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Clinical relevance of innovative immunoassays for serum ustekinumab and anti-ustekinumab antibody levels in Crohn's disease

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Key words
ustekinumab, anti-ustekinumab antibody, therapeutic drug monitoring.

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Abstract

Background and Aim: Ustekinumab is a human monoclonal antibody to the p40 subunit of human IL-12/IL-23. The purpose of this report is to verify the newly developed immunoassays for serum ustekinumab and anti-ustekinumab antibody (AUA) concentrations and assess their clinical utility.
Methods: Serum ustekinumab trough levels and AUA levels were measured using new immunoassays in 38 patients with Crohn's disease under ustekinumab maintenance injection.
Results: Mean ustekinumab trough levels were 2.54 ± 2.1 μg/mL, and 3 of 38 patients (7.9%) were positive for AUAs. There was no association between ustekinumab trough levels and AUA levels. The optimal trough level of ustekinumab to maintain negative C-reactive protein levels (≤ 0.3 mg/dL) was 1.67 μg/mL determined by receiver operating characteristic curve analysis. Ustekinumab trough level negatively but significantly correlated with C-reactive protein, erythrocyte sedimentation rate, and Crohn's disease activity index and positively and significantly correlated with serum albumin levels. Ustekinumab trough levels were significantly higher in biologics-naïve patients than in biologics-experienced patients, although there was no difference in AUA levels.
Conclusions: We developed new assays for serum ustekinumab trough and AUA levels. These assays might provide new insights into therapeutic drug monitoring-based management of Crohn's disease patients under ustekinumab therapy.

Introduction

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis, is a chronic relapsing inflammatory disorder of the gastrointestinal tract. Although various genetic, microbial, and environmental factors have been reported to be associated with intestinal inflammation, the precise etiology of IBD remains unclear.[1]

Interleukin (IL)-12 and IL-23 are heterodimeric pro-inflammatory cytokines that share a common p40 subunit, which pairs with the p35 subunit to form IL-12 and with the p19 subunit to form IL23.[2] IL-12 and IL-23 play pivotal roles in the pathogenesis of IBD through induction of Th1 and Th17 responses, respectively.[3] These cytokines represent attractive therapeutic targets for the treatment of IBD.[4] Ustekinumab is a human immunoglobulin (Ig)G1 monoclonal antibody that binds specifically to the p40 subunit of human IL-12/IL-23 and neutralizes their bioactivity.[5] Previous clinical studies have shown that ustekinumab is effective for the induction of clinical remission and clinical improvement in patients with moderate-to-severe CD.[6–10] Recently, its clinical effectiveness in inducing and maintaining remission in moderate-to-severe ulcerative colitis has been reported.[11]

Therapeutic drug monitoring (TDM) is the clinical practice of determining serum drug and/or anti-drug antibody levels to guide clinical decision making. The clinical usefulness of TDM has been reported mainly in IBD patients with tumor necrosis factor (TNF)-α antagonist therapy.[12–18] Adequate serum concentrations of TNF-α antagonists are associated with sustainable clinical responses,[12] and TDM is helpful in identifying mechanisms underlying a loss of response to biologics.[12] Proactive TDM has been shown to be associated with fewer surgeries, higher rates of mucosal healing, and a lower risk of treatment failure than conventional non-TDM-based care.[14,19,20] The TDM-based approach is more cost-effective than a clinically based approach in IBD patients with loss of response to TNF-α antagonists.[15,21]

There is an increasing number of reports concerning ustekinumab TDM in IBD patients.[18,22–27] However, the assays

for ustekinumab concentrations suitable for routine clinical use have not been generally available yet. Recently, Adedokun *et al*. reported using an assay proprietary to their company that an 8-week interval injection of ustekinumab induced threefold higher trough levels compared with a 12-week interval injection[23] and that a high trough of ustekinumab was associated with achievement of clinical remission and endoscopic improvement.[23] There are some available assays, but they are expensive and/or require special instruments and materials. Batta *et al*. reported using generally available homogeneous mobility shift assay[25] that the higher ustekinumab trough levels were associated with endoscopic response in CD patients. Verstocht *et al*. addressed a relationship between ustekinumab trough levels and endoscopic response in CD patients using an assay developed in their laboratory.[26] Their assay required a special material such as anti-ustekinumab idiotype antibodies.[26]

