Clinical Impact of Antibodies against Ustekinumab in Psoriasis: An Observational, Cross-Sectional, Multicenter Study

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Ustekinumab is an effective treatment for psoriasis, but response varies between patients. The formation of anti-drug antibodies (ADAs) may explain part of this variation by reducing the free ustekinumab level. Currently, published analyses of the clinical impact of ADAs are incomplete. In this observational cross-sectional multicenter study of 340 patients, we evaluated the impact of ADAs on ustekinumab level and clinical response as assessed by the PASI. Circulating ADA levels were measured using two assays: a drug-sensitive radioimmunoassay and a drug-tolerant ELISA. Circulating ustekinumab levels were measured using an ELISA. ADAs were detected in 3.8% (95% confidence interval [CI] = 3.2–4.2) and in 10.6% (95% CI = 7.9–13.9) of patients using the radioimmunoassay and drug-tolerant ELISA, respectively. At least 85% of the ADAs were neutralizing. Compared with patients negative for ADAs, ADA positivity in the radioimmunoassay and drug-tolerant ELISA were associated with lower median ustekinumab levels (–0.62 μg/ml [95% CI = −1.190 to −0.30] and –0.74 μg/ml [95% CI = −1.09 to −0.47], respectively) and higher absolute PASI (6.6 [95% CI = 3.0–9.9] and 1.9 [95% CI = 0.4–4.0], respectively). Absence of detectable ustekinumab regardless of ADA status correlated with poor clinical outcome (median sample PASI 10.1, 6.5 [95% CI = 3.9–8.8] compared with patients positive for ustekinumab). In conclusion, substantially reduced drug exposure resulting from ADAs formation is associated with impaired clinical response.


INTRODUCTION

Ustekinumab is an antagonistic human monoclonal IgG1 antibody directed against the p40 subunit of IL-12 and IL-23 and is used in the treatment of psoriasis, psoriatic arthritis, and Crohn’s disease. The clinical effectiveness of ustekinumab in psoriasis varies widely between patients. Approximately one-third of patients will not achieve good clinical response (at least 75% improvement in PASI, that is, PASI75), and only half will achieve good clinical response (>PASI 90) (Leonardi et al., 2008; Papp et al., 2008). For TNF inhibitors such as adalimumab and infliximab, it is well-established that poor response in some patients with psoriasis is related to low circulating drug levels (Mahil et al., 2013; Mostafa et al., 2017; Takahashi et al., 2013; Torii et al., 2017; Wilkinson et al., 2019). For ustekinumab, direct differences in drug level between responders and nonresponders have not been identified (De Keyser et al., 2019; Menting et al., 2015), but model predictions suggest that lower ustekinumab levels decrease the probability of good clinical response (Blauvelt, 2019; Tsakok et al., 2019). Moreover, it has been demonstrated that suboptimal clinical responses can be improved by dose adjustment (Langley et al., 2015).

Low drug levels may, in part, result from the formation of anti-drug antibodies (ADAs) by the patient. These ADAs can reduce the active drug level by directly blocking the drug–antigen interaction and by accelerating drug clearance...
through the formation of immune complexes, consequently impairing clinical response. Currently, the incidence of ADAs against ustekinumab in adult patients with psoriasis is unclear. The original phase III clinical studies (PHOENIX I and II) reported relatively low ADA incidence (5.1% and 5.4%) (Leonardi et al., 2008; Papp et al., 2008), which was supported by later studies (4.4–8.7%) (Chiu et al., 2015a; De Keyser et al., 2019; Menting et al., 2015; Tsai et al., 2011). However, the assays used in these studies were the so-called drug-sensitive assays, in which detection of ADAs can be hampered when a drug is present in the sample owing to the presence of a drug–anti-drug complexes (van Schouwenburg et al., 2010), thus, leading to the underestimation of the numbers of patients developing ADAs. Indeed, subsequent phase III studies in adults on maintenance dosing (PSTELLAR) and adolescent patients (CADMUS, partly receiving a modified dosing schedule) used a drug-tolerant ADA assay (in which the presence of a drug does not interfere with the measurement of ADA) and reported higher ADA incidences (13.8% and 8.2%, respectively) (Blauvelt et al., 2017; Landells et al., 2015). However, to date, studies have either not reported the clinical effects of ADAs or were insufficiently powered to draw conclusions.

