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ARTICLE

4β-Hydroxycholesterol Level in Patients With Rheumatoid Arthritis Before vs. After Initiation of bDMARDs and Correlation With Inflammatory State

BM Wollmann[1], SW Syversen[2], E Lie[2], C Gjestad[1], LL Mehus[3], IC Olsen[2] and E Molden[1,4,*]

Systemic inflammation has been linked to suppressed CYP3A(4) activity. We determined 4β-hydroxycholesterol (4βOHC), an endogenous CYP3A4 metabolite, in patients with rheumatoid arthritis (RA) before and after treatment with biological disease-modifying antirheumatic drugs (bDMARDs). The 4βOHC was compared in 41 patients before and 2–5 months after initiating TNFα inhibitors ($n = 31$), IL-6 inhibitors ($n = 5$), or B-cell inhibitors ($n = 5$). Correlations between 4βOHC and inflammatory markers (C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)) were also tested before and after bDMARDs. 4βOHC did not differ following bDMARD treatment ($P = 0.6$), nor in patients who started with IL-6 inhibitors (median 51.6 vs. 50.6 nmol/L). The 4βOHC and CRP/ESR did not correlate before treatment ($P > 0.5$), but correlated significantly after bDMARDs (CRP = Spearman r -0.40; $P < 0.01$; ESR = r -0.34; $P = 0.028$) suggesting that mainly non-CYP3A4-suppressive cytokines were reduced during treatment. Thus, this study does not support a generally regained CYP3A4 phenotype in patients with RA following initiation of bDMARDs.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✔ Systemic inflammation is associated with downregulation of drug-metabolizing enzymes, and initiation of bDMARDs has been highlighted as potential disease-drug-drug interaction involving CYP3A4 substrates. Two previous studies have reported reduced exposure of CYP3A4 substrates following initiation of IL-6 inhibitors in RA patients with a high inflammatory state, but knowledge is lacking whether use of IL6 inhibitors and other bDMARDs alters CYP3A4 phenotype in more heterogeneous RA populations.

WHAT QUESTION DID THIS STUDY ADDRESS?

✔ Does 4βOHC, an endogenous CYP3A4 metabolite, change in patients with RA after treatment with different types of bDMARDs, and is 4βOHC correlated with inflammatory markers?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✔ Two to 5 months of bDMARD treatment, regardless of drug subtype, does not alter 4βOHC levels in RA patients with generally modest pretreatment inflammatory state. Correlations between 4βOHC and inflammatory markers after treatment, but not before, may indicate a main effect on non-CYP3A4-suppressive cytokines.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✔ Dose adjustments of CYP3A4 substrates may generally not be required in patients with RA following initiation of bDMARDs, but interindividual variability in phenotype change implies close treatment monitoring when starting bDMARDs. The exclusive correlation between biochemical inflammatory markers and 4βOHC after initiation of bDMARDs indicates enrichment of interleukins with potential as dosing markers of drugs metabolized by CYP3A4 in patients with systemic inflammation.

Cytochrome P450 (CYP) 3A4 is the most important drug-metabolizing enzyme due its abundant expression in both liver and intestine, and broad substrate selectivity.[1] There is a large interindividual variability in CYP3A4 phenotype, which is reflected by > 10-fold range in enzyme expression in biopsies of human liver and intestine,[2] and a similar degree of variability in clearance of midazolam,[3] a CYP3A4 probe substrate. The extensive interindividual variability in CYP3A4-mediated metabolism is ascribed to a combination of genetic and environmental factors. So far, genetics have not been proven to be of substantial relevance for the variability in CYP3A4-mediated metabolism, and non-genetic factors, such as use of enzyme inhibitors or inducers, systemic inflammation, and hormonal state, are likely to be important contributors to the interpatient differences in CYP3A4 phenotype.[4]

[1]Center for Psychopharmacology, Diakonhjemmet Hospital, Oslo, Norway; [2]Department of Rheumatology, Diakonhjemmet Hospital, Oslo, Norway; [3]Department of Medicinal Biochemistry, Diakonhjemmet Hospital, Oslo, Norway; [4]Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Norway. *Correspondence: E Molden (espen.molden@farmasi.uio.no)
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Among the nongenetic factors, systemic inflammation has recently attracted great interest as a mechanism associated with downregulated expression of CYP enzymes and drug transporters.[5,6] It has been shown in cell, animal, and human experiments that inflammation suppresses CYP3A4 expression.[7,8] In a clinical study, exposure (area under the curve (AUC)) of verapamil, a combined CYP3A4/P-glycoprotein substrate, was shown to be three to fourfold increased in patients with rheumatoid arthritis (RA) compared with healthy controls.[9] This provides evidence that systemic inflammation in patients with RA could significantly alter pharmacokinetics, but, in the case of verapamil, it is difficult to interpret to what extent increased exposure is because of downregulation of CYP3A4 and/or P-glycoprotein phenotype.