In this study, we report new immunoassays for measurement of serum ustekinumab and anti-ustekinumab antibody (AUA) concentrations. Prevention of non-specific IgG binding to the plate is a key step in the development of a measurement system for serum ustekinumab concentrations, because ustekinumab is a drug of human IgG preparation. In this study, we successfully prevented non-specific serum IgG binding using a specially coated plate. In addition, we developed an immunoassay for AUA concentration that works even in the presence of free ustekinumab (drug-tolerant assay). These assays are relatively low cost and need no special materials such as radioisotope and/or anti-ustekinumab idiotype antibodies and no expensive measurement devices. These methods can be built with reproducibility in any laboratory and might allow the introduction of ustekinumab TDM into routine clinical care of IBD patients.

Methods

Patients. Thirty-eight patients with CD were enrolled. These patients were treated with ustekinumab at five academic hospitals (the Shiga University of Medical Science Hospital, the Hyogo Medical College Hospital, the Fujita Health University Hospital, the Ofuna Chuo Hospital, and the National Hospital Organization Higashi-Ohmi General Medical Center). The demographic characteristics of the study patients are described in Table 1. Healthy volunteers ($n = 25$) were enrolled to determine the background levels of assays.

Ustekinumab was introduced by a one-time intravenous infusion according to the patient's bodyweight (260 mg for patients < 55 kg, 390 mg for patients between 55 and 85 kg, and 520 mg for patients > 85 kg). The patients then received ustekinumab subcutaneous injection (90 mg per body) every 8 weeks. Blood was collected before the next injection (trough concentration). The average number of ustekinumab injections received by the patients was 5.5 ± 1.4 times (mean ± SD).

Ethics. The study protocol was approved by the institutional review boards of the Shiga University of Medical Science (permission no. R2017-136) and all institutes included in this study. All patients gave their written informed consent before their inclusion in this study. The registration number of the University Hospital Medical Information Network Center (UMIN) was 000033552.

Table 1 Baseline demographic and disease characteristics of the patients

Healthy controls ($n = 25$)	
Female/male	5/20
Age [years: median (range)]	33 (26–46)
Crohn's disease ($n = 38$)	
Female/male	20/18
Age [years; median (range)]	38 (21–72)
Type of Crohn's disease	
L1 (ileal)	10 (26.3%)
L2 (colonic)	7 (18.4%)
L3 (ileocolonic)	21 (55.3%)
Medication	
5-ASA	29
Azathioprine/6-MP	19
Prednisolone	5
Enteral nutrition	15
Biologics naïve	13
Switched from IFX	11
Switched from ADA	14
Duration of ustekinumab treatment [weeks: median (range)]	48 (24–64)

6-MP, 6-mercaptopurine.

Labeling of recombinant human IL-12 p40 and ustekinumab. Biotin-labeling of recombinant human IL-12 p40 (Pepro Tech, Rocky Hill, NJ) was performed using a commercially available biotin-labeling kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Horseradish peroxidase (HRP) labeling of ustekinumab was performed using a commercially available HRP conjugation kit (Solulink, San Diego, CA).

Measurement of serum ustekinumab concentrations. Serum ustekinumab levels were determined by an immunoassay, constructed according to the method described previously.[28] We used an avidin ELISA plate® (blocking-less type; Sumitomo Bakelite Co., Ltd, Tokyo, Japan), which is ready to use with a special coating to minimize non-specific protein binding. This plate was coated with biotinylated-IL-12 p40 (100 μL of 0.5 μg/mL) by incubation for 2 h. After extensive washing, a further blocking was performed with Block Ace® (DS Pharma Biomedical, Co., Ltd., Suita, Japan). After washing, samples (100 μL of 100-fold diluted serum) were incubated for overnight at 4 °C. Finally, the reacted ustekinumab was detected by HRP-labeled F $(ab')_2$ fragments of chicken antihuman IgG (×20 000 diluted; Thermo Fisher Scientific Co., Ltd., Waltham, MA). 3,3′,5,5′-Tetramethylbenzidine (Nacalai Tesque, Kyoto, Japan) was used for color development.