Furthermore, the clinical relevance of drug-sensitive versus drug-tolerant ADA assays is not well-established. This gap in knowledge is especially important given the continued pressure from regulatory to use drug-tolerant assays for immunogenicity testing in clinical trials. Appreciating the relevance of ADAs and selecting the correct tools for their detection may help identify the cause of nonresponse to ustekinumab treatment in individual patients and, in turn, guide clinical decision making.

The aim of this study was to determine the clinical relevance of ADAs in patients with psoriasis treated with ustekinumab. ADAs were detected and quantified using two assays in parallel; the previously described drug-sensitive radioimmunoassay (RIA) and a newly developed drug-tolerant bridging ELISA (dtELISA). We then investigated the clinical impacts of ADAs using two large cohorts, the real-world multicenter biomarkers of systemic treatment outcomes in psoriasis study and its nested psoriasis stratification to optimize relevant therapy discovery study. In a cross-sectional set of samples taken 60–120 days after treatment initiation, the qualitative and quantitative effect of ADA titer on both circulating ustekinumab level and same-day disease activity was assessed. The attribution of ADA status to the variation in ustekinumab level and disease activity in the presence of covariables was estimated using multivariable analyses.

RESULTS
Higher ADAs prevalence using the dtELISA
To assess the prevalence of ADAs in patients who had received ustekinumab for the treatment of psoriasis, we analyzed serum samples taken from 340 patients (characteristics in Table 1) using two immunogenicity assays: a drug-sensitive RIA, designed as a practical assay for use in routine clinical diagnostics, and a dtELISA (schematics in Supplementary Figure S1a and b, respectively), conforming to current US Food and Drug Administration (2019) guidelines for immunogenicity testing. The RIA had a sensitivity of 3.0 arbitrary units (AU)/ml in the absence of ustekinumab, but ustekinumab levels as low as 0.4 μg/ml blocked ADAs detection even in samples with high ADA titers (up to 600 AU/ml; Supplementary Figure S1c). The dtELISA had a sensitivity of 1.16 AU/ml and achieved high drug tolerance at all ustekinumab levels within the expected therapeutic range (detection of ≥3.0 AU/ml in the presence of 0.4 μg/ml ustekinumab and ≥75 AU/ml at 10 μg/ml ustekinumab; Supplementary Figure S1d). Serum samples had been taken 60–120 days after treatment initiation, at which stage treatment adherence is generally high (Iskandar et al., 2018; Warren et al., 2015), irrespective of ustekinumab dosing. A lower prevalence of ADAs was found in the RIA (13 of 340, 3.8%, 95% CI = 3.2–4.2; Figure 1a) than in the dtELISA (36 of 340, 10.6%, 95% CI = 7.9–13.9; Figure 1b). No difference in ADAs prevalence in the RIA or the dtELISA was found between biologic experienced or naive patients (both P > 0.05, Fisher’s exact test). Of the four RIA-positive samples that were negative in the dtELISA, one was also positive in the screening tier of the dtELISA (2.11 AU) but not in the confirmation tier (14% inhibition) (Figure 1c, red symbol). A pretreatment sample from this individual had similar reactivity in the dtELISA (3.63 AU, −8% inhibition), indicating false positivity. Overall, good concordance of ADA titers between the RIA and the dtELISA was observed for the double-positive samples (Figure 1c). The four patients who received methotrexate at the time of sampling were negative for ADAs in both assays.

ADAs are associated with reduced ustekinumab levels
Ustekinumab levels were analyzed in all 340 samples using an assay selective for free ustekinumab, that is, not bound to ADAs. The median ustekinumab level was 0.82 μg/ml (interquartile range [IQR] = 0.35–1.92 μg/ml; Figure 2a). No difference in median ustekinumab level was observed between patients on 45 mg or 90 mg doses (1.01 vs. 0.77 μg/ml, P = 0.25; Supplementary Figure S2a) nor was ustekinumab level correlated with bodyweight within these groups (r = 0.03 and r = 0.002, respectively; Supplementary Figure S2b).

