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**Associations between cytokine levels and CYP3A4 phenotype in patients with
rheumatoid arthritis**

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Running title page

Cytokines associated with CYP3A4 phenotype in RA patients

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Abbreviations:

4 β OHC, 4 β -hydroxycholesterol

CYP, cytochrome P450

IL, interleukin

RA, rheumatoid arthritis

TNF, tumor necrosis factor

Abstract

Systemic inflammation has been linked to suppressed CYP3A4 activity. The aim of this study was to examine associations between levels of a broad selection of cytokines and CYP3A4 phenotype in patients with rheumatoid arthritis (RA). The study included 31 RA patients treated with tumor necrosis factor (TNF)- α inhibitors. CYP3A4 phenotype was measured as serum concentration of 4 β -hydroxycholesterol (4 β OHC) by UPLC-MS/MS in samples collected prior to and 3 months after initiation of treatment with TNF- α inhibitors. Serum levels of the following 21 cytokines were determined in the same samples using a bead-based multiplex immunoassay (Luminex technology): CCL2, CCL3, CXCL8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN- γ), interleukin (IL)-1 β , IL-1 receptor antagonist (ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-18, IL-23 and TNF- α . Correlations between levels of cytokines and 4 β OHC were assessed by Spearman's rank correlation tests. Among the investigated cytokines, three were negatively correlated with CYP3A4 phenotype during treatment with TNF- α inhibitors: i.e. IL-1ra (r -0.408, P = 0.023), IL-6 (r -0.410, P = 0.022) and CXCL8 (r -0.403, P = 0.025) (P >0.3 for all other cytokines). None of the analysed cytokines were correlated with CYP3A4 phenotype prior to TNF- α inhibitor treatment (P >0.1 for all cytokines). These findings suggest that immune responses associated with increased levels of IL-1ra, IL-6 and CXCL8 may suppress CYP3A4 metabolism. Further studies are required to evaluate these preliminary findings in different patient populations and also examine the possible molecular mechanisms behind our observations.

Introduction

Cytochrome P450 (CYP) 3A4 is abundantly expressed in human liver and intestine, and is generally regarded as the most important enzyme in drug metabolism (Wilkinson, 2005).

There is a large inter-individual variability in CYP3A4 phenotype (Wilkinson, 2005), as reflected by a >10-fold range in enzyme expression in biopsies of human liver and intestine (Ulvestad et al., 2013) and clearance of CYP3A4 probe substrate midazolam (He et al., 2005). The extensive inter-individual variability in CYP3A4-mediated metabolism is attributed to a combination of genetic and environmental factors (Klein and Zanger, 2013; Hole et al., 2017); however, the clinical relevance of *CYP3A4* genetics seems to be limited, and non-genetic factors are likely the most important determinant of the inter-patient differences in CYP3A4-mediated metabolism (Klein and Zanger, 2013).

Systemic inflammation has been shown to downregulate the expression of multiple CYP enzymes, and has attracted great interest as a mechanism associated with suppressed CYP3A4 phenotype in patients (Christensen and Hermann, 2012). A number of clinical studies have demonstrated suppressed CYP3A4 phenotype during inflammatory conditions (Mayo et al., 2000; Rivory et al., 2002; Molanaei et al., 2012), and several cytokines (interleukin (IL)-1 β , IL-1 α , IL-6 and tumor necrosis factor (TNF)- α) have been reported to downregulate activity of multiple CYP enzymes in rodent and cell models (Renton, 2005; Aitken and Morgan, 2007). Cytokines, which are formed as part of the immune response during various inflammatory conditions, have received most focus as potential enzyme suppressors (Christensen and Hermann, 2012; Christmas, 2015), but other mediators of immune responses could be of potential importance for suppressed CYP metabolism as well.

Midazolam is considered the gold standard probe drug for CYP3A4-phenotyping. However, 4 β -hydroxycholesterol (4 β OHC), a cholesterol metabolite mainly formed by CYP3A4 (Bodin

et al., 2002), has over several years attracted great interest as an alternative biomarker (Bodin et al., 2001; Diczfalusy et al., 2008). Compared to midazolam, an advantage with 4 β OHC is its status as an endogenous marker, and the level of 4 β OHC has in previous studies been shown to respond to both inducers and inhibitors of CYP3A4 (Josephson et al., 2008; Hole et al., 2017). Moreover, 4 β OHC seems to reflect both hepatic and intestinal CYP3A4 phenotype (Gjestad et al., 2016), and the reported correlations between 4 β OHC levels and concentrations of drugs metabolized by CYP3A4 (Vanhove et al., 2016; Gjestad et al., 2017), supports its usefulness as a CYP3A4 biomarker.