Measurement of serum anti-ustekinumab antibody concentrations. An immunoassay for AUAs that works in the presence of ustekinumab (drug-tolerant assay) was developed according to the methods described previously.[28,29] Immune

complexes of ustekinumab and AUA in samples were dissociated by treatment with 0.1-M glycine-HCl buffer (pH 2.7), and IgG fraction was isolated using protein G beads. IgG was eluted, and the concentration was adjusted to 20 μg/mL IgG with a carbonate–bicarbonate buffer (pH 9.6). Each well of a 96-well ELISA plate was coated with diluted IgG containing AUAs (100 μL) overnight. AUAs on the plate were detected by 3-h incubation with HRP-labeled ustekinumab (100 μL of 2.0 μg/mL). 3,3′,5,5′-Tetramethylbenzidine was used for color development. The values were reported in μg/mL-calibrated (μg/mL-c) according to calibration standards using polyclonal goat antihuman IgG (MP Biomedicals, LLC, Solon, OH).

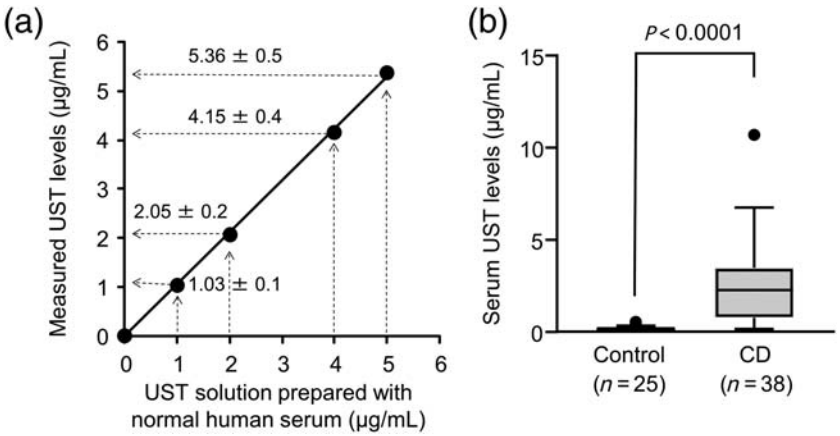

Figure 1 Accuracy of developed assay for ustekinumab. (a) In order to confirm effective prevention of non-specific serum IgG binding, we prepared ustekinumab standards (0, 1, 2, 4, and 5 μg/mL) using normal human serum. The obtained results coincided with the prepared concentrations, indicating accurate measurement of this assay. Each point represents the mean of measured values (n = 25). (b) The background levels determined by samples from healthy individuals were 0.14 ± 0.13 μg/mL (mean ± SD, n = 25), and serum ustekinumab trough levels in CD patients were 2.5 ± 2.1 μg/mL (n = 38).

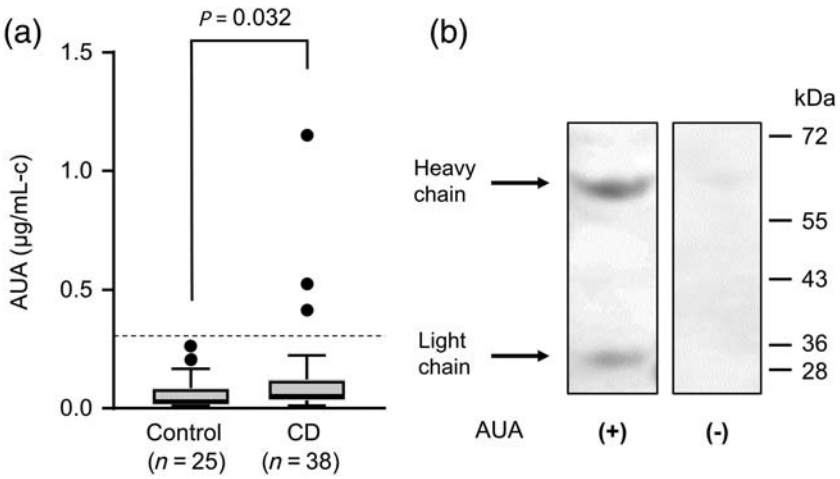

Figure 2 Assays for serum anti-ustekinumab antibody (AUA). (a) The cut-off value for a positive result of AUAs was determined as 0.27 μg/mL-c (mean + 3SD of healthy controls), based on the results of healthy controls (0.064 ± 0.071 μg/mL-c, n = 25). Three of 38 CD patients (7.9%) were positive for AUAs. (b) Western blot analysis showed that IgG isolated from an AUA-positive patient reacted with ustekinumab immobilized on membrane.

