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The in vitro effect of antirheumatic drugs on platelet function

Several antirheumatic drugs lower the cardiovascular risk among rheumatoid arthritis patients. It is however unknown whether inhibition of platelet function contribute to this risk reduction. Only few studies have investigated the potential role of platelets as a target of antirheumatic drugs. In this study, platelet function was tested in vitro in samples from 24 healthy individuals spiked with antirheumatic drugs in clinically relevant concentrations or vehicle. Platelet aggregation was tested with 96-well light transmission aggregometry (LTA), and when an effect ≥20% compared to vehicle was observed, flow cytometric platelet aggregation and activation were evaluated and closure time was measured by Platelet Function Analyzer (PFA-200). When evaluated by LTA, teriflunomide (the active metabolite of leflunomide), tocilizumab, and prednisolone reduced ADP- and collagen-induced platelet aggregation ≥20%, while adalimumab increased TRAP-induced platelet aggregation ≥20%. Using flow cytometry, agonist-induced platelet aggregation with teriflunomide or vehicle was mean ± standard deviation (SD); 30.7% ± 5.8 vs. 41.7% ± 6.5, p=0.02 using ADP, and 34.7% ± 13.9 vs. 55.8% ± 3.9, p=0.01 using collagen. Results indicate that teriflunomide, prednisolone and tocilizumab inhibit, and adalimumab increases platelet aggregation.

The study suggests that the majority of antirheumatic drugs mainly reduced cardiovascular risk through indirect effects (e.g. reducing inflammation).

Keywords: Platelet function; Rheumatoid arthritis; Cardiovascular disease; Pharmacology; Experimental study.
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease affecting 0.5-1% of the adult western population\(^1\). RA patients have increased mortality compared to the general population, mainly because of a higher incidence of cardiovascular disease (CVD)\(^2\). However, the risk of CVD in patients with RA cannot be fully explained by traditional cardiovascular risk factors\(^3\). Evidence supports that inflammation contributes to accelerated atherosclerosis and affect the composition of atherosclerotic plaques, which render the plaques more unstable and increases the risk of rupture and thus, arterial thrombosis\(^3\). Additionally, enhanced platelet activation has been observed in RA patients\(^4\). This is important as presence of active platelets is associated with increased risk of arterial thrombosis regardless of whether the patient has prevalent CVD\(^5\).

Several studies have indicated that anti-inflammatory treatment with disease-modifying antirheumatic drugs (DMARDs), both conventional synthetic DMARDs and newer biological DMARDs, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) inhibitors, reduces the risk of CVD\(^6-8\). It is not clear whether this CVD risk reduction is solely due to an anti-inflammatory effect. Alternatively, DMARDs may affect platelet function directly, but only few antirheumatic drugs have been investigated for their effect on platelet function. Hydroxychloroquine, sulfasalazine, and high dose methotrexate have been shown to inhibit platelet aggregation\(^9-11\). However, the information is relevant because a platelet inhibitory effect of antirheumatic drugs may partly explain why the drugs reduce the risk of CVD among RA patients.

Our hypothesis is that antirheumatic drugs have an inhibitory effect on platelet function, which we tested in vitro among 13 different antirheumatic drugs.
Methods

Participants

Participants were healthy volunteers recruited at the Department of Clinical Immunology at Odense University Hospital, Denmark. Inclusion criteria were age ≥18 years, no chronic disease, no thrombocytopenia (mean platelet count > 150 x 10⁹/l), and no intake of medicine in the preceding 14 days that could affect platelet function, including NSAID and aspirin. A total of 24 participants were included.

Blood samples

Blood was drawn by venipuncture of a peripheral vein with a 21-gauge needle and no venostasis into BD vacutainer tubes with sodium citrate (Becton Dickinson, New Jersey, USA). Platelet-rich plasma (PRP) was generated by centrifugation of whole blood at 200 g for 10 minutes. The remaining sample was recentrifuged at 10,000 g for 10 minutes, and platelet-poor plasma (PPP) was collected. Platelet counts were determined in whole blood using a Sysmex XN-9000 analyzer (Sysmex, Kobe, Japan).

Antirheumatic drugs

The following 13 antirheumatic drugs were tested: Methotrexate, sulfasalazine, hydroxychloroquine sulphate, teriflunomide (the active metabolite of leflunomide), and prednisolone, all from Selleckchem (Houston, Texas, USA), tocilizumab and rituximab (both from Roche Registration Limited, Welwyn Garden City, Great Britain), abatacept (Bristol-Myers Squibb Pharma EEIG, Uxbridge, Great Britain), and the TNF-α inhibitors; infliximab (Celltrion Healthcare Hungary Kft., Budapest, Hungary), etanercept (Pfizer Limited, Kent, Great Britain), adalimumab (AbbVie LTD, Maidenhead, Great Britain), certolizumab (UCB Pharma S.A., Brussels, Belgium), and golimumab (Janssen Biologics B.V., Leiden, Holland). As recommended by the manufacturer, methotrexate, sulfasalazine, teriflunomide, and prednisolone were dissolved in dimethyl sulfoxide (DMSO), and hydroxychloroquine sulphate was dissolved in water.