A previous study showed that 4 β OHC levels in patients with rheumatoid arthritis (RA) were significantly lower (~20 %) compared to healthy controls, both before and after initiation of biological disease-modifying antirheumatic drugs (bDMARDs) (Wollmann et al., 2017). To obtain knowledge on inflammatory mediators possibly suppressing CYP3A4 metabolism in RA patients, the aim of this pilot study was to investigate the associations between serum levels of 4 β OHC and cytokines representing different immune responses in patients treated with TNF- α inhibitors in a real-life clinical setting.

Materials and methods

Patients

RA patients (n=31) starting treatment with TNF- α inhibitors were included from the NOR-DMARD study (ClinicalTrials.gov identifier NCT01581294). The study protocol, which was previously described in detail (Wollmann et al., 2017), included serum samples collected at baseline (before start of TNF- α inhibitor treatment) and after 3 months of treatment. In the present study these samples were used for analysis of cytokines, 4 β OHC and total cholesterol. The serum samples were stored at -20 °C until analysis.

Information about C-reactive protein (CRP) concentrations, Disease Activity Score 28 (DAS28) (Prevoo et al., 1995) and Clinical Disease Activity Index (CDAI) (Aletaha et al., 2005) were available from the NOR-DMARD study database. This database also provided details about gender, age and disease duration.

The study was carried out in accordance with the Declaration of Helsinki and approved by the Regional Committee for Medicinal and Health Research Ethics and the Hospital Investigational Review Board.

4 β OHC and cholesterol measurements

Serum concentration of 4 β OHC was determined by an ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method previously described in detail (Gjestad et al., 2016). Briefly, 10 μ L purified sample, also containing the internal standard (deuterium labelled 4 β OHC; 4 β OHC-d7), was analysed on a Waters Acquity Quattro Micro UPLC-tandem mass spectrometry system (Milford, MA) with a Waters Acquity UPLC BEH Shield RP18 column (1.7 μ m, 1 x 100 mm). A gradient elution with a mix of water and methanol (85-95%) was used as mobile phase for chromatographic separation, while MS/MS detection was obtained by an atmospheric pressure chemical ionisation probe operated in positive mode. The transitions that were used were 385 \rightarrow 367 and 392 \rightarrow 374 for 4 β OHC and

4 β OHC-d7, respectively. Total run time was 10 minutes and the retention time of 4 β OHC was 3 minutes.

The concentration data of 4 β OHC applied in the present investigation was also included in a recent investigation (Wollmann et al., 2017), where patients treated with TNF- α inhibitors comprised the major subgroup. In this follow-up project, additional concentration measurements of cholesterol, the precursor of 4 β OHC, and analyses of a range of different cytokines were performed in the same serum samples. Total serum cholesterol was determined by a standard enzymatic method based on hydrolysis of cholesterol esters to free cholesterol (Roche Diagnostics GmbH, Mannheim, Germany).

Quantification of cytokines

Serum concentration of the following 21 cytokines was measured in the patients' samples: CCL2, CCL3, CXCL8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN- γ), IL-1 β , IL-1 receptor antagonist (ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-18, IL-23 and TNF- α . IL-23 was analysed as a single-plex assay, whereas the remaining 20 cytokines were analysed as a multiplex assay, both bead-based immunoassays (Bio-Rad, Hercules, USA) based on xMAP technology (Luminex, Austin, USA). The cytokines were selected based to represent a broad repertoire of immune responses, including Th1, Th2, Th17 and macrophage activation, and based on previously documented role in the immunology in RA and response to TNF- α inhibition. The manufacturer's analytical protocol was followed. For values below the lower limit of quantification (LLOQ), samples with fluorescence values above blank were set to 50 % of LLOQ, while samples with fluorescence values at blank or lower were regarded as 0 pg/mL.

Statistics

The potential associations between the various cytokines, CRP and 4 β OHC levels were assessed using Spearman's rank correlation tests both at baseline and at 3 months follow-up. These correlations were performed both for the whole patient population and in females only. The Pearson's correlation test was used to investigate the association between unadjusted and cholesterol-adjusted 4 β OHC levels, while the Wilcoxon signed rank test was used to compare biomarker levels after vs. before initiation of TNF- α inhibitor treatment.

All statistical analyses were conducted using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). In all tests, $P < 0.05$ was considered significant.

Results

Patient characteristics

Demographic and clinical characteristics of the patients, including CRP levels and composite disease activity measures (DAS28 and CDAI) are summarized in Table 1. Regarding the various TNF- α inhibitors that the patients were treated with, the patient majority (n= 21) started treatment with certolizumab pegol. Five and four patients started treatment with golimumab and etanercept respectively, whereas one patient started treatment with adalimumab.