Among cytokines upregulated in RA, interleukin 6 (IL-6) is thought to be most important for CYP3A4 downregulation, but tumor necrosis factor α (TNFα), IL-1β, and interferon γ could also play a role in phenotype suppression.[10] Because systemic inflammation may suppress CYP3A4 activity, anti-inflammatory treatment could potentially cause regained CYP3A4 phenotype in patients with RA. This hypothesis is supported by two small studies ($n = 12$) in which patients with RA obtained significantly lower exposure (AUC) of the CYP3A4 substrates simvastatin and midazolam after initiation of the IL-6 inhibitors tocilizumab and sirukumab, respectively.[11,12] In both studies, pharmacokinetic assessments were only performed during a 5–6 week period after administration of IL-6 inhibitors. Moreover, the studies included patients with RA with high disease activity (CRP three to fourfold upper limit of normal). Along with the fact that other biological disease-modifying antirheumatic drugs (bDMARDs) than IL-6 inhibitors are more commonly used in patients with RA, additional studies with more heterogeneous patient groups are warranted to increase our understanding of the impact of both systemic inflammation and anti-inflammatory treatment on CYP3A4 metabolism in RA.

Midazolam is considered the golden standard CYP3A4 probe drug for assessing alterations in CYP3A4 phenotype (e.g., when evaluating the interaction potential of new drugs). Recently, endogenous CYP3A4 biomarkers have also attracted great interest,[13] and among these 4β-hydroxycholesterol (4βOHC) seems to be the most promising one.[14,15] 4βOHC is a cholesterol metabolite (oxysterol) almost exclusively formed by CYP3A4,[16] and, in previous studies, it has been shown to respond to both inducers and inhibitors of CYP3A4.[17–19] Although 4βOHC is more sensitive in detecting CYP3A4 induction than inhibition, possibly due to its long elimination half-life,[20] it is a promising endogenous biomarker, which seems to reflect both intestinal and hepatic CYP3A4 phenotype.[21] 4βOHC has not been fully validated as a CYP3A4 biomarker, but the reported significant correlation with oral and intravenous midazolam clearance in healthy volunteers supports its usefulness as an endogenous CYP3A4 metric.[22] In addition to the practical advantages with an endogenous biomarker, 4βOHC is not a P-glycoprotein substrate and displays limited intrasubject variability under stable conditions.[20]

A previous study, including patients with antibody deficiency or increased susceptibility to respiratory infections, reported a significant negative correlation between C-reactive protein (CRP) and 4βOHC,[23] but 4βOHC levels have not previously been characterized in relation to inflammation status and use of bDMARDs in patients with RA. The aim of this study was, therefore, to characterize and compare 4βOHC levels in patients with RA before and after initiation of different bDMARDs in a prospective clinical study.

MATERIALS AND METHODS

Patients

The study included the first 41 patients with RA enrolled in the Norwegian multicenter study, "NOR-DMARD," a prospective observational study of patients with rheumatic joint diseases starting bDMARD treatment. In the NOR-DMARD study, a wide range of clinical measures, including assessments of disease activity, had been collected in order to examine the real life effectiveness of bDMARDs. In addition, serum samples had been stored for analysis of biomarkers at baseline and follow-up visit, which was planned 3 months after initiation of bDMARD treatment. In order to obtain a noninflammatory reference level of 4βOHC serum level, samples of 52 randomly selected levetiracetam-treated patients with CRP levels $<$ 5 mg/L, retrieved from a therapeutic drug monitoring service, were additionally analyzed in the project. The Regional Committee for Medicinal and Health Research Ethics and the Hospital Investigational Review Board approved the study.

In the patients with RA, information about the type of bDMARD being initiated, inflammatory status (CRP and erythrocyte sedimentation rate (ESR)), clinical disease activity measures (Disease Activity Score 28 (DAS28) and Clinical Disease Activity Index (CDAI)), and use of synthetic DMARDs and inflammatory drugs, were registered both at baseline and follow-up. Demographic details, such as gender, age, RA diagnosis (International Classification of Disease-10), and time of RA diagnosis, were also registered in the study, but information about use of non-RA medications was not recorded.

As the reference group to reflect general 4βOHC level among persons without systemic inflammation, defined as CRP $<$5 mg/L, the study additionally included 4βOHC measurements in serum samples from 52 randomly selected levetiracetam-treated patients recruited from the therapeutic drug-monitoring service at the Center for Psychopharmacology, Diakonhjemmet Hospital (Oslo, Norway). In these serum samples, absence of enzyme-inducing antiepileptic drugs (carbamazepine, phenytoin, phenobarbital, and/or oxcarbazepine) was confirmed by a routine ultraperformance liquid chromatography (UPLC) tandem mass spectrometry method detecting all clinically used antiepileptic drugs in Norway.[21] Information about gender, age, and comedication of the reference subjects were obtained from the respective requisition forms. CRP measurements were anonymously performed in residual serum samples of the levetiracetam-treated reference subjects at the Department of Medical Biochemistry, Diakonhjemmet Hospital (Oslo, Norway).

