Comparison of Two Different Techniques to Assess Adalimumab Trough Levels in Patients with Crohn’s Disease

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INTRODUCTION

Tumor necrosis factor (TNF) alpha is likely the most important cytokine involved in the pathogenesis of inflammatory bowel diseases (IBD), and is the main target molecule of IBD monoclonal antibody therapy [1-5]. Anti-TNF antibody treatment with either infliximab or adalimumab (ADA) is extremely effective in patients with IBD, although approximately 30% of Crohn’s disease (CD) patients who respond to infliximab need a dose escalation or an interval adjustment in order to maintain a long-term clinical response. Treatment discontinuation due to loss of response (LOR) occurs in approximately 10% of patients per year [6-11]. As far as ADA treatment is concerned, Billioud et al. showed that the annual risk for dose intensification was 24.8% per patient-year, while the mean LOR rate was approximately 20% per year [12].

There are several reasons for LOR to anti-TNF treatment, although one of the main causes of LOR is decreased drug levels due to the development of anti-drug antibodies (AA) [13]. Anti-drug antibodies - typically IgG antibodies - bind the drug, thereby neutralizing its effect, and the resulting immune complexes have a faster clearance in the reticulo-endothelial system than the drug alone, thus resulting in an altered drug pharmacokinetics and in a reduction of its therapeutic efficacy [14, 15]. Recent studies have emphasized the importance of...
assessing serum trough levels of anti-TNF drugs and AA in order to predict the clinical efficacy of biological therapy [16-19]. In this regard, Fasanmade et al. observed that early treatment responders with higher infliximab trough levels had a greater CD activity index decline than patients with lower concentrations, while patients with lower infliximab trough levels were less likely to achieve complete fistula response [20]. Lastly, Colombel et al. showed that the rates of corticosteroid-free clinical remission were greater in patients with higher infliximab trough levels at week 30 [21]. In these studies, serum trough levels were measured by the enzyme-linked immunosorbent assay (ELISA) based on the principle that the biological drug is captured via its ability to bind TNF [22, 23]. As far as ADA is concerned, some studies have shown that low serum drug concentrations assessed by means of homogeneous mobility shift assay (HMSA) were associated with high C-reactive protein (CRP) values, while higher drug levels were associated with increased rates of mucosal healing [24]. However, there are no studies that have assessed ADA trough levels with both ELISA and HMSA methods.

In this study, our aim was to compare two different techniques - ELISA and HMSA - in assessing ADA trough levels and antibodies in a series of patients with CD, and to evaluate their diagnostic accuracy for LOR during long-term follow-up.

PATIENTS AND METHODS

In this study we enrolled 23 patients with moderate to severe CD [Harvey-Bradshaw Index (HBI) >8], consecutively treated at our Unit between June, 2011 and June, 2013 and who had obtained clinical remission with ADA mono-therapy. Adalimumab was administered at an induction dose of 160/80 mg and then at standard doses of 40 mg every 2 weeks [25]. Inclusion criteria were: male and female patients between the ages of 18 and 80, diagnosis of CD (confirmed by endoscopic, radiologic and histologic evaluation), anti-TNF drug naïve patients. Exclusion criteria were: diagnosis of ulcerative colitis, symptomatic obstructive disease, ileostomy, extensive small bowel resection (as determined by the investigator) or a short bowel syndrome, positive Clostridium difficile stool assay, azathioprine or methotrexate concomitant administration. Moreover, before starting ADA therapy all patients were evaluated to exclude latent tuberculosis (purified protein derivative skin test or QuantiFERON test, chest radiography, and careful history taking), hepatitis C and hepatitis B infections.

All subjects underwent careful history taking and a physical examination. At the beginning of the study, all patients underwent routine biochemistry, lower gastrointestinal endoscopy, and Magnetic Resonance Imaging or Computed Tomography of the small bowel. The modified Vienna classification was used to assess CD location and behavior [26].

