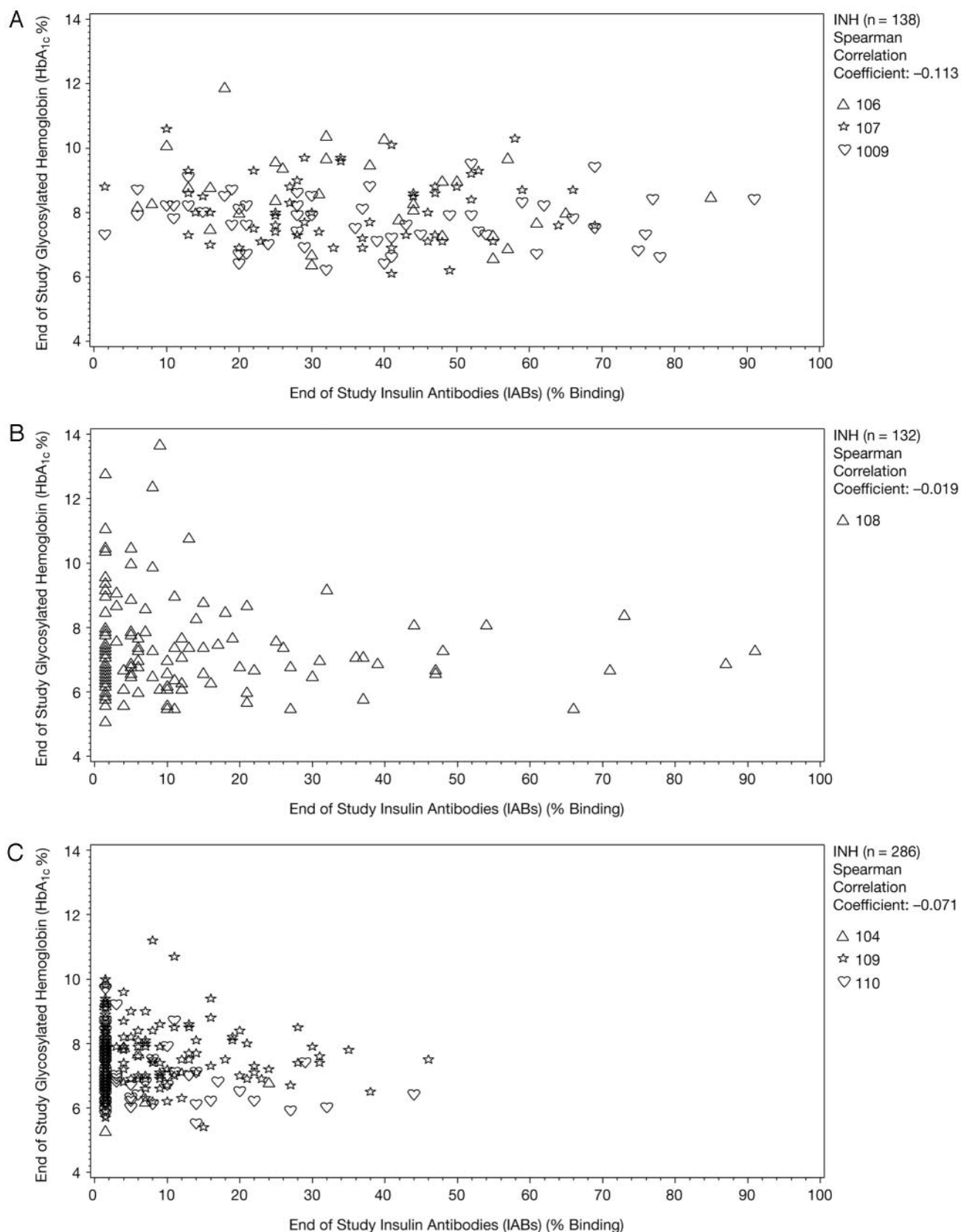


FIG. 3. Scatterplots of insulin antibody responses (percent binding) *vs.* end of study HbA_{1c} (percent) in type 1 patients (A), insulin-using type 2 patients (B), and non-insulin-using type 2 patients (C) for subjects treated with inhaled insulin (INH).