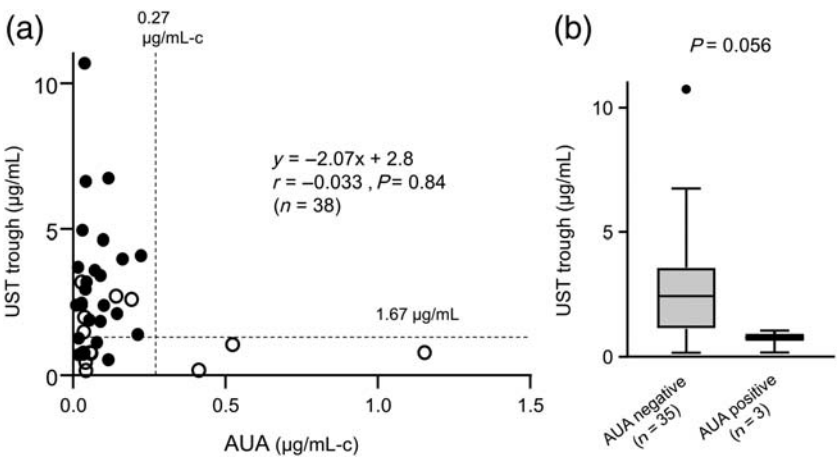

Figure 3 Association of serum AUA levels with ustekinumab trough levels. (a) There was no association between serum AUA and ustekinumab trough levels (y = −2.07x + 2.8, r = −0.03, P = 0.84, n = 38). (b) Ustekinumab trough levels tended to be lower in the patients positive for AUAs (mean 0.66 μg/mL, n = 3) than in the patients negative for AUAs (2.7 ± 2.1 μg/mL, n = 35). •, CRP ≤ 0.3 mg/dL; ○, CRP, > 0.3 mg/dL.

Table 2 The clinical utility of ustekinumab trough levels depicted as normal CRP levels (≤ 0.3 mg/dL)

n, CRP ≤ 0.3/CRP > 0.3	25/13
AUC (95% CI)	0.79 (0.64–0.94)
P value	0.004
Odds ratio (95% CI)	7.13 (1.6–31.7)
Sensitivity (95% CI)	0.76 (0.57–0.89)
Specificity (95% CI)	0.69 (0.42–0.87)
Ustekinumab cut-off (μg/mL)	1.67

CI, confidence interval.

Western blotting. Western blotting was performed according to the method described previously.[28,29]

Statistical analyses. The chi-squared test or Mann–Whitney *U*-test was used to evaluate the association between two independent groups. The cut-off values of ustekinumab concentration associated with normal C-reactive protein (CRP) value (≤ 0.3 mg/dL) were determined using receiver operating characteristic (ROC) curve analysis. All statistical testing was performed at the 0.05 significant level.

Results

Accuracy of newly developed immunoassays for ustekinumab. Because ustekinumab is a human IgG1 monoclonal antibody, prevention of non-specific binding of serum IgG is a critical step for the measurement of serum ustekinumab levels. In order to check an effective prevention of non-specific serum IgG binding, we prepared ustekinumab standards (0, 1, 2, 4, and 5 μg/mL) using dilution by normal human serum. These standards were put on the current assay, and agreement between the prepared standards and the measurement results was confirmed (Fig. 1a). This means that the developed system can be used for the measurement of serum ustekinumab levels. Background levels obtained from the samples of healthy individuals were 0.14 ± 0.13 μg/mL (mean ± SD, n = 25) (Fig. 1b), and mean ustekinumab trough levels in CD patients were 2.5 ± 2.1 μg/mL (n = 38) (Fig. 1b).

Determination of anti-ustekinumab antibody. We constructed an immunoassay for serum AUAs according to the previously reported method for anti-infliximab antibodies in our laboratory.[29] This is a so-called drug-tolerant assay, which allows