After achieving clinical remission, defined as an HBI <4, patients were included in a prospective follow-up program in order to assess the maintenance of CD clinical remission. Patients underwent routine biochemistry evaluation every 8 weeks, or earlier in the case of relapse or suspicion of relapse. At baseline, at week 48, and at week 96 we also planned to withdraw 12 ml of peripheral blood to dose ADA and AA. Samples were collected just before ADA routine injection (every other week). A relapse was defined by an HBI >7 over two consecutive weeks.

The study was performed according to the Declaration of Helsinki. All patients were asked to give written informed consent at study inclusion.

Therapeutic drug monitoring of ADA and AA

The ADA trough concentration was assessed by means of both HMSA (Prometheus® Anser™ ADA Assay, Prometheus Laboratories, San Diego, CA, USA) and ELISA test (Shikari® Q-ADA Matriks Biotek, Ankara, Turkey).

The performance characteristics of the Prometheus® Anser™ ADA Assay standard curve was assessed in 29 experimental runs by multiple analysts using different instruments over various days [27]. In this study, the coefficient of variation for all concentrations except the lowest one was <25%, and the dynamic range was two orders of magnitude; the intra-assay precision was <20% and the accuracy was <3%, whereas the inter-assay precision was <12% and the accuracy was <22%. Cut-point for ADA values with the Prometheus® Anser™ were determined from 100 serum samples collected from ADA-naïve, healthy subjects (value, 0.676 mcg/mL: 100% sensitivity, 97% specificity). The AA were assessed using the same assay, and AA positivity was defined as the presence of a value >1.7 U/mL. This assay is able to measure AA serum concentrations in the presence of drugs. More in detail, the Prometheus® Anser™ is able to detect AA levels as low as 10 U/mL in the presence of an ADA concentration of 20 μg/mL [27].

The second method used to evaluate ADA concentrations in serum is a solid phase ELISA based on the sandwich principle. Adalimumab concentration standards and diluted samples are incubated in the microtiter plate coated with the reactant for ADA. After incubation, the wells are washed, a horse radish peroxidase conjugated probe is added and binds to ADA captured by the reactant on the surface of the wells. Following incubation, wells are washed and the bound enzymatic activity is detected by the addition of chromogen-substrate. The color developed is proportional to the amount of ADA in the sample or standard. Results of samples can be determined directly using the standard curve.

Statistical analysis

Data are presented as median and range. Differences between independent groups were assessed by means of the Mann–Whitney U test. Receiver Operating Characteristic curves were used to identify the ADA trough levels with the highest accuracy for the identification of patients in remission. A P-value <0.05 was considered statistically significant.

RESULTS

The baseline demographic and clinical characteristics of the 23 CD patients included in the study are summarized in Table I. As far as disease location is concerned, the majority of patients had ileal disease (n=15, 65.3%), while disease behavior was equally distributed among non-constricting/non-penetrating (n=8, 34.8%), stricturing (n=8, 34.8%), and penetrating (n=7, 30.4%).
The median HBI before ADA treatment was 10 (range, 5-17) and after ADA induction dose declined to 4 (range, 3-8) (P<0.0001).