4βOHC analysis

The 4βOHC concentrations were determined in all serum samples, which had been stored at -20°C until analysis.

A validated UPLC-tandem mass spectrometry assay was used to determine 4βOHC concentrations in the remaining volumes of the serum samples. Deuterium labelled 4βOHC (Toronto Research Chemicals, Toronto, Ontario, Canada) was used as the internal standard and 4βOHC was quantified from calibration curves prepared in methanol. The assay was based on a method previously published by van de Merbel *et al.*,[24] with a few modifications and performed as described by Gjestad *et al.*[21] Briefly, 500 μL serum was mixed with 50 μL 10 μM internal standard (deuterium labelled 4βOHC) dissolved in methanol and 1 mL of a 1 mol/L sodium methoxide solution for alkaline hydrolysis of steroidal esters. Then, 1 mL of water and 4 mL of hexane was added and the liquid phase extraction was carried out. To ensure complete phase separation, the sample was extracted for 2 min in ambient temperature, centrifuged at 2,500 g for 5 min at 20°C, and then stored at -80°C for 20 min. After 20 min, the aqueous layer was frozen and the upper organic phase was transferred to new tubes, evaporated to dryness under nitrogen at 37°C, and reconstituted in 500 μL methanol. Because of precipitation of hydrophobic particles after reconstitution, the tubes were stored at -20°C for 15 min to induce precipitation and then transferred to centrifuge filters (Costar Spin-x, HPLC Micro Centrifuge Filter, 0.2 μm Nylon Filter) and centrifuged at 2,500 g for 6 min at 2°C. The extracts were transferred to ultraperformance liquid chromatography (UPLC) sample vials and stored at 5°C in the autosampler until injection.

The samples (10 μL) were analyzed on a Waters Acquity Quattro Micro UPLC-tandem mass spectrometry system (Milford, MA) with a Waters Acquity UPLC BEH Shield RP18 column (1.7 μm, 1 × 100 mm) at 40°C. The mobile phase was a mix of water and methanol (85–95%, gradient elution) and flow rate 0.15 mL/min. Total run time was 10 min and the retention time of 4βOHC was ~3 min. The mass spectrometer was operated in positive atmospheric pressure chemical ionization mode (cone voltage 30 V, collision energy 14 eV) and multiple-reaction monitoring at 385.25→367.45 and 392.30→374.50 for 4βOHC and deuterium labelled 4βOHC (internal standard), respectively. The intraday (within-day) and interday (between-day) accuracy was < 8% at 25 nmol/L, whereas the intraday and interday precision was < 15%. The lower limit of quantification was 25 nmol/L.

All samples were analyzed twice and the mean values were applied in the statistical calculations.

End Points and statistics

Initially, measured 4βOHC concentrations were tested for normality by Kolmogorov-Smirnov, D'Agostino-Pearson omnibus, and Shapiro-Wilk tests, but none of the normality tests were passed. Therefore, nonparametric statistical tests were used for end-point assessment.

Paired serum concentrations of 4βOHC, as well as biochemical inflammatory markers (CRP/ESR) and clinical disease measurements (DAS28/CDAI), were compared in patients with RA before and after initiation of bDMARD treatment using the Wilcoxon signed rank test (primary analysis of the study). Secondary, a Mann-Whitney *U* test analysis was applied to compare 4βOHC serum concentrations between patients with RA and noninflammatory reference subjects. The same test was used to compare the age distribution between patients with RA and noninflammatory reference subjects, whereas the Fisher's exact test was applied to compare the gender distribution between the same groups. Within the population of patients with RA, after vs. before ratios of 4βOHC were compared nominally between subgroups/clusters starting treatment with different types of bDMARDs (i.e., TNFα inhibitors, IL-6 inhibitors, or B-cell inhibitors). For assessment of the potential association between baseline 4βOHC values and its relative change (after:before 4βOHC ratio) following initiation of bDMARDs, a visual (not statistical) inspection of the two variables plotted against each other was performed. The same approach was applied to assess potential associations between treatment response and change in 4βOHC (i.e., individual βOHC ratios were plotted against the respective after:before ratios of various disease measures (CRP, ESR, DAS28, and CDAI)).

The potential associations between inflammatory markers (CRP/ESR) and 4βOHC levels in patients with RA at baseline and following initiation of bDMARDs were assessed using Spearman's rank correlation tests. All statistical analyses were conducted using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). In all tests, $P < 0.05$ was considered significant.