The drugs were diluted in 0.1 M phosphate buffered saline (PBS) to three different concentrations, resulting in a final concentration corresponding to the
maximum concentration in blood plasma ($C_{\text{max}}$), and $C_{\text{max}} +/ - 1$ SD. PRP or whole blood was incubated with drug or vehicle for one hour at $37 \, ^\circ\text{C}$.

First, all 13 antirheumatic drugs was screened with 96-well light transmission aggregometry (LTA). Positive initial testing was defined as a 20% difference in 96-well LTA results between samples with drug or vehicle. For drugs with positive initial findings, we tested flow cytometric platelet aggregation and platelet activation and closure time with PFA-200.

**Maximum plasma concentrations**

The $C_{\text{max}}$ for therapeutic use of antirheumatic drugs among RA patients were identified in the literature. For our experiments, we prioritised the highest $C_{\text{max}}$ measured in RA patients after intake of the highest recommended dose (Table 1) as identified in the Product Information published by the European Medicines Agency, [http://www.ema.europa.eu/ema/](http://www.ema.europa.eu/ema/) (search date 04.04.16). For certolizumab and golimumab, the plasma concentration was identified among patients with Crohn’s disease and in healthy subjects, respectively, because no data for RA patients were available. For sulfasalazine, hydroxychloroquine, leflunomide and certolizumab, only values for the mean plasma concentrations were identified and used for experiments instead of $C_{\text{max}}$-values.

**96-well LTA**

Half-area 96-well microtiter plates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) were prepared prior to the test of platelet function. Wells were precoated with 5 µl PBS with or without agonist. The plates were sealed and stored at $-80 \, ^\circ\text{C}$ until used. Platelet agonists were arachidonic acid (AA) (the final concentration in the sample with PRP) 0.5 mM, thrombin-receptor activator peptide (TRAP)-6 10 µM, adenosine diphosphate (ADP) 6.4 µM and collagen type 1 6.4 µg/ml. ADP was from Sigma Aldrich (St. Louis, Missouri, USA), and the other agonists were from Roche (Mannheim, Germany).

On the test day, PRP from six participants was pooled before drug incubation. The microtiter plates were thawed, and 45 µl of PRP with and without antirheumatic drug were added to different wells with agonists. As reference, 45 µl of PRP or PPP were added to wells with PBS alone.
Plates were shaken (900 rpm) for 10 minutes at 37 °C and the optical density (OD) in each well was determined using a Victor X5 (Perkin Elmer, Turku, Finland). All tests were completed within 4 hours from blood sampling.

Platelet aggregation was calculated from OD in wells with agonists in reference to PRP in PBS (set to 0 % platelet aggregation) and PPP (set to 100 % platelet aggregation), as 100 % x (OD_{PRP} – OD_{sample})/(OD_{PRP} - OD_{PPP})^{12,13}. The intraassay coefficient of variation (CV%) of this assay is ≤7%^{12}. Ten experiments were conducted per setting using pools from six healthy individuals.

**Platelet aggregation by flow cytometry**

Platelet aggregation was determined using flow cytometry as previously described^{14}. PRP was divided into two fractions; an undiluted fraction labelled with the fluorescent dye Calcein-AM Ultrapure grade (CAMU) (San Diego, CA, USA) 1 µM, and a fraction diluted 1:10 with dilution buffer (NaCl 134 nM, KCl 2.9 mM, MgCl$_2$ 1 mM, glucose 5.6 mM, 4-(-2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.4) and labelled with the fluorescent dye Calcein-AM Violet 450 (CV450) (San Diego, CA, USA) 21 µM. From the working stock, 5 µl dye was added per 100 µl PRP, and incubated in the dark for 15 min at 37°C. Then, 70 µl HEPES and 35 µl of each of the labelled platelet fractions were mixed, and the mix was incubated with drug or vehicle. Next, 3.5 µl of platelet agonist was added, and the samples were shaken at 1000 rpm for 5 min in an Eppendorf Thermomixer® comfort (Eppendorf, Hamburg, Germany). The final concentration of agonists was ADP (Sigma-Aldrich, St Louis, MO, USA) 244 µM, TRAP (SFLLRN, JPT Peptide Technologies, Berlin, Germany) 244 µM, and collagen (Chrono-Par collagen, Chrono-log Corp., USA) 9 µg/ml. Finally, samples were transferred into fixation buffer (0.2% formaldehyde in PBS) and run on the FACSCanto II flow cytometer, and data was analysed with FACS Diva software (Becton Dickinson, Franklin Lakes, New Jersey, USA). Platelet aggregation was reported as the percentage of Calcein-AM double-positive events (positive for both CAMU and CV450) in proportion to all CV450 positive events. The experiments were performed on six healthy individuals per drug. The CV% of this method is ≤3% for the used agonists^{14}.