During a median follow-up period of 96.4 weeks, we collected a total of 191 serum samples. At 48-week, 10 patients (43.5%) were in remission, while 13 patients (56.5%) experienced a relapse. Among relapsing patients, 9 developed a mild disease requiring medical treatment with antibiotics and/or a short steroid course in order to recover the remission status, while 4 patients discontinued the ADA treatment (2 underwent surgical treatment, 1 switched to infliximab with benefit, and 1 withdrew ADA therapy due to the occurrence of a perineal abscess). The ADA trough levels were significantly lower in patients who experienced relapse compared to those in remission, using both ELISA and HMSA methods: 4.4 mcg/mL (range, 2.4-7.2 mcg/mL) vs. 7.55 mcg/mL (range, 6.6-8.4 mcg/mL) (P=0.01, Fig. 1A) and 6.9 mcg/mL (range, 3-10 mcg/mL) vs. 10.1 mcg/mL (range, 7-16.2 mcg/mL) (P=0.004, Fig. 1B), respectively. There was a significant, positive correlation between ELISA and HMSA ADA trough levels at 48 weeks (r=0.691, P=0.0003, Fig. 2A). ROC curves identified an ADA trough level of 7.4 mcg/mL (ELISA) and of 5.8 mcg/mL (HMSA) as the cut-offs with the highest accuracy for the identification of patients in remission (ELISA AUROC = 0.854; HMSA AUROC = 0.819), and Table II shows the diagnostic accuracy characteristics of both methodologies at 48 weeks. At this time-point, median ADA trough levels were lower in patients with positive AA (ELISA, 3.4 vs. 6.8 mcg/mL, P=0.156; HMSA, 3.7 vs. 9.3 mcg/mL, P=0.006).

At the end of follow up (i.e., 96 weeks), 17 patients (73.9%) were continuing ADA therapy: between week 48 and week 96, 2 patients switched to infliximab due to loss of response, 8 patients (47.1%) were still in remission, while 9 (52.9%) patients with previous surgical resection 9 (39.1)

Patients with fistula 3 (13.1)

Patients with perianal disease 4 (17.4)

Patients with previous use of azathioprine 10 (43.5)

### Table 1. Baseline demographic and clinical features of the 23 patients with Crohn’s disease

<table>
<thead>
<tr>
<th>Baseline features</th>
<th>n (%)</th>
<th>Median value (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male</td>
<td>14 (60.9)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>41 (21-66)</td>
<td></td>
</tr>
<tr>
<td>Age &gt;40 years</td>
<td>13 (56.5)</td>
<td></td>
</tr>
<tr>
<td>Body mass index, Kg/m²</td>
<td>22 (16-50)</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>12.9 (6.8-55.8)</td>
<td></td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>10 (0-32)</td>
<td></td>
</tr>
<tr>
<td>Disease localization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1-terminal ileum</td>
<td>15 (65.3)</td>
<td></td>
</tr>
<tr>
<td>L2-colon</td>
<td>1 (4.3)</td>
<td></td>
</tr>
<tr>
<td>L3-ileocolon</td>
<td>7 (30.4)</td>
<td></td>
</tr>
<tr>
<td>L4-upper gastrointestinal tract</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Disease behavior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1, non-constricting non-penetrating</td>
<td>8 (34.8)</td>
<td></td>
</tr>
<tr>
<td>B2, stricturing</td>
<td>8 (34.8)</td>
<td></td>
</tr>
<tr>
<td>B3, penetrating</td>
<td>7 (30.4)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>12 (52.2)</td>
<td></td>
</tr>
<tr>
<td>Past-smokers</td>
<td>7 (30.4)</td>
<td></td>
</tr>
<tr>
<td>No smokers</td>
<td>4 (17.4)</td>
<td></td>
</tr>
<tr>
<td>Patients with previous surgical resection</td>
<td>9 (39.1)</td>
<td></td>
</tr>
<tr>
<td>Patients with fistula</td>
<td>3 (13.1)</td>
<td></td>
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<tr>
<td>Patients with perianal disease</td>
<td>4 (17.4)</td>
<td></td>
</tr>
<tr>
<td>Patients with previous use of azathioprine</td>
<td>10 (43.5)</td>
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</tbody>
</table>