Table 1 Baseline characteristics of the patients with RA, including age/gender distribution, levels of inflammatory markers, clinical disease activity measures (DAS28 and CDAI), and numbers treated with different synthetic DMARDs, and numbers starting on various biological DMARDs

Characteristics	
Age, years median (range)	56 (19–76)
Gender, no. male/female	6/35
CRP[a], mg/L, median (range)	5.5 (1–69)
ESR, mm/h, median (range)	22 (3–84)
DAS28[b], median (range)	3.9 (2.4–7.8)
CDAI[b], median (range)	12.8 (4.0–60.4)
Time since RA diagnosis[c], months, median (range)	71 (3–359)
Synthetic DMARDs, no.	
Methotrexate	30
Sulfasalazine	8
Leflunomide	6
Hydroxychloroquine	3
Cyclosporine A	1
Biological DMARDs, no.	
TNFα inhibitors	31
IL-6 inhibitors	5
B-cell inhibitors	5

CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DMARD, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; DAS28, Disease Activity Score 28; IL-6, interleukin 6; RA, rheumatoid arthritis; TNFα, tumor necrosis factor α.
[a]CRP available for $n = 40$.
[b]DAS28 available for $n = 39$ and CDAI available for $n = 37$.
[c]Time since RA diagnosis available for $n = 24$.

RESULTS

Baseline characteristics of the included patients with RA are summarized in **Table 1**. The majority of the patients (33 of 41) were rheumatoid factor positive. There was an

extensive variability in baseline inflammatory markers in the population with CRP levels and ESRs ranging >20-fold. Generally, biochemical parameters (CRP/ESR) and clinical disease measures (DAS28/CDAI) were only moderately elevated in patients with RA prior to initiation of bDMARDs (**Table 1**). In serum samples of levetiracetam-treated subjects included to obtain a noninflammatory reference level of 4βOHC, the CRP concentration (median 0.99 mg/L, range < 0.30–4.54 mg/L) was significantly lower than in patients with RA at baseline (median, 5.5 mg/L; range, 1–69; $P < 0.0001$). In this parallel, noninflammatory reference group, the proportion of women was significantly lower than in patients with RA (i.e., 56% vs. 85%; $P < 0.01$). Observed age also differed (not significantly) between noninflammatory subjects and patients with RA (median, 43.5 vs. 56 years; $P = 0.3$).

Most of the patients with RA (30 of 41) were using methotrexate (**Table 1**), and all synthetic DMARDs registered at baseline were continued to follow-up. Prescription of nonsteroidal anti-inflammatory drugs and systemic corticosteroids was registered in 11 and 25 patients, respectively, at baseline. Regarding the type of bDMARD that was initiated, the patient majority ($n = 31$; 75.6%) started treatment with TNFα inhibitors (adalimumab $n = 1$, certolizumab pegol $n = 21$, etanercept $n = 4$, and golimumab $n = 5$), whereas five patients each started treatment with IL-6 inhibitors (only tocilizumab) and B-cell inhibitors (only rituximab), respectively.

The median time between measurements at baseline (bDMARD initiation) and follow-up was 93 days (range, 53–150). Between initiation and follow-up, median DAS28 was reduced from 3.90 (range, 2.4–7.8) to 3.25 (range, 0.5–7.2; $P = 0.02$), whereas CDAI was reduced from 12.8 (range, 4–60.8) to 9.4 (range, 0.1–49.4; $P = 0.0614$). In parallel with the clinical improvement, median CRP and ESR values were reduced from 5.5 mg/L (range, 1–69) to 2 mg/L (range, 1–63; $P < 0.05$), and from 22 mm/h (range, 3–84) to 14 mm/h (range, 2–69; $P < 0.05$), respectively. Individual changes in clinical measures and inflammation markers after treatment initiation with bDMARDs varied from more than twofold elevation to > 90% reduction (data not shown).

Although the 4βOHC level in patients with RA did not differ before and after initiation of bDMARD treatment (median, 50 vs. 52 nmol/L; $P = 0.6$), it was significantly lower in patients with RA than the noninflammatory reference value measured in levetiracetam-treated subjects (median, 68 nmol/L). The difference in 4βOHC level between the noninflammatory reference group and the patients with RA was statistically significant at baseline and follow-up ($P < 0.01$). In patients with RA, there was a 10-fold variability in 4βOHC levels, which largely overlapped with the noninflammatory reference subjects (**Figure 1a**). For the bDMARD subgroups, there were not any tendencies of change in 4βOHC level following initiation of bDMARD treatment (**Figure 1b**). When plotting the individual baseline 4βOHC levels against the respective paired after:before 4βOHC ratios, a negative trend between the two variables was apparent (i.e., those with lowest baseline obtained highest ratios and vice versa; **Figure 1c**). After:before ratios of 4βOHC were also plotted against the after:before ratios of all treatment response variables (DAS28, CDAI, CRP, and ESR), but no trends of