Table 1. Patient Characteristics (n = 340)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
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<tbody>
<tr>
<td>Demographics</td>
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<tr>
<td>Median age, y (IQR)</td>
<td>45 (35–55)</td>
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<tr>
<td>Median weight, kg (IQR)</td>
<td>90 (77–105)</td>
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<tr>
<td>Male sex, n (%)</td>
<td>223 (66)</td>
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<tr>
<td>Baseline status</td>
<td></td>
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<tr>
<td>Median disease duration, y (IQR)</td>
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<tr>
<td>Median PASI (IQR)</td>
<td>12.7 (9.3–17.7)</td>
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<tr>
<td>Biologic naive, n (%)</td>
<td>180 (52.9)</td>
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<tr>
<td>Treatment</td>
<td></td>
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<tr>
<td>Dose regimen 45 mg/90 mg, n/n</td>
<td>197/143</td>
</tr>
<tr>
<td>Concomitant methotrexate, n (%)</td>
<td>4 (1.2)</td>
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Abbreviation: IQR, interquartile range.

ADAs prevalence was similar between the 45 mg and 90 mg dose groups (RIA: 4.1% and 3.5%, respectively; dtELISA: 9.1% and 12.6%, respectively). No detectable ustekinumab (<0.02 μg/ml) was present in 9 of 13 RIA-positive samples.
Of the remaining four samples, three had very low ADA titers (<6 AU, Figure 2b), and one was false-positive as outlined above (Figures 1c and 2b, red symbol). In the dtELISA-positive samples, the median ustekinumab level was significantly lower (0.12 μg/ml) than ADA-negative samples (1.03 μg/ml; difference 0.74 μg/ml; 95% CI = 0.47–1.09 μg/ml; Figure 2a) but varied considerably between samples (IQR <0.02–0.42 μg/ml). No detectable ustekinumab was found in the samples with high dtELISA ADA titer (> 25 AU; Figure 2c). In total, 20 of 340 samples (5.9%) had no detectable ustekinumab level at the time of sampling. Of these samples, 9 of 20 (45%) were positive for ADAs in the RIA and 15 of 20 (75%) in the dtELISA (both \( P < 0.001 \), Fisher’s exact test), demonstrating that the absence of a detectable ustekinumab level in a large fraction of patients was associated with the presence of ADAs.

To assess the extent to which variance in drug level is attributable to ADAs status in the presence of other covariables (see Methods), we performed separate multivariable analyses of variance (MANOVA) for ADA status as measured
with the RIA or the dtELISA. In both analyses, ADA status was the variable explaining the largest fraction of variance in drug level (11.1% for RIA ADA status and 21.1% for dtELISA ADA status). Additional significant parameters days since the last dose, biologic naive status, days after treatment initiation, and past or present alcohol use explained 6.6%, 2.6%, 1.5%, and 1.5% of the variance in drug level in the RIA MANOVA, respectively, and days since the last dose, biologic naive status, days after treatment initiation, and baseline PASI explained 4.9%, 2.8%, 1.4%, and 1.1% in the dtELISA MANOVA, respectively.

Impaired clinical response in patients is associated with ADAs
To establish whether the presence of ADAs may have impacted the clinical response in patients, we evaluated disease activity PASI on the day of sampling (available for 335 patients). Overall, there was a substantial reduction in disease severity from baseline, with a median PASI of 3.0 on the day of sampling (IQR = 1.5–5.8; Figure 3a) compared with a median PASI of 12.7 at baseline (IQR = 9.3–17.7; Supplementary Figure S3), where a PASI < 2 is considered clear and/or almost clear (Mahil et al., 2019). Considerably higher disease activity was observed for patients positive for ADAs in the RIA (median PASI 9.3) than patients negative for RIA (median PASI 3.0, difference 6.6, 95% CI = 3.0–9.9). Patients positive in the dtELISA also showed higher disease activity (median PASI 4.7, score available for 35 of 36 patients) than patients negative for dtELISA (median PASI 3.0, difference 1.9, 95% CI = 0.4–4.0). A weak-positive relationship was observed between the ADA titer determined using either the RIA (r = 0.32) or the dtELISA (r = 0.38; Supplementary Figure S4) and disease activity but reached significance only for the dtELISA (P = 0.02). The extent to which variance in PASI on the day of sampling is attributable to ADA status in the presence of other covariables was estimated using MANOVA. Only a small fraction of the PASI variance could be explained by ADA status determined using the RIA (2.5%) or the dtELISA (1.5%). Other significant covariables also had low explanatory power (gender 1.7% in both analyses, and baseline weight 0.8% and 0.7%, respectively). After stratifying patients positive for ADAs into those who had detectable versus undetectable circulating ustekinumab levels, we found a significant association with the worse clinical outcome only in the latter group for both the RIA (median PASI 11.7, difference 8.4, 95% CI = 5.6–12.0, Figure 3b) and the dtELISA (median PASI 11.7, difference 8.4, 95% CI = 5.7–11.2, Figure 3b) compared with patients