After 3 months of treatment with TNF- α inhibitors median CRP level was <5 mg/L (median 2 mg/L, range 1-63 mg/L), which is the reference range of normal. The low inflammation activity was also reflected by the modest scores in composite disease activity measures (Table 1). CRP values were lower than before treatment with TNF- α inhibitors, but the difference was not significant (median 5 mg/L, range, 1-52 mg/L, $P > 0.2$). Three months of treatment with TNF- α inhibitors did not alter the 4 β OHC levels in the patients (median 49 vs. 52 nmol/L; $P > 0.9$, Wilcoxon signed rank test).

Correlations analyses

Spearman's correlation r and P values for the correlation analyses between all the cytokines, 4 β OHC and CRP after 3 months of treatment with TNF- α inhibitors are presented in Table 2. More than 50 % of the samples had undetectable fluorescence values of CCL3, and this cytokine was therefore excluded from the statistical analyses.

Significant negative correlations were observed between 4 β OHC and IL-1ra (Fig. 1A, Spearman r -0.408, $P = 0.023$), IL-6 (Fig. 1B, r -0.410, $P = 0.022$) and CXCL8 (Fig. 1C, r -0.403, $P = 0.025$). Correlations between 4 β OHC and the remaining cytokines, were not statistically significant (P values were >0.2).

IL-6 and CXCL8 were also correlated positively with CRP, i.e. IL-6 vs. CRP (r 0.541, P = 0.002) and CXCL8 vs. CRP (r 0.402, P = 0.025). A positive, non-significant correlation between IL-1ra and CRP was also observed (r 0.340, P = 0.061). Prior to TNF- α inhibitor treatment, none of the analysed cytokines were correlated with CYP3A4 phenotype (P > 0.1; Supplementary Table 1). None of the cytokines were correlated with CRP before treatment with TNF- α inhibitors (P > 0.1; Supplementary Table 1), apart from IL-6 vs. CRP (r 0.372, P = 0.043).

In separate correlation analyses between 4 β OHC and cytokine levels in female RA patients, the same three cytokines were significantly correlated with CYP3A4 activity during treatment with TNF- α inhibitors, i.e.: IL-1ra (r -0.441, P = 0.027), IL-6 (r -0.506, P = 0.010) and CXCL8 (r -0.418, P = 0.037) (Table 3). IL-1ra, IL-6 and CXCL8 were also the only cytokines that were negatively correlated with 4 β OHC in separate analyses of the patients treated with certolizumab pegol; which was the TNF- α inhibitor the majority of the patients (68 %) were treated with (IL-1ra (r 0.631, P = 0.002), IL-6 (r -0.507, P = 0.019) and CXCL8 (r -0.461, P = 0.061) (Supplementary Table 2)).

Unadjusted vs. cholesterol-adjusted 4 β OHC levels

For patients who had sufficient serum sample volumes available for measurement of both cytokine levels and total cholesterol (C) concentration, Pearson's correlation analysis was used to investigate the correlation between unadjusted and cholesterol-adjusted 4 β OHC levels both prior to and during use of TNF- α inhibitors. Twenty-five of 31 samples prior to and 28 of 31 samples during treatment with TNF- α inhibitors had sufficient serum volumes available for total cholesterol measurements. Significant, almost linear positive correlations between absolute 4 β OHC (unadjusted) concentration and 4 β OHC/C ratio were observed both prior to and during stable treatment with TNF- α inhibitors (Pearson's r >0.9; P < 0.0001, Supplementary Figure 1).

Discussion

CYP3A4 metabolism is suppressed during systemic inflammation, but little is known about the potential mechanism(s) behind the reduced enzyme activity. In this study we investigated the correlation between CYP3A4 phenotype and 21 cytokines reflecting different immune responses in RA patients, and observed significant negative correlations between CYP3A4 activity (4 β OHC level) and IL-1ra, IL-6 and CXCL8, respectively, during treatment with TNF- α inhibitors. These preliminary findings might suggest that IL-1ra, IL-6 and/or CXCL8 are mediators or indirect measures of the suppressed CYP3A4 metabolism in RA patients.

The causality regarding the observed correlations is unclear, but a possible hypothesis is that one or more of the cytokines directly or indirectly suppress CYP3A4 activity by modulating levels of transcription factors in the liver (Morgan et al., 2008). Posttranslational inhibition of enzyme function and/or destabilisation of enzymes through formation of nitric oxide are also potential mechanisms (Morgan et al., 2008).

Among the cytokines studied in relation to suppression of activity of drug-metabolizing enzymes, IL-6 has been subjected to most interest. Suppression of CYP3A mRNA by IL-6 has been reported in several *in vitro* studies (Abdel-Razzak et al., 1993; Sunman et al., 2004; Aitken and Morgan, 2007; Dickmann et al., 2011). Aitken and Morgan et al. showed that IL-6 reduced the mRNA expression of CYP3A4 and CYP3A4 protein level to 5% and 50% of the control, respectively, in human hepatocytes (Aitken and Morgan, 2007). mRNA expression of other isoenzymes was also tested in the same study, and IL-6 was the only cytokine or molecule associated to immune response which consistently showed a reduction in all isoenzymes (apart from CYP2C18).