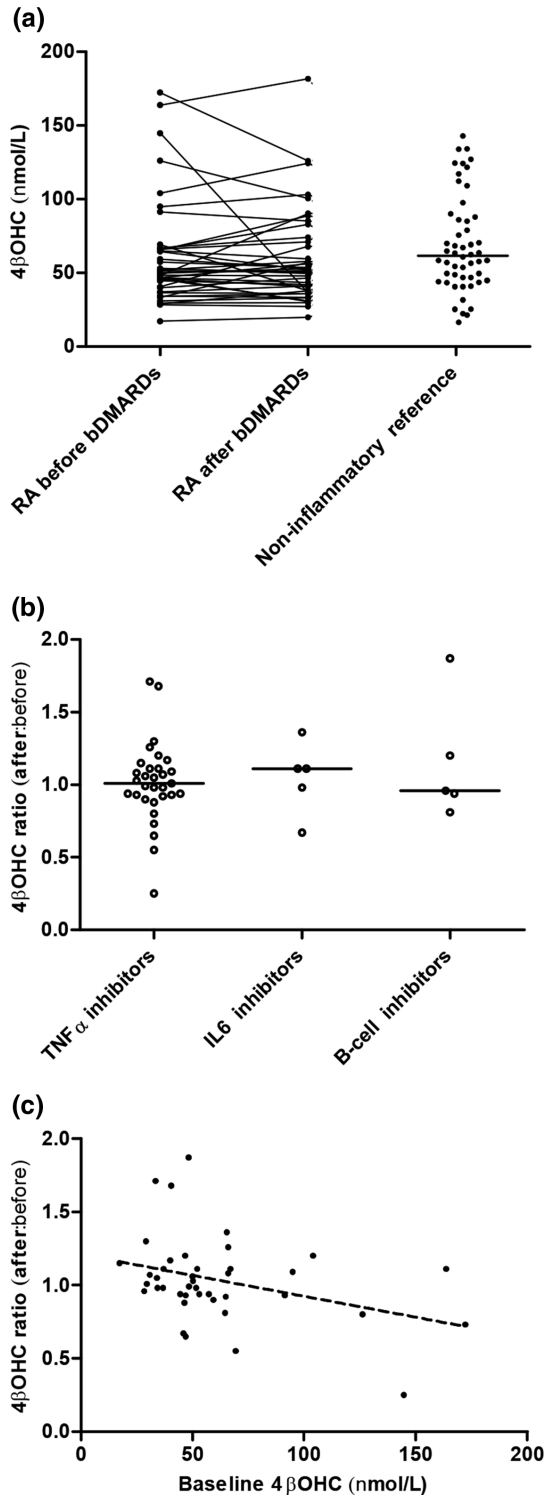

Figure 1 (**a**) Individual 4β-hydroxycholesterol (4βOHC) concentrations in patients with rheumatoid arthritis (RA; $n = 41$) before vs. after disease-modifying antirheumatic drugs (bDMARDs) treatment and in noninflammatory reference subjects (right panel; $n = 52$), (**b**) relative changes in 4βOHC (paired after:before ratios) in patient subgroups treated with tumor necrosis factor alpha (TNFα) inhibitors ($n = 31$), interleukin (IL)-6 inhibitors ($n = 5$) and B-cell inhibitors ($n = 5$), and (**c**) simple plot of relative changes in 4βOHC according to individual baseline values in the whole RA population (dotted linear trend line added for visual purpose).