Figure 3. Inferior clinical response in patients positive for ADA. (a) PASI on the day of sample collection stratified based on ADA status. Each dot represents a single patient. (b) PASI for patients negative and positive for ADAs using the RIA or the dtELISA grouped according to the presence or absence of detectable circulating ustekinumab. (c) PASI for patients with or without detectable circulating ustekinumab. Significance was calculated using a Mann-Whitney U test. ADA, anti-drug antibody; dtELISA, drug-tolerant bridging ELISA; LOD, limit of detection; ns, nonsignificant; PASI, psoriasis area and severity index; RIA, radioimmunoassay.
negative for ADAs. As a predictor for clinical outcome, the absence of detectable ustekinumab had similar strength (median PASI 10.1, difference 6.5, 95% CI = 3.9–8.8; Figure 3c) to either the RIA alone or the dtELISA in combination with ustekinumab level. Similar trends were observed when analyzing the change in PASI relative to baseline rather than absolute PASI (Supplementary Figure S5).

Together, these data are indicative of an adverse effect of ADAs on clinical outcomes, mediated by a reduction in free ustekinumab level, meaning that the ustekinumab level represents a good independent predictor of outcome.

Antibodies against ustekinumab are predominantly neutralizing

Direct blocking of the ustekinumab–IL-12 interaction by neutralizing ADAs could explain the strong relationship between ADA positivity and the reduced level of free ustekinumab resulting in poor clinical response. To assess the fraction of neutralizing ADAs within the ADA-positive samples, we tested the competition between IL-12 and ADAs for ustekinumab binding in an IL-12 competition assay using samples selected to have an RIA titer > 75 AU/ml. To increase the power of this analysis, samples from additional patients positive for ADAs from the same cohort, but outside our 60–120-day time window, were also tested (Tsakok et al., 2019). Inhibition by IL-12 resulted in the loss of ADAs detection in a dose-dependent manner, with the highest concentration of IL-12 reducing ADA detection to the assay background level (Figure 4a). In all patients, most ADA was neutralizing, as demonstrated by almost complete loss of ADA detection by inhibition with excess IL-12 (median 93% inhibition, range 85–96%; Figure 4b). There was no correlation between the fraction of neutralizing ADAs and the ADAs titer (Figure 4c).

DISCUSSION

This study demonstrates that higher disease activity on ustekinumab treatment for psoriasis is, in a proportion of patients, related to ADAs formation and the associated decrease in circulating ustekinumab level. Assessment of drug levels and ADAs may, therefore, be a useful tool to help identify the cause of clinical nonresponse to ustekinumab. Observations in this study are in line with studies of other biologics used in the treatment of psoriasis. Although ADA prevalence is relatively low for ustekinumab (in this study, 3.8% using a drug-sensitive RIA assay and 10.6% using the drug-tolerant dtELISA) than the TNF inhibitors adalimumab and infliximab (reported incidences as high as 50%) (Blauvelt et al., 2018; Menting et al., 2014; Reich et al., 2005), the consequence of ADA positivity appears similar: a decrease of the effective drug level, down to undetectable levels in the case of high ADA titers, and associated loss of clinical response (Chiu et al., 2015b; Hsu et al., 2014; Kui et al., 2016; Menting et al., 2014). This study, therefore, generalizes the notion that ADA-associated loss of drug exposure is likely to result in impaired clinical response. In addition, we also report that ADAs formation without substantially reduced drug exposure is unlikely to cause any noticeable effect on treatment efficacy.