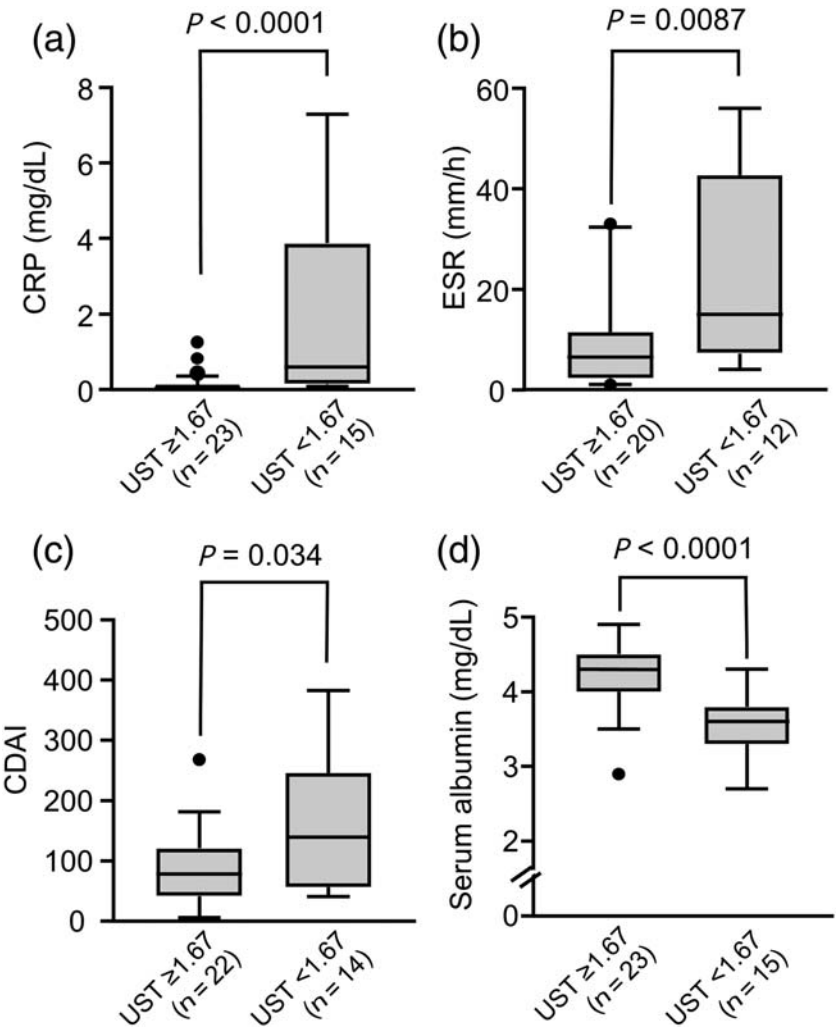

Figure 4 Relationship between ustekinumab trough levels and clinical markers. (a, b, and c) CRP, ESR, and CDAI (Crohn's disease activity index) were significantly lower in patients with ustekinumab trough levels ≥ 1.67 μg/mL than in patients with ustekinumab trough levels < 1.67 μg/mL. (d) Serum albumin was significantly higher in patients with ≥ 1.67 μg/mL than in patients with ustekinumab trough levels < 1.67 μg/mL.

the measurement of AUA in the presence of free ustekinumab. As shown in Figure 2a, the results of healthy controls (n = 25) were 0.064 ± 0.071 μg/mL-c (mean ± SD), and the cut-off value was set at 0.27 μg/mL-c (mean + 3SD). A result of 0.27 μg/mL-c or more was judged to be AUA-positive, and three of 38 CD patients (7.9%) were positive for AUAs. Western blot analysis revealed that IgG fraction isolated from an AUA-positive patient reacted with ustekinumab immobilized on nitrocellulose membrane (Fig. 2b).

Relationship between serum anti-ustekinumab antibody levels and ustekinumab trough levels. Ustekinumab trough levels and AUA levels were with whether CRP level is positive or not (CRP cut-off, 0.3 mg/dL) (Fig. 3a). There was no association between ustekinumab trough levels and AUA levels (y = −2.07x + 2.8, r = −0.033, P = 0.84, n = 38). Three patients positive for AUAs were positive for CRP (≥ 0.3 mg/dL). As shown in Figure 3b, ustekinumab trough levels tended to be lower in AUA-positive patients (mean, 0.66 μg/mL, n = 3) than in AUA-negative patients (mean ± SD; 2.7 ± 2.1 μg/mL, n = 35).

The cut-off values of ustekinumab concentration predicting normal CRP value (≤ 0.3 mg/dL) were determined using ROC curve analysis (Table 2). The cut-off value identifying whether CRP is ≤ 0.3 mg/dL was 1.67 μg/mL of ustekinumab.