### Figure 1

**Fig. 1.** Box-and-whisker and dot plots of adalimumab trough levels assessed either by means of ELISA (Figure 1A) or HMSA (Figure 1B) method and subdivided according to response to treatment at week-48.
experienced relapse. At this time-point, both ELISA and HMSA assessments of median ADA trough levels showed significantly lower levels in patients who relapsed compared to patients in remission: 4.1 mcg/mL (range, 1.6-6.6 mcg/mL) vs. 7.4 mcg/mL (range, 5.7-9.3 mcg/mL) (Fig. 3A, P=0.009) and 5.9 mcg/mL (range, 3.3-8.5 mcg/mL) vs 13.1 mcg/mL (range, 9.4-16.2 mcg/mL) (Fig. 3B, P=0.001), respectively. Figure 2B shows that ELISA and HMSA ADA trough levels at 96 weeks had a significant, positive correlation (r=0.822, P=0.0001). At 96 weeks, only 2 of the 17 patients who were still on ADA had positive AA, and a comparison of ADA trough levels was not carried out due to the very small number of patients.

The ADA trough level cut-offs with the highest accuracy for the identification of patients in remission were 8.7 mcg/mL (ELISA) and 4.2 mcg/mL (HMSA), and the corresponding AUROCs were 0.958 and 0.875, respectively. Their diagnostic accuracy characteristics at 96 weeks are reported in Table II.

At 96 weeks, 8 (34.8%) patients were still in remission on ADA while 15 (65.2 %) experienced a disease relapse. Analyzing ADA trough levels at the time of disease relapse, median ADA serum concentration was significantly higher in patients in remission as compared to patients who relapsed both using ELISA and HMSA (ELISA: 8.05 mcg/ml [4.9-9.4] vs 4.1 mcg/ml [0.0-8.4], P=0.0024; HMSA: 11.88 mcg/ml [7.18-16.12] vs 4.97 mcg/ml [0.14-9.26], P=0.0004). Moreover, AA were negative in all patients in remission during the whole follow up, while 4 patients who relapsed had positive AA. The ADA serum concentration was significantly lower in AA positive patient vs. AA negative patients using HMSA (3.82 mcg/ml [0.22-4.97] vs 8.67 mcg/ml [0.14-16.12], P=0.02) but not with ELISA test (3.35 mcg/ml [0-8.4] vs. 5.4 mcg/ml [1.1-9.4], P=0.22).

**DISCUSSION**

During anti-TNF therapy, the occurrence of LOR to treatment is a well-characterized phenomenon and represents a challenge in the management of IBD patients. The annual risk of LOR was calculated to be 13% for infliximab and 24% for ADA across CD trials and clinical case-series [10-12]. The management of a LOR was empirically based on an intensified dose regimen or change to another biological drug, or to a different class of drugs. Indeed, LOR may be secondary to several clinical issues such as non-inflammatory mechanisms, uncontrolled IBD inflammation, and decreased circulating drug levels. Recently, low trough levels of biological drugs, mainly related to the development of AA, have been identified as the most important mechanism underlying LOR, and its more accurate characterization has helped greatly in the

**Table II.** Main accuracy characteristics of ELISA and HMSA methods at the various study time-points.

<table>
<thead>
<tr>
<th></th>
<th>48-week</th>
<th>96-week</th>
<th></th>
<th>48-week</th>
<th>96-week</th>
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<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>HMSA</td>
<td>ELISA</td>
<td>HMSA</td>
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<tr>
<td>Cut-off (mcg/mL)</td>
<td>7.4</td>
<td>5.8</td>
<td>8.7</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>90</td>
<td>100</td>
<td>87.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>76.9</td>
<td>76.9</td>
<td>100</td>
<td>66.7</td>
<td></td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>75</td>
<td>76.9</td>
<td>100</td>
<td>72.7</td>
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<tr>
<td>Negative predictive value</td>
<td>90.9</td>
<td>100</td>
<td>90</td>
<td>100</td>
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</table>

![Fig. 2. Scatter diagram and line of identity of adalimumab trough levels measured with ELISA and HMSA at 48-weeks (Figure 2A) and 96-weeks (Figure 2B).](image)

![Fig. 3. Box-and-whisker and dot plots of adalimumab trough levels assessed either by means of the ELISA (Figure 3A) or HMSA (Figure 3B) method and subdivided according to the response to treatment at week-96.](image)
improvement of patient management [28-30]. As a result, clinical decisions in these patients are often driven by the assessment of anti-TNF serum concentrations.