Absence of circulating ustekinumab, as determined using the ustekinumab level assay, identified the most patients with potentially insufficient drug exposure and associated impaired clinical response. However, because the absence of circulating drug could be due to factors other than ADA alone, measurement of ADA may add value by pointing to the underlying mechanism. Taken alone, neither of the ADA assays used in our study appeared to be optimal in identifying potential nonresponders. The drug-sensitive RIA erred on the side of caution, likely underestimating the portion of patients whose clinical response was negatively affected by ADAs. In contrast, the dtELISA identified a portion of patients with seemingly adequate drug exposure, thereby, not negatively affecting clinical response. However, the combination of undetectable ustekinumab level and ADA positivity seemed to accurately identify the cause of nonresponse in a subset of patients, with a marginally higher proportion identified using the dtELISA.

For patients with a poor clinical response and low ustekinumab level, with or without detectable ADAs, two major
options may be considered in principle: dose adjustment or switching medication. Incrementing the drug dose may be sufficient to resolve low circulating ustekinumab levels in patients with zero and/or low ADA titer, although this option is at present confined to on-label dosing. Dose adjustment is less likely to sufficiently compensate for drug loss resulting from a high ADA titer, and in this scenario, it may be appropriate to switch treatment. Indeed, several biologics targeting alternative pathways have shown clinical efficacy in psoriasis (e.g., TNF-, IL17-, and IL-12/23-targeting antibodies) (Sbidian et al., 2017). To evade ADAs established against an ongoing treatment, switching to a biologic targeting the same or a different pathway can be considered. By contrast, the poor clinical response in the presence of circulating drug may indicate that the wrong disease pathway was targeted, so it may be preferable to switch to a biologic targeting a different pathway. However, switching biologics should be considered with care because drug survival for most biologics is limited (Lin et al., 2018). Uninformed switching may prematurely or unnecessarily use up alternative treatment options. One might consider an approach where incrementing drug dose is combined with continuous monitoring of ustekinumab level and clinical response and followed by switching of treatment only in patients who do not show clinical improvement despite achieving adequate drug levels. Additional studies will be required to establish precise levels of ADAs that may indicate a poor chance of regaining response on dose increase.

The prevalence of ADAs in our cohort is comparable with previous reports using either drug-sensitive or drug-tolerant assays (Chiu et al., 2015a; De Keyser et al., 2019; Landells et al., 2015; Langley et al., 2015; Leonardi et al., 2008; Menting et al., 2015; Papp et al., 2008; Tsai et al., 2011). Nevertheless, ADA prevalence was slightly lower in this study than the previous smaller studies that used the same drug-sensitive RIA (Chiu et al., 2015a; De Keyser et al., 2019; Menting et al., 2015). This likely stems in part from the significantly higher median drug level in our cohort (0.82 μg/ml) than those studies (0.2–0.4 μg/ml), in combination with the drug-sensitivity of the RIA. Samples in our cohort were taken irrespective of the dosing schedule, whereas, in the aforementioned studies samples were taken at the trough. A further explanation for the difference in ADA prevalence is the design of our study. Our cross-sectional selection targeting the early immune response will have missed the formation of ADAs at later time points (De Keyser et al., 2019; Menting et al., 2015). Survival bias may also have contributed to differences between studies, with the earliest samples being analyzed at week 4 (Menting et al., 2015), then at week 16 (Keyser et al., 2019), and then at week 28 (Chiu et al., 2015a). Reported ADA incidence for ustekinumab is lower than adalimumab and infliximab. Despite the use of comparable assay methodologies and similar time points for sample collection, ADA incidences cannot be directly compared between biologics. However, the reported differences in incidence are also reflected in the reported proportions of patients in whom ADAs impacted drug level and clinical outcome, indicating that true differences in immunogenicity of these biologics are at play. Immunogenicity of a given drug has proven difficult to predict but is determined by factors such as the primary drug structure (e.g., presence of T cell and B cell epitopes), target interaction, the route of administration, and dosing (Garçès and Demengeot, 2018; Jullien et al., 2015; Krieckaert et al., 2012). However, ADA formation may be reduced by coadministration of methotrexate, which is being actively investigated in patients with psoriasis who are receiving adalimumab treatment (Busard et al., 2017).