No studies have so far reported a correlation between levels of IL-6 and CYP3A4 metabolism in patients with chronic inflammation. However, indirect evidence for the importance of IL-6

for CYP3A4 activity *in vivo* have been obtained in two studies measuring metabolism after vs. before use of IL-6 inhibitors (Schmitt et al., 2011; Zhuang et al., 2015). In the two small studies with RA patients (n=12), initiation of treatment with IL-6 inhibitors tocilizumab and sirukumab, resulted in significant lower exposure (AUC) of CYP3A4 substrates simvastatin and midazolam, respectively. Together with our novel data, these studies support that IL-6 is a central cytokine for regulation of CYP3A4 activity in patients with inflammation-driven diseases.

The two other cytokines correlating with CYP3A4 activity in our study, i.e. IL-1ra and CXCL8, have not previously been implicated with a decline in CYP3A4 activity in RA patients. However, *in vitro* studies have demonstrated that IL-1 (α and β) reduces CYP3A expression in human hepatocytes (Abdel-Razzak et al., 1993; Sunman et al., 2004; Aitken and Morgan, 2007) and this effect has been shown to be significantly reversed by IL-1ra in human hepatoma cell lines (Mimura et al., 2015). IL-1ra is a competitive antagonist to the IL-1-receptor, thereby blocking the inflammatory properties of IL-1 α and IL-1 β , and levels of these three cytokines are often increased simultaneously (Arend, 2002). Although the effect of CXCL8 on CYP3A4 activity has not been investigated, these *in vitro* studies might explain why levels of IL-1ra were found to be negatively correlated with 4 β OHC in the present study. Nevertheless, future studies are required to clarify the roles of IL-1ra and CXCL8 in regulating CYP3A4 phenotype *in vivo*.

As IL-6, CXCL8 and IL-1 β are both cytokines reflecting T-helper 17 (Th17) cell responses (Tesmer et al., 2008), which are considered to be central in mediating the disease process in RA (McInnes and Schett, 2007). IL-6, IL-1 β , TNF- α and IL-23 are key inflammation promoting cytokines, released as a part of the Th17 cell immune response, while induction of CXCL8, among other chemokines, leads to recruitment of T cells, B cells, monocytes and

neutrophils to the inflamed joint (Tesmer et al., 2008). Thus, our results indicate a role for Th17 cells in suppression of CYP3A4 activity.

IL-1ra, IL-6 and CXCL8 were also the only cytokines that were correlated with 4 β OHC during TNF- α inhibitor treatment in the separate correlation analysis of the female RA patients. Interestingly, the correlations were stronger in females than in the whole population. The reason for this is unclear, but a factor of importance could be that females generally exhibit higher CYP3A4 activities than males (Gjestad et al., 2017; Hole et al., 2017). Additionally, more cytokines were positively correlated with CRP when analysing the female RA patients than in the analysis of the whole population. Thus, it is crucial that future studies investigating the effects of inflammation on CYP3A4 metabolism include sex as variable.

Previous *in vitro* studies have reported downregulated expression of CYP3A4 by TNF- α (Aitken and Morgan, 2007; Mimura et al., 2015). However, this was not supported by our study, where we did not observe any correlations between TNF- α and 4 β OHC, neither prior to or after 3 months of treatment with TNF- α inhibitors. Furthermore, initiation of treatment with TNF- α inhibitors did not alter CYP3A4 phenotype after 3 months. The discrepancy between the results in our study and the *in vitro* studies might be explained by the fact that the most pronounced effect on CYP3A4 expression in *in vitro* studies were shown for IL-1, IL-6 and lipopolysaccharide (LPS) (Aitken and Morgan, 2007; Mimura et al., 2015), but further studies are needed to examine the potential roles of TNF- α and TNF- α inhibition on CYP3A4 expression in RA patients.

During treatment with TNF- α inhibitors, 4 β OHC and CRP levels were significantly negatively correlated, as also reported in a previous study (Bjorkhem-Bergman et al., 2013). In our patient population, IL-6 and CXCL8 ($P < 0.05$), and IL-1ra ($P = 0.061$) were positively correlated with CRP. Thus, we consider it likely that CRP reflects levels of these cytokines in

the patient population rather than mediating the downregulation of CYP3A4 activity per se. This is supported by the fact that levels of the cytokine CCL2, significantly correlated with CRP, but not with 4 β OHC.