Currently, there are various methods to measure anti-TNF trough levels. Most studies have been performed using a solid-phase, double-antigen ELISA assay. Another recently developed technique is the HMSA using size-exclusion high-performance liquid chromatography, which has a high sensitivity and specificity [27]. The current literature on this topic emphasizes that performing clinical decisions on the basis of serum drug concentrations is a cost-effective method, although there is a lack of concordance regarding the most accurate method to assess ADA trough levels, and a possible cut-off value for LOR [31-34].

In our study, we compared two different assays to evaluate anti-TNF trough levels in a cohort of infliximab-naïve CD patients followed for a long-period of time (i.e., 96 weeks) during ADA treatment. In our cohort of 23 patients, 17 (73.9%) were still on ADA therapy with a sustained clinical benefit at the end of follow-up. Out of these 17 patients on ADA treatment, 8 (34.8%) were still considered responders (HBI<4) and these data are consistent with previously published studies [35-38].

We demonstrated a significant association between ADA serum concentrations and patients’ clinical outcome using both ELISA and HMSA assays. Indeed, we found that ADA trough levels in patients who relapsed were significantly lower as compared to patients who maintained a response to ADA treatment, both at 48 and 96 weeks. Furthermore, we found a close correlation between ADA trough levels assessed by either ELISA and HMSA at the main study time-points. Moreover, ROC curve analyses showed that both assays had excellent accuracy in the identification of LOR, with c-statistics ranging between 0.854 to 0.958 for the ELISA assay, and between 0.819 to 0.875 for the HMSA assay. Lastly, ADA serum trough levels at 48 weeks were lower in patients who had positive AA, although during follow-up we obviously “selected” a cohort of patients who responded to treatment, and therefore at 96 weeks only 2 patients had AA positivity, thus making the comparison of trough levels unfeasible due to the very small numbers.

Recently, Mazor et al. identified ADA trough serum concentrations – but not AA – as the most important parameter linked to response to treatment, thus indirectly emphasizing how drug trough levels may be the result of a multi-factorial process [39]. Although several studies highlighted the clinical relevance of serum drug concentration in the management of patients with IBD, these studies used various assays to assess ADA trough levels, therefore making the comparison of results challenging. As far as the identification of a possible trough level cut-off, we observed that the two methodologies evaluated in this study report different thresholds, with the ELISA assay reporting consistently lower ADA trough levels at the various study time-points. This finding underlines the need to report the assay used to assess ADA trough levels, to apply a standardized and consistent methodology, so as to “personalize” clinical decision algorithms. In clinical practice, this highlights the need to use the same assay to monitor patients longitudinally, as a change in drug trough levels may be erroneously attributed to a change in drug metabolism.

Our study undoubtedly has some limitations. Firstly, the sample size we evaluated was relatively small, but patients were consistently assessed throughout the whole study and we collected a large series of samples along an adequate period of follow-up (i.e., 96 weeks). Secondly, although the HMSA technique may appear more cumbersome, it permits the assessment of a single sample without the need of multiple samples to run an ELISA session, and this may have an impact on the turn-around time of patients’ management. Lastly, the potential economic impact of the different cost of the two assays was not taken into consideration.

CONCLUSION

Our study found that both the ELISA and HMSA assays are accurate methods to assess ADA trough levels in patients with CD and those who experience loss of response. Preferential use of a technique should be based on local availability and the physician’s experience.

Conflicts of interest: No conflicts of interest.

Authors’ contribution: G.B.: planning and conducting the study, collecting and interpreting data, and drafting the manuscript. E.G.: interpreting data and drafting the manuscript. M.F., E.M., L.D.N., L.A. and A.M.: collecting data. I.B.: laboratory analyses. V.S. and E.S.: planning the study, interpreting data and drafting the manuscript. All authors have approved the final draft submitted.

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