A limitation of this study's cross-sectional design is the potential for underestimating the clinical impact of ADA formation. Samples were selected from week 8 onward when most of the initial clinical response is expected to have already occurred (Leonardi et al., 2008; Papp et al., 2008), but negative effects of ADAs on clinical response may take months to become apparent, as shown by the slow loss of response in the placebo arm of the cross-over study by Leonardi et al. (2008). Longitudinal studies assessing large patient populations are likely needed to estimate the full clinical consequences of ADAs formation throughout this lifelong disease. In contrast, loss of drug exposure at later time points, whether or not related to ADAs, may not affect a proportion of patients who achieve stable remission, as demonstrated by dose tapering studies in rheumatoid arthritis and as suggested by the data from PSTELLAR (Blauvelt et al., 2017; Verhoef et al., 2019). Another limitation, true for ADA assays in general as a result of the methodology used to determine the limit of detection or cut-point, is the potential for generating false-positive samples. Because the fraction of positive samples in this study is low, a larger proportion of these might be false-positive, potentially affecting reported correlations.

In conclusion, ADAs against ustekinumab are associated with low drug exposure and poor clinical response in a proportion of patients treated for psoriasis. Combined results from drug level and ADA assays may be used to guide ad hoc clinical decisions such as switching biologic or adjusting dose but should be interpreted with care so as to prevent unnecessary switching.

METHODS

Patients and samples

This study included a cross-sectional selection of samples collected 60–120 days after the initiation of ustekinumab treatment in patients with psoriasis recruited into the biomarkers of systemic treatment outcomes in psoriasis or psoriasis stratification to optimize relevant therapy discovery study, between June 2009 and December 2016, reported in detail elsewhere (Burden et al., 2012; Griffiths et al., 2015). All patients had provided written informed consent before enrollment. Biomarkers of Systemic Treatment Outcomes in Psoriasis was approved by The South East London REC 2 Committee (11/ H0802/7) and psoriasis stratification to optimize relevant therapy discovery by the London Bridge Research Ethics Committee (14/LO/1685). Patients included were aged ≥16 years, had a dermatologist's diagnosis of psoriasis, and were enrolled in the British Association of Dermatologists Biologic and Immunomodulators Register. No minimum PASI was required. All patients received 45 mg (≤100 kg bodyweight at baseline) or 90 mg (>100 kg) ustekinumab (Stelara, Janssen-Cilag International NV, Belgium) subcutaneously at weeks 0 and 4, and 12-weekly thereafter. Venous blood samples were
collected during routine clinical review irrespective of the dosing schedule (Supplementary Figure S6). Blood was collected in serum separating clot activator tubes (Vacutte, Greiner Bio-One, Kremsmünster, Austria), processed, and serum stored at −80 °C. Only patients who were ustekinumab-naive before enrollment were analyzed. A total of 10 patients had multiple samples within the selected time frame, and only the last sample was analyzed.

**Drug level assay**

Ustekinumab level in patient sera was measured using an ELISA validated for clinical diagnostics at Sanquin diagnostics services (Menting et al., 2015). Briefly, recombinant IL-12p70 (R&D systems, Minneapolis, MN) was immobilized with anti–IL-12p35 (Diaclone, Besancon, France) onto MaxiSorp ELISA plates (Thermo Fisher Scientific, Waltham, MA). Patient serum was serially diluted in high-performance ELISA buffer (Sanquin, Amsterdam, Netherlands) and incubated in the plate for 1 hour at room temperature. Next, plates were incubated with biotinylated rabbit anti–ustekinumab-idiotype (generated in a similar way to that described previously) (Rispens et al., 2011), followed by polymerized horseradish peroxidase–conjugated streptavidin (Sanquin) and developed using 3,3',5,5'-tetramethyi benzidine. Between each step, plates were washed five times with PBS-Tween (0.02% tween-20 (Merck, Darmstadt, Germany) in PBS). Absorption was measured at 450 nm, and ustekinumab serum levels were calculated from a two-fold serially diluted titration curve of ustekinumab. The lower limit of quantification of this assay was 20 ng/ml.