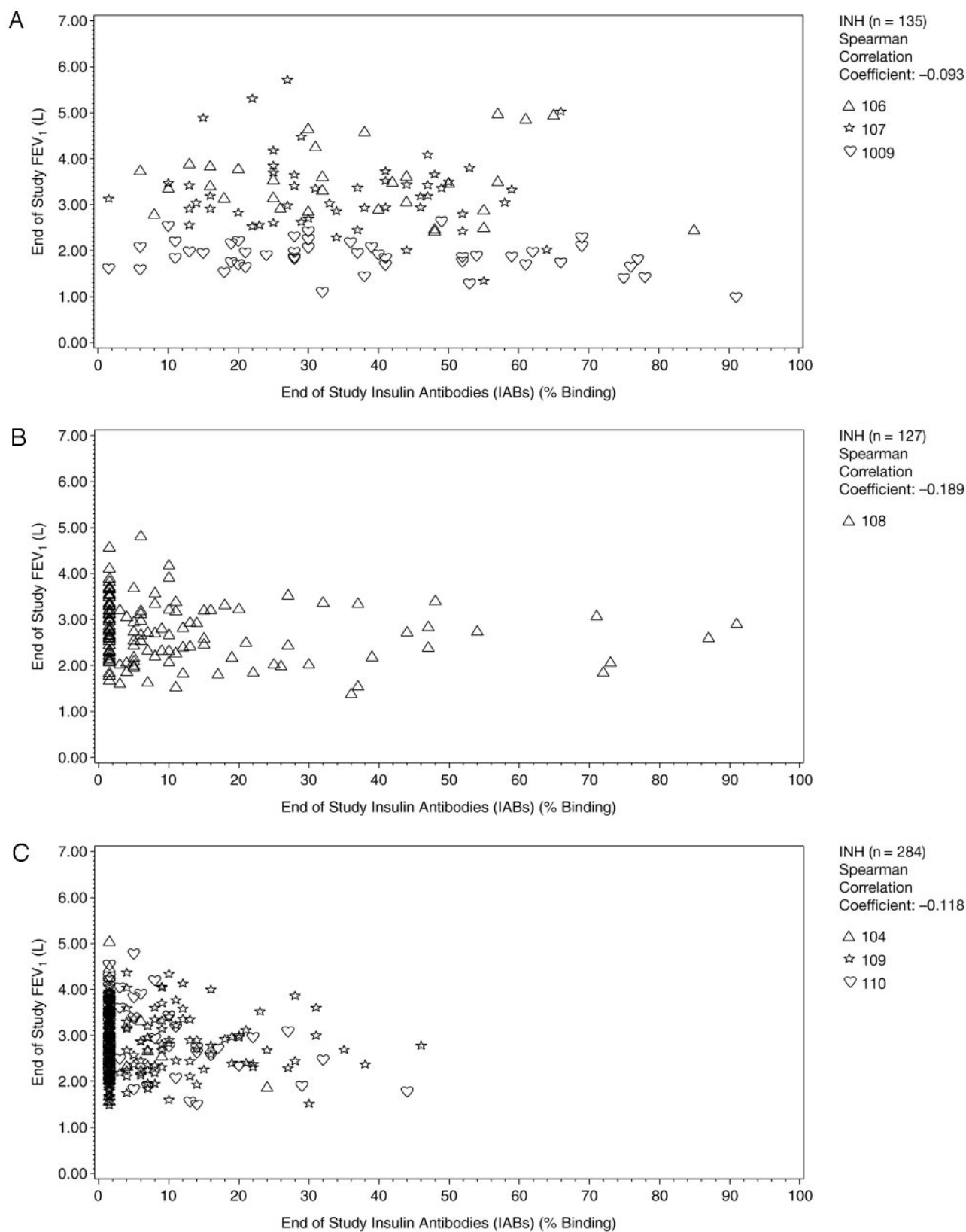


FIG. 4. Scatterplots of insulin antibody responses (percent binding) *vs.* end of study FEV₁ (liters, L) in type 1 patients (A), insulin-using type 2 patients (B), and non-insulin-using type 2 patients (C) for subjects treated with inhaled insulin (INH).