Prior to TNF- α inhibitor treatment, none of the analysed cytokines were correlated with CYP3A4 phenotype. The RA patients constitute, at this time point, a quite heterogeneous patient population due to variability in disease state and various anti-inflammatory treatment regimens prior to start with of TNF- α inhibitor treatment in this study. This heterogeneity is a complicating factor when investigating correlations between CYP3A4 activity and inflammation markers, and we therefore focused mainly on the correlations between cytokine levels and CYP3A4 phenotype during treatment with TNF- α inhibitors in the current study. In future studies it is important to examine the correlations between CYP3A4 and these cytokines in a larger population, with newly diagnosed patients and over a longer time span after initiation of treatment.

In this study we examined the correlations between cytokine levels and CYP3A4 activity, an important drug metabolizing enzyme with extensive inter-individual variability in phenotype. However, since systemic inflammation has shown to downregulate multiple CYP enzymes (Christensen and Hermann, 2012), it is important that the impact of inflammation on other CYP enzymes is examined. A combination of a cocktail approach, which enables multiple enzymes to be tested at once through concurrent administration of several probe drugs, and multiplex immunoassays, as used in this study, would allow a high-throughput method for investigation of the immune responses involved in suppression of CYP phenotypes.

Limitations of the current study includes the heterogeneity of the patient population in terms of variable time of RA diagnosis, variability in disease activity and comorbidity, and different RA treatment regimens and non-RA comedication, which may affect levels of both 4 β OHC

and cytokines. Another possible limitation may be the increase likelihood of type I errors since multiple testing was performed. In addition, pre-analytical sample handling procedures have shown to affect CXCL8 levels in serum samples prior to analysis and this could have affected the reliability of the CXCL8-measurements in our study (Skogstrand et al., 2008; de Jager et al., 2009). However, it is unlikely that systematic differences in sample handling procedures have affected our results.

In conclusion, this is the first study to examine the associations between serum levels of a range of cytokines and CYP3A4 metabolism in patients with chronic inflammation. The significant and negative correlations of 4 β OHC levels with IL-1ra, IL-6 and CXCL8 suggest that these cytokines and associated immune responses might be associated with suppressed CYP3A4 metabolism in RA patients. These preliminary findings imply that these cytokines may be mediators or indirect measures of the suppressed CYP3A4 metabolism in RA patients with chronic inflammation, but additional studies are required both to confirm these findings and to examine the possible molecular mechanisms behind our observations.

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Author Contributions

Participated in research design: Wollmann, Syversen, Vistnes, Lie, and Molden

Conducted experiments: Wollmann, Vistnes, and Mehus

Analysed the data: Wollmann, Syversen, Vistnes, and Molden

Wrote or contributed to the writing of the manuscript: Wollmann, Syversen, Vistnes, Lie, and Molden

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Figure Legends

Figure 1. Correlations between 4 β -hydroxycholesterol levels (4 β OHC) and levels of interleukin (IL)-1ra (A), IL-6 (B) and CXCL8 (C) during treatment with tumor necrosis factor- α inhibitors in 31 patients with rheumatoid arthritis. Estimated r and P values from Spearman's rank correlation test are added on each illustration. On illustration (A) and (C) 1 and 2 data points are outside the respective axis limits.

Tables

Table 1. Patient characteristics Baseline characteristics of the 31 patients with rheumatoid arthritis (RA). The same patients were also included in a previously published paper (Wollmann et al., 2017)

Characteristics	
Age, years median (range)	56 (19-76)
Gender, no. male/female	6/25
CRP, mg/L, median (range)	5 (1-52)
DAS28 ^a , median (range)	3.9 (2.4-5.8)
CDAI ^a , median (range)	11.6 (4.6-39.9)
Time since RA diagnosis ^b , months, median (range)	57 (3-291)
Synthetic DMARDs, no.	
Methotrexate	26
Sulfasalazine	7
Leflunomide	5
Hydroxychloroquine	3
Nonsteroidal anti-inflammatory drugs / systemic corticosteroids	10 / 18
TNF- α inhibitor initiated, no.	
Certolizumab pegol	21
Golimumab	5
Etanercept	4
Adalimumab	1

TNF, tumor necrosis factor; CRP, C-reactive protein; DMARDs; disease modifying anti-rheumatic drugs

DAS28, Disease Activity Score 28; CDAI, Clinical Disease Activity Index

^aDAS28 available for $n = 28$ and CDAI available for $n = 29$, ^bTime since RA diagnosis available for $n = 19$.