**Drug-sensitive RIA**

The drug-sensitive RIA was performed as previously described and is validated for clinical diagnostics (Menting et al., 2015). Briefly, 1 μl serum diluted in freeze medium (Sanquin) was incubated overnight with sepharose-immobilized protein A (1.0 mg/test; Pharmacia, Uppsala, Sweden) in the presence of 1.0 ng/test biotin-conjugated ustekinumab (F[antibody]). Nonbound serum components were removed by washing five times before 50 μl 125I-labeled streptavidin was added in 500 μl PBS-AT (0.3% BSA [Millipore, Burlington, MA], 0.01 M EDTA [Scharlau, Spain], 0.004% tween-20, 0.05% sodium azide). After overnight incubation, samples were washed five times, and sepharose-bound radioactivity was measured using a gamma counter (2480 WIZARD2, PerkinElmer, Waltham, MA). Assay results were converted to AU/μl from a two-fold serially diluted calibration curve of rabbit anti–ustekinumab-idiotype diluted in 2% human serum, range 1,500–12 AU/μl extrapolated to 0 AU (blank serum), using logit regression. The limit of detection of 3 AU/μl for this assay was calculated as the mean ±3 SD assay signal from 48 sera from patients with ustekinumab-naive psoriasis after outlier analysis, which corresponds to a false positivity rate of 0.1%. In this assay, 1.0 ng of affinity-purified ustekinumab-specific rabbit anti–idiotype antibody yielded approximately 1.2 AU. Drug tolerance of the assay was assessed by spiking ustekinumab within (maximum 10 μg/ml) and above (50 μg/ml) the expected therapeutic range into a pool of healthy donor sera containing a titration of ADAs (polyclonal rabbit anti-ustekinumab).

dtELISA

**Screening assay.** To dissociate potential ustekinumab-ADA complexes, we incubated 13.3 μl patient serum with 55.3 μl dissociation buffer (300 mM acetic acid [Merck] with 12.5% 100 mM hydrochloric acid [Merck]) at a final pH 3.25 in 96-well conical-bottom plates (Thermo Fisher Scientific) for 45 minutes at room temperature. Samples were supplemented with 33.3 μl buffer containing 33.3 (v/v) neutralization buffer (1.5 M Tris [Invitrogen, Carlsbad, CA] in hydrogen dioxide) and 66% detection buffer (0.1% tween-20, 1% BSA, 1.0 mg/ml IgV [Nanogram, Sanquin] in PBS) with 0.75 μg/ml biotinylated ustekinumab and 0.75 μg/ml cFLAG-conjugated (DYKDDDDKC, Dirs El Atmoui, Netherlands Cancer Institute, the Netherlands [van Beek et al., 2017]) ustekinumab and incubated for 1 hour at room temperature. Samples were transferred to MaxiSorp ELISA plates precoated with 1.0 μg/ml anti–FLAG antibody (clone M2, Sigma, St. Louis, MO) to capture ustekinumab-cFLAG/ADA/ustekinumab-h complex. After incubation for 1 hour at room temperature, plates were washed five times with PBS-Tween and incubated with polymerized horseradish peroxidase-streptavidin in detection solution, washed, and developed using 3,3',5,5'-tetramethyi benzidine. Absorbance was measured at 450 nm. Ustekinumab serum levels were calculated from a two-fold serially diluted calibration curve of human anti-ustekinumab (generated in a similar way to that described previously) (Rispens et al., 2011), followed by polymerized horseradish peroxidase–conjugated streptavidin (Sanquin) and developed using 3,3',5,5'-tetramethyi benzidine. Between each step, plates were washed five times with PBS-Tween (0.02% tween-20 (Merck, Darmstadt, Germany) in PBS). Absorption was measured at 450 nm, and ustekinumab serum levels were calculated from a two-fold serially diluted titration curve of ustekinumab. The lower limit of quantification of this assay was 20 ng/ml.