Association between clinical markers and ustekinumab trough levels. C-reactive protein and erythrocyte sedimentation rate (ESR) were significantly lower in CD patients with ustekinumab trough levels ≥ 1.67 μg/mL than in patients with trough levels < 1.67 μg/mL (Fig. 4a,b). Similarly, Chron's disease activity index (CDAI) was significantly lower in CD patients with trough levels ≥ 1.67 μg/mL (Fig. 4c). Serum albumin levels were significantly higher in patients with ustekinumab trough levels ≥ 1.67 μg/mL than in patients with trough levels < 1.67 μg/mL (Fig. 4d).

Negative significant correlations of ustekinumab trough levels with CRP, ESR, and CDAI were observed (Fig. 5a–c). There was a positive significant correlation between ustekinumab trough levels and serum albumin levels (Fig. 5d).

Effects of prior experience of anti-tumor necrosis factor-α antagonists. This study included anti-TNF-α antagonist-naïve (biologics-naïve) patients (n = 13) and patients who had experienced anti-TNF-α antagonists and had then switched to ustekinumab (biologics-switched patients) (n = 25). There was no significant difference in serum AUA levels between biologics-naïve and biologics-switched patients (Fig. 6a). However, ustekinumab trough levels were significantly higher in biologics-naïve patients than in biologics-switched patients (Fig. 6b). Serum albumin levels were significantly higher in

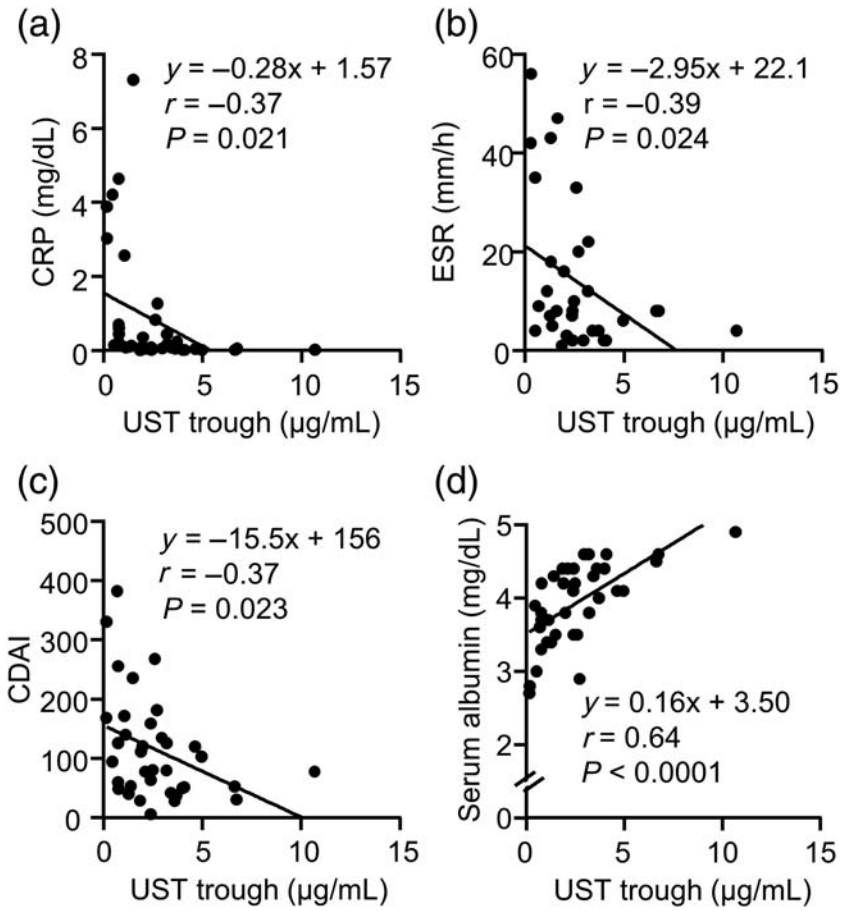

Figure 5 Association of ustekinumab trough levels with clinical markers. (a, b, and c) Serum CRP, ESR, and CDAI were negatively and significantly correlated with ustekinumab trough levels. (d) Serum albumin levels positively and significantly correlated with ustekinumab trough levels.