Table 2. Correlation analysis of serum levels of cytokines with 4 β -hydroxycholesterol and CRP Estimated *r* values, 95% confidence intervals (95% CI) and *P* values from Spearman’s rank correlation tests of the associations between cytokines and 4 β -hydroxycholesterol, and between cytokines and C-reactive protein during treatment with tumor necrosis factor- α inhibitors in 31 patients with rheumatoid arthritis

Cytokine	4 β -hydroxycholesterol		C-reactive protein	
	r (95%CI)	P value	r (95%CI)	P value
CCL2	-0.147 (-0.485– 0.229)	0.431	0.470 (0.128– 0.712)	0.008
CXCL8	-0.403 (-0.669– -0.046)	0.025	0.402 (0.045– 0.668)	0.025
G-CSF	-0.030 (-0.390– 0.338)	0.873	0.085 (-0.288– 0.435)	0.651
GM-CSF	-0.136 (-0.476– 0.240)	0.466	0.145 (-0.231– 0.484)	0.436
IFN- γ	0.007 (-0.357– 0.370)	0.968	0.034 (-0.334– 0.393)	0.856
IL-1ra	-0.408 (-0.672– -0.052)	0.023	0.340 (-0.027– 0.626)	0.061
IL-1 β	-0.099 (-0.447– 0.275)	0.596	0.087 (-0.286– 0.437)	0.642
IL-2	-0.110 (-0.456– 0.264)	0.555	0.092 (-0.282– 0.441)	0.623
IL-4	-0.084 (-0.435– 0.288)	0.651	-0.004 (-0.367– 0.361)	0.985
IL-5	-0.137 (-0.477– 0.239)	0.464	0.180 (-0.197– 0.510)	0.333
IL-6	-0.410 (-0.673– -0.054)	0.022	0.541 (0.221– 0.756)	0.002
IL-7	-0.193 (-0.520– 0.184)	0.299	0.214 (-0.163– 0.536)	0.249
IL-10	0.071 (-0.301– 0.424)	0.704	0.087 (-0.286– 0.437)	0.642
IL-12	-0.154 (-0.491– 0.222)	0.407	0.169 (-0.207– 0.502)	0.362
IL-13	-0.099 (-0.447– 0.275)	0.596	0.087 (-0.286– 0.437)	0.642
IL-15	-0.029 (-0.389– 0.338)	0.876	0.059 (-0.312– 0.414)	0.753
IL-17A	-0.073 (-0.426– 0.299)	0.695	-0.005 (-0.368– 0.359)	0.978

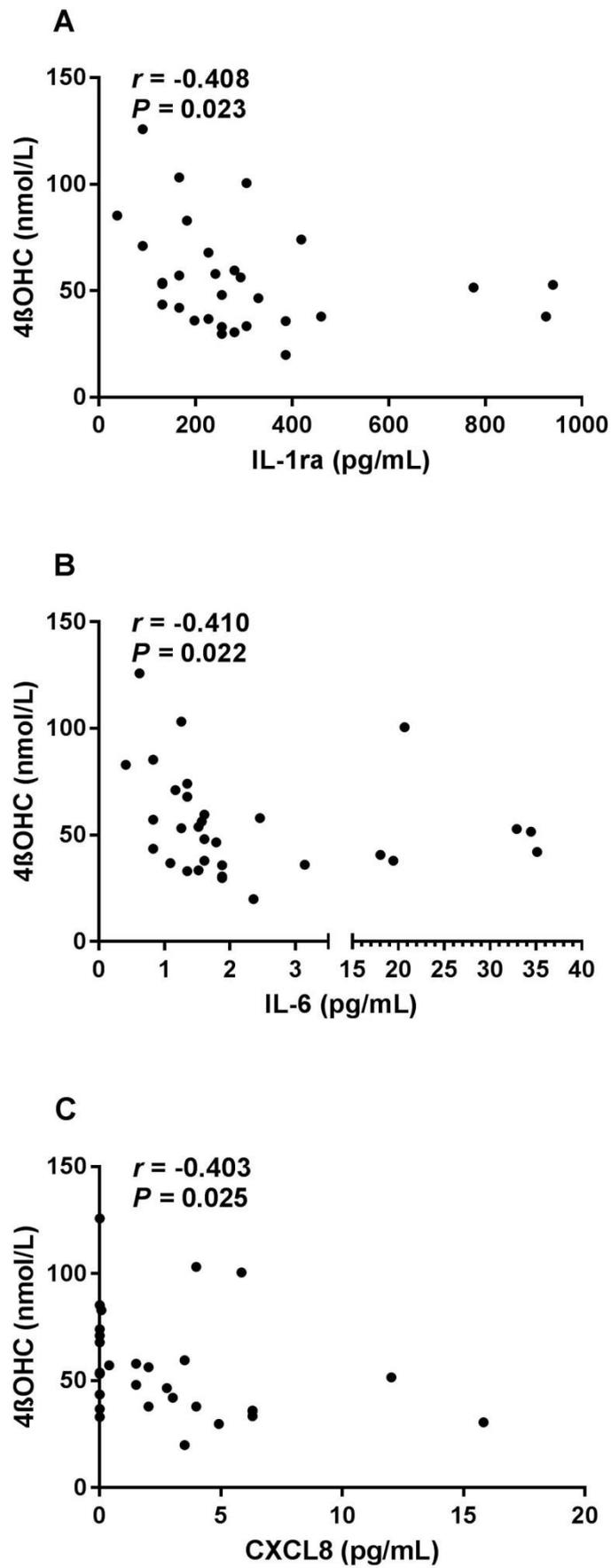
IL-18	-0.079 (-0.431– 0.293)	0.672	0.165 (-0.211– 0.499)	0.374
IL-23	0.039 (-0.329– 0.398)	0.833	-0.020 (-0.381– 0.346)	0.914
TNF- α	-0.109 (-0.455– 0.266)	0.561	0.325 (-0.044– 0.616)	0.075