**Confirmation assay.** The noninhibited condition was an exact copy of the screening assay. In the inhibited condition, a serum equivalent of 10 μg/ml unlabeled ustekinumab was added during sample neutralization. The percentage of signal inhibition between the inhibited and the noninhibited condition was calculated from the raw assay signal (optical density). The cut-point of 30% inhibition was calculated as 1 minus the antilog of the mean ±2.33 SD (1% false-positive rate) of the log-transformed absorbance of 32 samples from patients with ustekinumab-naive psoriasis after outlier analysis. The drug tolerance of the assay was assessed in a similar way to the RIA. Samples with an ADA titer above the cut-point were measured in the confirmation assay.

**Clinical assessment**

Disease severity was scored at baseline and on the day of sample collection using the gold-standard objective assessment tool, the PASI. Baseline was defined as the most recent PASI recorded before the start of ustekinumab treatment (median 13 days before the start, IQR = 0–31 days, maximum 178 days).

**IL-12 competition assay**

The IL-12 competition assay was performed in a similar way to the previously described TNF competition assay (van Schie et al., 2015). Only samples with >75 AU/ml ADAs as determined in the RIA were selected for this analysis to allow assessment of the percentage of inhibition with reasonable precision. Samples were diluted, such that each tube contained approximately 300 AU/ml ADAs. Samples were analyzed in the RIA as described above, but using Human Fc affinity Matrix (CaptureSelect, Thermo Fisher Scientific) for IgG capture and in the presence of 100 μl/test fetal calf serum (Bodinco, Alkmaar, The Netherlands), with and without preincubation of ustekinumab F(antibody)2 with 250 ng or a serial dilution from 500 ng to 7.2 ng IL-12/23p40 (Peprotech, London, United Kingdom). Assay signals were converted to ng/ml calculated from a two-fold serially diluted calibration curve of human anti-ustekinumab (HCA210, Bio-Rad, Hercules, CA) diluted in 2% human serum, range 40–0.08 ng/ml extrapolated to 0 ng/ml (blank serum), using
logit regression. The percentage of neutralization was calculated from the converted assay signals from IL-12/23p40 preincubated condition by the condition without IL-12/23p40.

**Statistical analyses**

Univariable analyses were performed with indicated tests using GraphPad Prism software version 8.0.2. 95% CI of differences in drug level and clinical outcome between patients with ADA-positive and ADA-negative were calculated using Hodges-Lehmann estimated medians for comparison. The relationship between ADA titer and clinical outcome was calculated by linear regression analysis. Outlier analysis for the limit of detection and cut-point calculation was performed using iterative Grubbs’ (α = 0.05). To explore the association between ADA status, determined using the RIA or the dtELISA and drug level or treatment outcome in the presence of other covariates (collected at baseline: gender, age, length, waist, weight, PASI, disease duration, biologic naive status, dose regimen, past alcohol use, past smoking; at the time point of sampling: days after treatment initiation, recorded days since last dose, waist, weight, alcohol use, smoking, ustekinumab level [only in disease outcome analysis]), MANOVA was performed using R (https://www.r-project.org). Covariates with a lognormal distribution were log-transformed after setting zero-values to half of the smallest nonzero entry. Covariates associated with drug level or treatment outcome at a significance level of P < 0.1 were stepwise taken forward to a MANOVA. Covariates with a significance level P > 0.05 were stepwise removed from the final model.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author, FCL, on reasonable request.

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**CONFLICT OF INTEREST**

CEMG reported receiving honoraria and/or research grants support (University of Manchester) from AbbVie, Bristol-Myers Squibb, Celgene, GlaxoSmithKline, Novartis, Pfizer, Regeneron, Roche, and an Medical Research Council consortium, which had several industry partners (see psort.org.uk). TR reported receiving honoraria for lectures from AbbVie, Pfizer, and Regeneron, a research grant from Genmab. The remaining authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: FCL, GJW, CHS, TR; Data Curation: FCL, TT, MD, BD, AR, ND, AdV, KB; Formal Analysis: FCL, TR; Funding Acquisition: CEMG, NJR, JB, AD, RBW, CHS, TR; Investigation: FCL, TT, LD, MHH, AdV; Methodology: FCL, LD, MHH, TR; Resources: CEMG, NJR, JB, AD, RBW, CHS, TR; Supervision: GJW, CHS, TR; Writing - Original Draft Preparation: FCL, TR; Writing - Review and Editing: FCL, TT, ND, RBW, CHS, TR

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.03.957.

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