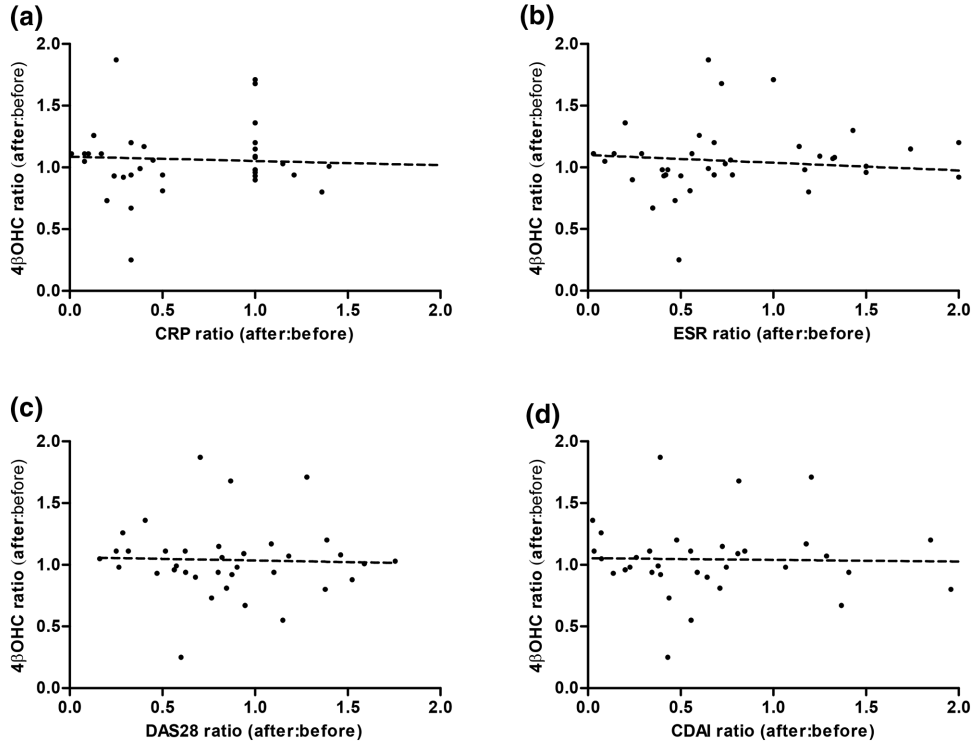

Figure 2 Visualization of individual changes in 4β-hydroxycholesterol (4βOHC) levels against the respective clinical responses on various disease measures (i.e., C-reactive protein (CRP) **a**; erythrocyte sedimentation rate (ESR) **b;** Disease Activity Score 28 (DAS28) **c**; and Clinical Disease Activity Index (CDAI) **c**). Relative changes in both 4βOHC level and disease measures presented as paired after:before disease-modifying antirheumatic drug (bDMARD) treatment ratios. Dotted linear trend lines added for visual purpose.

associations between relative changes in 4βOHC and individual responses to bDMARD therapy were observed in the present RA population (**Figure 2**).

Although there were no correlations between inflammatory markers and 4βOHC levels before treatment ($P > 0.5$ (CRP); $P > 0.9$ (ESR)), significant negative correlations were observed after bDMARD treatment (Spearman r -0.40, $P < 0.01$ (CRP) and Spearman r -0.34, $P = 0.028$ (ESR); **Figure 3**). For the clinical variables, DAS28 and CDAI, no significant correlations with 4βOHC levels were observed before or after bDMARD treatment ($P > 0.4$; data not shown).

DISCUSSION

To our knowledge, this is the first study to characterize and compare 4βOHC levels in patients with RA before and after initiation of bDMARD treatment. No change in circulating 4βOHC concentration was observed following initiation of bDMARDs. As 4βOHC is suggested to be an endogenous CYP3A4 biomarker, our findings do not support that initiation of bDMARDs generally regains CYP3A4 phenotype in patients with RA. However, there was a great intersubject variability in relative 4βOHC changes, and the observed tendency that patients with lowest baseline 4βOHC levels displayed highest relative increases in apparent CYP3A4 phenotype at follow-up may suggest that RA patients with sufficiently suppressed enzyme activity could achieve a substantial regain in metabolic phenotype after initiation of bDMARDs. One might also have expected that patients with the greatest relative response to bDMARD therapy typically would display the highest increase in apparent CYP3A4 phenotype, but we observed no tendency of covariance between 4βOHC ratios and individual changes in the respective disease activity measures. Due to the generally limited increase in disease activity measures before initiation of bDMARDs in the present study, these observations do not preclude that clinical effect of bDMARD therapy could be of relevance for a potential regain in drug-metabolizing phenotype in patients with a higher burden of disease at baseline.

The absence of a general increase in 4βOHC following treatment with bDMARDs in the present study population was in line with the observation that 4βOHC only correlated with inflammatory markers after initiation of bDMARDs (not before). In fact, switching from a statistically significant to a highly significant correlation between 4βOHC level and CRP before vs. after initiation of therapy clearly indicates that bDMARD treatment in our study mainly reduced cytokines not suppressing CYP3A4 phenotype. IL-6 is the cytokine that has been most closely linked to downregulation of CYP3A4 metabolism. In addition to published *in vitro* data,[25] this is supported by the two recent studies showing decreased exposure (AUC) of simvastatin and midazolam after initiation of IL-6 inhibitors.[11,12] In our study, about 75% of the included patients started treatment with TNFα inhibitors. Although TNFα has been shown to suppress CYP3A4 expression *in vitro*,[10] the apparent lack of increased CYP3A4 phenotype in the current population may suggest that cytokines suppressing CYP3A4 enzyme expression or activity are not