Table 3. Correlation analysis of serum levels of cytokines with 4 β -hydroxycholesterol and CRP in female RA patients Estimated *r* values, 95% confidence intervals (95% CI) and *P* values from Spearman’s rank correlation tests of the associations between cytokines and 4 β -hydroxycholesterol, and between cytokines and C-reactive protein during treatment with tumor necrosis factor- α inhibitors in 25 female patients with rheumatoid arthritis

Cytokine	4 β -hydroxycholesterol		C-reactive protein	
	r (95%CI)	P value	r (95%CI)	P value
CXCL8	-0.418 (-0.704– -0.015)	0.037	0.577 (0.224– 0.796)	0.003
IL-1ra	-0.441 (-0.718– -0.044)	0.027	0.497 (0.115– 0.751)	0.011
IL-2	-0.250 (-0.595– 0.173)	0.228	0.399 (-0.007– 0.693)	0.048
IL-6	-0.506 (-0.756– -0.126)	0.010	0.764 (0.519– 0.893)	<0.0001
IL-7	-0.250 (-0.595– 0.173)	0.228	0.417 (0.014– 0.704)	0.038
TNF- α	-0.162 (-0.533– 0.261)	0.439	0.533 (0.163– 0.772)	0.006

Figures

Figure 1



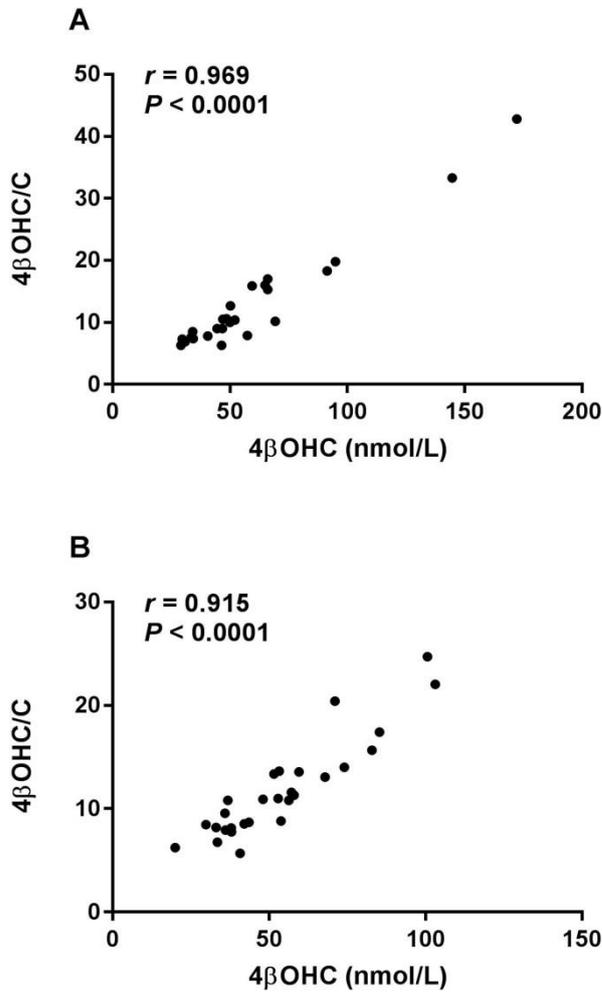
Associations between cytokine levels and CYP3A4 phenotype in patients with rheumatoid arthritis

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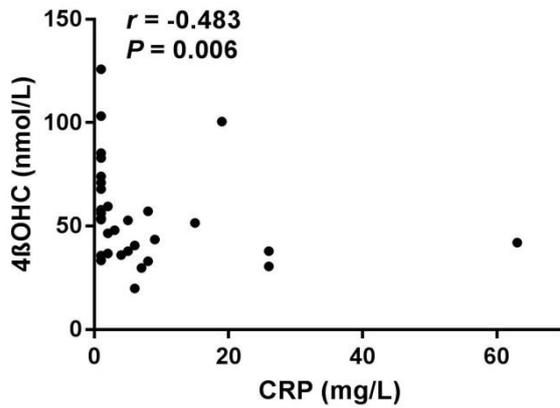
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Supplementary Figure 1



Supplementary Figure 1. Correlations between unadjusted 4β-hydroxycholesterol levels (4βOHC) and 4βOHC-to-cholesterol (C) ratios (4βOHC/C) prior to (A) and during (B) treatment with tumor necrosis factor (TNF)-α inhibitors. 25 of 31 samples prior to and 28 of 31 samples during treatment with TNF-α inhibitors had sufficient serum volumes available for analysis of both cytokines and C. Estimated r and P values from Pearson's correlation tests are added on each illustration.