TABLE 2. All-cause adverse events of an allergic nature from controlled phase II and III studies (COSTART dictionary terms)

	Inhaled insulin (n = 920)	Subcutaneous insulin (n = 602)
Subjects' months of exposure	3791	2939
Body as a whole (includes potential injection site reactions and allergic reactions)	40 (4.3)	33 (5.5)
Lipodystrophy	4 (0.4)	4 (0.7)
Respiratory (includes asthma)	107 (11.6)	81 (13.5)
Skin and appendages (includes nonspecific rashes and dermatitis)	64 (7.0)	28 (4.7)

Data represent number of adverse events (%). COSTART, Coding Symbols for Thesaurus of Adverse Reaction Terms.

is possible that the assay did not detect patients with very low levels of insulin antibodies, these are not the patients in whom clinical consequences might be expected.

Among possible explanations for increased immunogenicity of inhaled insulin include structural alterations in insulin during preparation of insulin powder or differences in immune responses related to different routes of drug delivery. Structural studies of insulin powder have revealed no evidence of altered molecular structure, and no differences in bioactivity have been demonstrated in *in vitro* studies (data on file). Although storage conditions may affect immunogenicity, the excipients [sodium citrate (dihydrate), mannitol, glycine, and sodium hydroxide] used in the powder formulation are not known to be immunogenic. A recent proof of concept study in insulin-using patients with type 2 diabetes showed that human insulin administered as liquid droplets via the pulmonary route also resulted in increased levels of insulin antibodies (22). Therefore, whether pulmonary insulin is delivered as a powder or as a liquid, increased antibody responses are observed, suggesting that the site of antigen delivery and the susceptibility of the recipient may significantly affect immune responses.

Animal and human studies have shown that the site of antigen delivery and the formulation of antigens can be important determinants of immune responses. In trials with purified porcine insulin, increased antibody responses were observed with continuous sc insulin infusion *vs.* multiple daily injection therapy, as well as during administration of intermediate and long-lasting insulins *vs.* short-acting insulins (23). The site of insulin delivery also affects antibody response; for example, ip delivery of insulin results in higher antibody levels than sc injections (24, 25).

The formulation and route of delivery also affect immunogenicity for various other proteins. In an *in vivo* study by Ge *et al.* (26), the introduction of human factor IX into mice resulted in a specific humoral response with no stimulated cellular immunity when administered by im injection and no humoral response when administered iv. Gutierrez *et al.* (27) compared the intranasal, sc, and oral routes of administering encapsulated BSA. The immune responses were similar via the sc and oral routes but higher for the intranasal route; 1000-nm particles elicited the highest antibody responses. Free and alum-linked antigen elicited lower antibody responses than encapsulated particle antigen (27). Brockstedt *et al.* (28) studied the immunologic responses to recombinant adeno-associated virus encoded for ovalbumin (AAV-Ova) delivered via the ip, iv, im, or sc routes; im injection led to minimal cytotoxic lymphocyte responses to AAV-Ova, whereas ip, iv, and sc administration resulted in both hu-

moral responses and the development of cytotoxic lymphocytes to AAV-Ova.

Animal models have also demonstrated that immunologic presensitization can result in subsequent immunologic responses (29, 30). Responses of respiratory tissue immunologically presensitized to other antigens have resulted in the development of allergy, hypersensitivity, hyperreactivity, and pulmonary inflammatory disease (31, 32). Therefore, presensitization with sc insulin may be a possible explanation for the increased immunogenicity in insulin-using patients with type 1 and type 2 diabetes relative to non-insulin-using patients with type 2 diabetes. The difference in immunogenicity between the initially non-insulin-using *vs.* insulin-using patients with type 2 diabetes was maintained over 2 yr of inhaled insulin exposure, despite the fact that sc basal insulin was added to inhaled insulin therapy in 32% of patients who were initially not using insulin. The diminished immune response seen in patients who were not using insulin at baseline, even after the addition of sc basal insulin, may reflect active suppression of the humoral insulin responses.

Antigens presented to the deep lung typically result in an IgG antibody response and may even lead to the induction of immunologic tolerance (31, 33). In these studies, no correlations were observed between insulin antibodies and FEV₁ or DL_{CO}.

Conclusion

Although inhaled insulin produces a greater antibody response than sc insulin, insulin antibody binding was not associated with adverse clinical consequences.

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