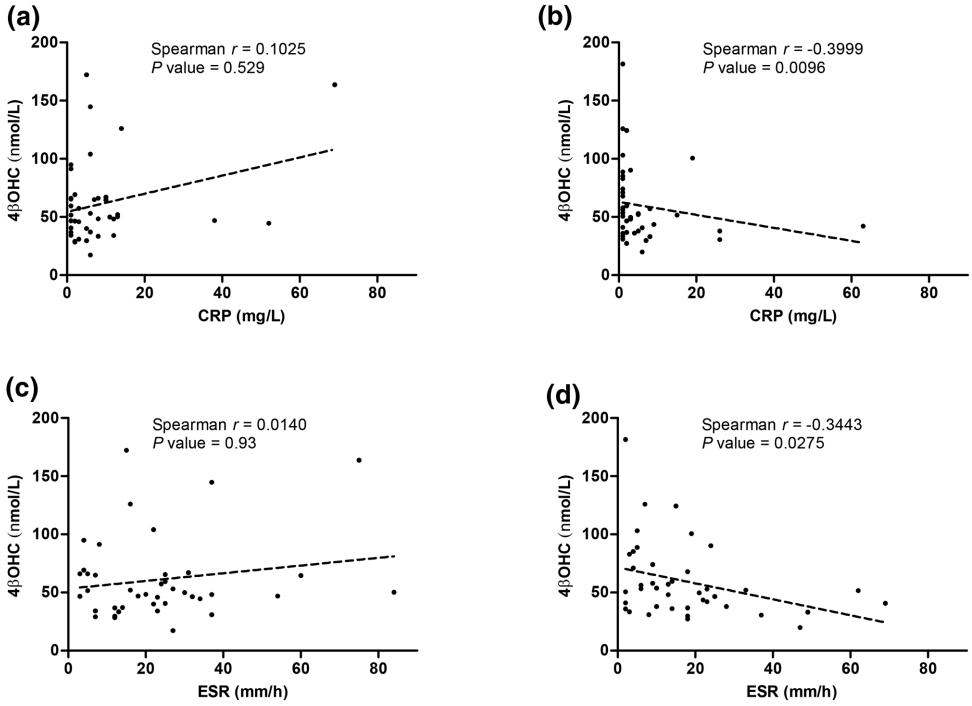

Figure 3 Correlations between 4β-hydroxycholesterol (4βOHC) and C-reactive protein (CRP) levels before (**a**) and after (**b**) initiation of disease-modifying antirheumatic drug (bDMARD) treatment in patients with rheumatoid arthritis (RA), and similarly between 4βOHC level and erythrocyte sedimentation rates (ESR) before (**c**) and after (**d**) initiation of DMARDs in patients with RA. Estimated *r* and *P* values from Spearman's rank correlation tests are added on each illustration (dotted linear trend lines added for visual purpose).

reduced *in vivo* by this subtype of bDMARD. However, nor was there a tendency of increased 4βOHC levels in the minority of patients (12%) starting IL-6 inhibitor therapy, which, in previous studies, has been associated with regained CYP3A4 phenotype in patients with RA.[11,12]

The underlying explanation for the apparent lack of increased CYP3A4 phenotype after bDMARD treatment in our study, including IL-6 inhibitors, is not obvious, but a factor of importance is probably the observation that median 4βOHC level in the patients with RA at baseline was only 30% lower than in the noninflammatory reference group. Thus, the potential of a regain in CYP3A4 activity seems to have been limited in the present patient population. Although statistically significant, the modest quantitative difference compared with the reference group might indicate that CYP3A4 phenotype in the current RA population was not particularly suppressed. The latter could have been due to a generally limited inflammatory activity among the included patients (i.e., median baseline CRP level just above the reference range). This is an important difference compared with previous studies investigating exposure of the CYP3A4 substrates simvastatin and midazolam following administration of tocilizumab and sirukumab, respectively, in which the patients' CRP levels were three to fourfold the upper limit of normal prior to initiation of bDMARD therapy.[11,12] The baseline inflammatory activity is definitely a factor that might affect the responsiveness toward a potential regain in CYP3A4 phenotype following bDMARD treatment, and could be an explanatory factor for the variable study outcomes.

It has been debated to what extent 4βOHC is a valid *in vivo* biomarker of CYP3A4 phenotype.[26] Basically, there is no doubt that 4βOHC primarily is produced by CYP3A4 from cholesterol,[16] but greater sensitivity toward enzyme inducers than inhibitors,[18] has questioned its suitability as a CYP3A4 biomarker. However, in healthy subjects, it has been reported as a significant correlation between 4βOHC level and oral/intravenous clearance of the CYP3A4 probe drug midazolam.[22] Moreover, there are strong indications that 4βOHC is formed by both intestinal and hepatic CYP3A4,[21] and unpublished data from our laboratory show a strong and significant, negative correlation between 4βOHC level and dose-adjusted exposure of orally administered quetiapine, a low-bioavailability CYP3A4 substrate (data provided in **Supplementary Materials**). Despite some limitations as a CYP3A4 metric for detecting drug interactions, possibly due to long elimination half-life (~17 days),[20] the latter supports that circulating 4βOHC level reflects basal *in vivo* CYP3A4 activity. Thus, we consider it likely that the measured 4βOHC levels in the current patients with RA reflected their actual CYP3A4 metabolic status.