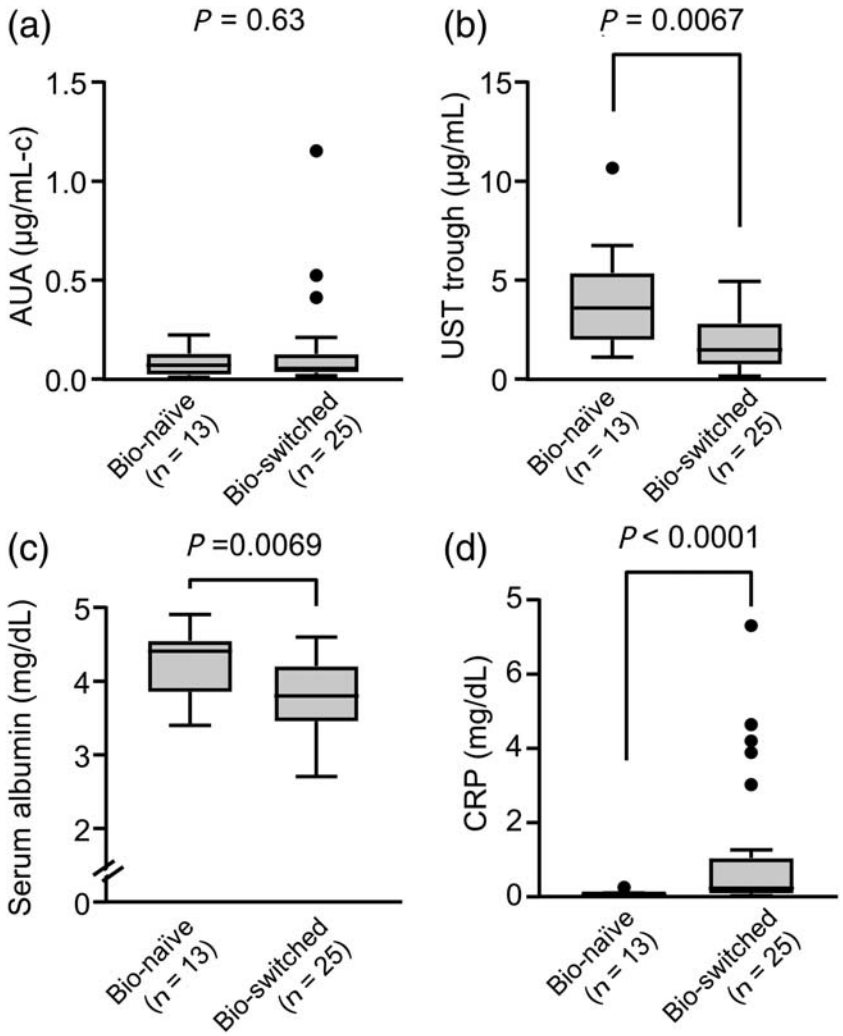

Figure 6 Influence of preceding TNF antagonists to AUA and ustekinumab trough levels. (a) There was no difference in serum AUA levels between biologics-experienced (bio-switched) patients and biologics-naïve (bio-naïve) patients. (b, c) Ustekinumab trough levels and serum albumin levels were significantly higher in biologics-naïve patients than in biologics-experienced patients. (d) CRP levels were significantly higher in biologics-experienced patients than in biologics-naïve patients.

biologics-naïve patients (Fig. 6c), and CRP levels were significantly lower in biologics-naïve patients (Fig. 6d).

Discussion

The introduction of TNF-α antagonists reduced the risk of irreversible bowel complications and caused a paradigm shift in the natural history of CD with reduced necessity for surgery and hospitalization. However, one third of patients do not have an initial response to TNF-α antagonists and one third of patients experience a loss of response associated with insufficient drug levels due to the development of anti-drug antibodies.[30] In such circumstance, ustekinumab was shown to be effective in patients with moderate-to-severe CD, particularly among patients who previously received anti-TNF-α antagonists.[8]

In this study, we reported new assays for serum ustekinumab and AUA levels that might be useful for routine clinical care. These assays are relatively low cost and need no special materials such as radioisotopes and/or anti-ustekinumab idiotype antibodies and no expensive measurement devices. Because ustekinumab is human IgG, the most important step of our assay was complete prevention of non-specific binding of serum IgG. For this purpose, we used a commercially available special ELISA plate, which enable to use HRP-labeled antihuman IgG antibodies as a detection antibody. The background values in control serum were negligible (Fig. 1a), and this means that serum IgG did not interfere with ustekinumab measurement. Our result that ustekinumab trough level to predict negative CRP was 1.67 μg/mL, and this was coincided with the values in a recent report described by Addokun *et al*.[23] where ustekinumab trough levels of 0.8 to 1.4 μg/mL was required to maintain a clinical remission.[23]