Supplementary Figure 2



Supplementary Figure 2. Correlation between 4β-hydroxycholesterol (4βOHC) and C-reactive protein (CRP) levels in 31 patients with rheumatoid arthritis during treatment with tumor necrosis factor-α inhibitors. Estimated r and P values from Spearman's correlation test are added on the illustration.

Supplementary Table 1

Supplementary Table 1. Correlation analysis of serum levels of cytokines with 4 β -hydroxycholesterol and CRP Estimated *r* values, 95% confidence intervals (95% CI) and *P* values from Spearman's rank correlation tests of the associations between cytokines and 4 β -hydroxycholesterol (n=31), and between cytokines and C-reactive protein (n=30) prior to treatment with tumor necrosis factor- α inhibitors in patients with rheumatoid arthritis

Cytokine	4 β -hydroxycholesterol		C-reactive protein	
	r (95%CI)	P value	r (95%CI)	P value
CCL2	0.270 (-0.104 – 0.577)	0.142	0.074 (-0.304 – 0.432)	0.698
CXCL8	-0.105 (-0.452 – 0.269)	0.573	-0.178 (-0.514 – 0.206)	0.348
G-CSF	0.056 (-0.315 – 0.411)	0.766	-0.023 (-0.390 – 0.350)	0.904
GM-CSF	-0.162 (-0.496 – 0.215)	0.385	0.005 (-0.365 – 0.375)	0.977
IFN- γ	-0.086 (-0.436 – 0.287)	0.645	-0.010 (-0.378 – 0.362)	0.960
IL-1ra	-0.272 (-0.579 – 0.102)	0.138	-0.049 (-0.412 – 0.327)	0.797
IL-1 β	-0.185 (-0.514 – 0.192)	0.320	0.080 (-0.299 – 0.437)	0.674
IL-2	-0.115 (-0.459 – 0.260)	0.540	-0.030 (-0.395 – 0.344)	0.876
IL-4	-0.217 (-0.538 – 0.160)	0.241	-0.120 (-0.469 – 0.262)	0.528
IL-5	-0.058 (-0.414 – 0.312)	0.755	0.039 (-0.335 – 0.404)	0.836
IL-6	-0.257 (-0.568 – 0.118)	0.163	0.372 (0.002 – 0.652)	0.043
IL-7	0.024 (-0.343 – 0.385)	0.898	-0.254 (-0.570 – 0.128)	0.176
IL-10	0.081 (-0.291 – 0.432)	0.664	0.074 (-0.304 – 0.432)	0.697
IL-12	-0.107 (-0.454 – 0.267)	0.566	0.017 (-0.356 – 0.384)	0.931
IL-13	-0.067 (-0.421 – 0.304)	0.719	-0.013 (-0.381 – 0.359)	0.948
IL-15	-0.092 (-0.441 – 0.281)	0.622	0.016 (-0.356 – 0.384)	0.934

IL-17A	-0.125 (-0.468 – 0.250)	0.503	-0.046 (-0.409 – 0.330)	0.809
IL-18	0.085 (-0.288 – 0.435)	0.650	0.145 (-0.238 – 0.489)	0.444
IL-23	-0.051 (-0.407 – 0.319)	0.785	-0.134 (-0.480 – 0.248)	0.480
TNF- α	-0.213 (-0.535 – 0.164)	0.251	0.075 (-0.303 – 0.433)	0.693

Supplementary Table 2

Supplementary Table 2. Correlation analysis of serum levels of cytokines with 4 β -hydroxycholesterol and CRP Estimated *r* values, 95% confidence intervals (95% CI) and *P* values from Spearman's rank correlation tests of the associations between cytokines and 4 β -hydroxycholesterol, and between cytokines and C-reactive protein during treatment with the tumor necrosis factor- α inhibitor; certolizumab pegol, in 21 patients with rheumatoid arthritis

Cytokine	4 β -hydroxycholesterol		C-reactive protein	
	r (95%CI)	P value	r (95%CI)	P value
CXCL8	-0.416 (-0.725 – 0.033)	0.061	0.219 (-0.248 – 0.603)	0.341
IL-1ra	-0.631 (-0.839 – -0.261)	0.002	0.291 (-0.174 – 0.650)	0.200
IL-6	-0.507 (-0.776 – -0.083)	0.019	0.357 (-0.102 – 0.691)	0.112