It has been claimed that individual differences in cholesterol levels might be of importance for the formation of 4βOHC, but a study has reported that variability in cholesterol levels only explains about 9% of the variability in 4βOHC concentrations.[27] The interindividual variability in cholesterol levels, and potential intraindividual change between bDMARD initiation and follow-up, is therefore unlikely to be a relevant limitation in the present study. Moreover, it has previously been shown that the apparent recovery of

CYP3A4 expression/activity is achieved already 1 week after administration of an IL-6 inhibitor (tocilizumab) in patients with high inflammatory activity.[11] With an elimination half-life of ~17 days,[20] this implies that a new steady-state concentration of 4βOHC is likely to have been obtained 2 months following initiation of bDMARDs, which was the shortest period between baseline and follow-up in the present study. Although this supports the use of 4βOHC as a CYP3A4 metric in our study, it would clearly have been of value to include additional CYP3A4 biomarkers (e.g., urinary 6β-hydroxycortisol:cortisol) to strengthen the negative findings of bDMARD treatment initiation on apparent CYP3A4 phenotype. The lack of effect on 4βOHC following initiation of bDMARD treatment in patients with RA should therefore be verified in future studies, where it will be of particular interest to study the potential change in relation to differences in baseline disease activity using different CYP3A4 biomarkers.

The significantly lower 4βOHC level found in patients with RA compared with the noninflammatory reference group supports that chronic, systemic inflammation suppress CYP3A4 phenotype, which may lead to elevated serum concentrations of CYP3A4-metabolized drugs in patients with RA. Although the quantitative difference in median 4βOHC level between patients with RA and the noninflammatory reference group was only about 30% both before and after bDMARDs, it highlights that lower doses of CYP3A4 substrate drugs with a narrow therapeutic index may be required in patients with RA and other systemic inflammation diseases. Patients with RA with the most suppressed metabolic activity would be most vulnerable of potential concentration-dependent side effects, but there is no standard test to identify patients' CYP3A4-metabolizing phenotype in clinical practice. Because systemic inflammation is associated with suppressed CYP3A4 metabolism, inflammatory markers could potentially be surrogate measures of CYP3A4 phenotype. A significant negative correlation between inflammation and CYP3A phenotype, measured by CRP and 4βOHC, respectively, has actually been reported in a previous study with a group of patients ($n = 116$) with antibody deficiency or increased susceptibility to respiratory infections.[23] However, in our study, we only detected a significant correlation between biochemical inflammatory markers (CRP/ESR) and 4βOHC levels after initiation of bDMARDs. A likely mechanistic explanation behind this finding is that bDMARD treatment in the present population probably enriched CRP/ESR measurements with interleukins exhibiting a regulatory effect on CYP3A4 phenotype. Thus, the finding has a translational potential because it may lead to the identification of clinical biomarkers of *in vivo* CYP3A4 activity, and, hence, dose requirements of many drugs in patients with systemic inflammatory diseases. An approach to further investigate the translational potential of this observation would be to determine more specific inflammation biomarkers than CRP/ESR as possible surrogate measures of CYP3A4 phenotype in future studies, including patients with RA or other inflammatory diseases.

The present study has some methodological limitations. One issue is that the two included populations (i.e., patients with RA and noninflammatory reference subjects), were not matched in terms of gender and age distribution, which might have biased the comparison of 4βOHC levels between the populations.[21,27] However, the most important limitation is probably the lack of information regarding comedication with non-RA drugs in patients starting bDMARDs. Potential changes in drug use between baseline and follow-up, and particularly initiation or withdrawal of CYP3A4 inducers or inhibitors, might have overruled possible alterations in 4βOHC levels following initiation of bDMARDs. Another scientific limitation includes the real-life study design (e.g., variable time of RA diagnosis and different RA treatment regimens). However, the naturalistic setting of the study could also be viewed as a strength because the findings reflect real-life and could be directly translated into clinical practice.

In conclusion, 4βOHC levels in patients with RA did not change following initiation of bDMARD treatment in the present study. Moreover, 4βOHC levels were only significantly associated with inflammatory biomarkers after (not before) administration of bDMARDs, suggesting that mainly non-CYP3A4-suppressive cytokines were reduced during treatment. Thus, our findings do not support a generally regained CYP3A4 phenotype in patients with RA following initiation of bDMARDs, but there is a great interindividual variability in relative 4βOHC changes after bDMARDs, which might be related to baseline CYP3A4 phenotype.

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Conflict of Interest. All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: i) no support from any organization for the submitted work; ii) no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; and iii) no other relationships or activities that could appear to have influenced the submitted work.

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