Almost all previously reported assays for anti-drug antibodies are based on a two-site immunoassay in which the drug is used for both capture and detection.[29,31] The major problem in such systems is interference of detection by the drug presenting in patients' serum (drug-sensitive assay). The drug in the patients' serum forms an immune-complex with anti-drug antibodies and interferes with *in vitro* detection by the enzyme-labeled drug.[29] A critical step of the current system for AUAs is acidic buffer treatment of the ustekinumab-AUA immune complexes.[29] This process dissociates AUA from ustekinumab and recovers the binding capacity of enzyme-labeled ustekinumab.[29] The cut-off value of AUAs was determined as 0.27 μg/mL-c based on the results for healthy individuals, and 7.9% (3 of 38) of CD patients on

ustekinumab maintenance therapy were positive for AUAs. This positive rate is slightly higher than in the findings of Addokun *et al*. (2.3%).[23] This may be due to differences in the assay system and/or study design. Using similar assay systems to the current study, we have previously reported that the positive rates for anti-infliximab and anti-adalimumab antibodies were 27.6% and 35%, respectively.[28,29] These observations indicate a lower immunogenicity of ustekinumab compared with infliximab and adalimumab. Furthermore, previous studies of infliximab and adalimumab demonstrated a negative but significant correlation between drug trough levels and anti-drug antibody levels.[28,29,32,33] In contrast, there was no association between ustekinumab trough levels due to low immunogenicity of ustekinumab. Three patients positive for AUAs also exhibited low ustekinumab trough levels and positive CRP, suggesting a neutralizing activity of detected AUAs.

We investigated ustekinumab trough level that distinguishes patients with or without a normal CRP value (≤ 0.3 mg/dL). ROC and AUC analyses identified an optimal trough cut-off level of 1.67 μg/mL for negative CRP levels. ESR, serum albumin levels, and CDAI were significantly improved in patients with ustekinumab ≥ 1.67 μg/mL. In addition, significant (negative or positive) correlations between ustekinumab trough levels and clinical markers (CRP, ESR, serum albumin, and CDAI) were observed. These findings suggest that a ustekinumab cut-off of 1.67 μg/mL may be optimal as a predictor of favorable responses to ustekinumab.

In this study, ustekinumab was more effective in biologics-naïve patients than in biologics-experienced patients, and this was accompanied by lower trough levels. A similar observation has been reported in global clinical trials of ustekinumab induction and maintenance therapy in CD patients (UNITI-1, UNITI-2, and IM-UNITI).[8,10,34] In general, biologics are more effective in biologics-naïve patients than in biologics-experienced patients. For example, adalimumab has been shown to be more effective for biologics-naïve patients compared with infliximab-experienced patients.[23,35] As one of the mechanisms underlying such a phenomenon, we have previously suggested that lower trough levels due to easy development of anti-drug antibodies may contribute to lower response to next biologics.[23] In this study, the ustekinumab trough level was lower in biologics-experienced patients than in biologics-naïve patients, but AUA levels did not increase in biologics-experienced patients. This means that immunogenicity of ustekinumab did not drive lower trough levels in biologics-experienced patients. Although the precise mechanism underlying lower trough levels of ustekinumab in biologics-experienced patients remains unclear, a higher consumption of ustekinumab associating with remaining mucosal inflammation may be responsible for this response.

This study has some limitations based on the small number of patients. First, although the main goal to confirm the accuracy of the developed assays was achieved, the clinical usefulness of the developed assays should be re-evaluated in a larger patient group. Second, the ability of the cut-off value of serum ustekinumab levels to predict efficacy should be improved by evaluation in a much greater number of patients. Finally, although the incidence of AUAs was low and had an impact on efficacy, this finding should be treated with caution due to a few AUA-positive patients in this study.

In conclusion, we developed improved immunoassays for the accurate measurement of ustekinumab trough and AUA levels without special materials. The immunogenicity of ustekinumab was very low compared with TNF-α antagonists such as infliximab and adalimumab, suggesting a low incidence of loss of response to ustekinumab. In order to confirm such a clinical character of ustekinumab, we are proceeding with the verification of more patients.

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