**Platelet activation by flow cytometry**


Platelet activation was examined using flow cytometry. First, 2.5 μl whole blood with drug or vehicle was incubated for 15 min at room temperature in PBS with fluorophore-conjugated monoclonal antibodies (mAbs) and agonists to a final sample volume of 32.5 μl. The mAbs include phycoerythrin (PE)-conjugated glycoprotein (GP) Ib (CD42b, clone HIP1) (dilution factor in final sample (DF) 78), and allophycocyanin (APC)-conjugated P-selectin (CD62p, clone Psel.KO2.3) (DF 325) (both from eBioscience, San Diego, CA, USA), PE-cyanine 7 (PE-Cy7) granulophysin (CD62, clone H5C6) (DF 13) and fluorescein isothiocyanate (FITC)-conjugated PAC-1 (DF 13) (both from Becton Dickinson Bioscience, San Jose, CA, USA). The agonists were ADP (Sigma-Aldrich, St Louis, MO, USA) (FC: 12.8 μM), TRAP (SFLLRN, JPT Peptide Technologies, Berlin, Germany) (FC: 10 μM) or collagen-related peptide (Dr. Richard W. Farndale, University of Cambridge, United Kingdom) (FC: 0.46 μg/ml). A negative sample was incubated with mAbs against CD63, CD42b, and PAC-1, and with an anti-P-selectin matched APC-conjugated isotype control (from eBioscience, San Diego, CA, USA), and EDTA was added to inhibit specific binding of PAC1 and CD63 mAbs. Incubation was stopped by fixation buffer, and the samples were analysed on the FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Data was analysed using Kaluza software 1.3 (Beckman Coulter, California, USA). Platelets were identified by GPIb expression and scatter pattern. The results were reported as mean fluorescence intensities (MFI) and as the percentage of platelets positive for P-selectin, CD63 and/or activated GPIIb/IIIa compared to the negative sample. The experiments were performed on six healthy individuals per drug. The CV% of this method is <11% for all activation markers\textsuperscript{15}.

**PFA-200**

Samples of 800 μl citrated whole blood preincubated with drugs or vehicle were analysed using the platelet function analyzer PFA-200\textsuperscript{®} (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) with use of the collagen/ADP cartridge according to the instructions from the manufacturer. The result obtained was “closure time” defined as the time until the blood flow through a fictive capillary is arrested\textsuperscript{16}. The experiments were performed on six healthy individuals per drug. According to previous data, the CV% for this method is 15-25%\textsuperscript{16}. 
**Statistics**

Continuous data was reported as means and SD. To compare the control group and the three groups with different concentrations of an antirheumatic drug, one-way analysis of variance (ANOVA) and Tukey’s post-test were used. If only two groups were compared, the Student’s t-test was used. All tests were based on normally distributed data. P-values were two-sided, and a p-value < 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

We designed the present study based on 96-well LTA and used collagen-induced aggregation for calculation as it showed the highest variation (7%) \(^{12}\). In the present study, we conducted ten experiments per agonist concentration to be able to detect a 20% difference in platelet aggregation based on mean platelet aggregation 81\% ± 5.7 with collagen, power = 0.9, and \( \alpha = 0.05 \).

**Ethics**

The study was approved by the Regional Scientific Ethical Committees of Southern Denmark (Project ID: S-20160016) and the Danish Data Protection Agency (Journal no.: 16/13649) and was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent before the data was collected.
Results

With 96-well LTA, we identified four drugs, which affected the platelet aggregation response ≥ 20% (Figure 1-4). For all other drugs, 96-well LTA results are shown in Supplementary Figure S1.

Teriflunomide

Tested with 96-well LTA, teriflunomide exerted a dose-dependent inhibition of platelet aggregation after stimulation with collagen (Figure 1). Furthermore, the highest concentration of teriflunomide reduced ADP-induced platelet aggregation compared to samples without teriflunomide. No effect was seen on TRAP- and AA-induced platelet aggregation. Congruently, platelet aggregation tested with flow cytometry was for samples with teriflunomide lower than samples without teriflunomide after stimulation with collagen; 34.7% ± 13.9 vs. 55.8% ± 3.9, p = 0.01, and after stimulation with ADP; 30.7% ± 5.8 vs. 41.7% ± 6.5, p = 0.02. With regard to platelet activation tested with flow cytometry, there was no effect of teriflunomide on the percentage of platelets positive for GPIIb/IIIa, P-selectin or CD63 after stimulation with agonists and no effect on the expression levels of the same platelet activation markers (Supplementary Figure S2). With PFA-200, the closure time was equal for samples with and without teriflunomide; 84.8 seconds ± 7.9 and 84.0 seconds ± 14.1, respectively, p = 0.77.

Prednisolone

The highest concentration of prednisolone had an inhibitory effect on ADP-induced platelet aggregation with 96-well LTA, while there was no effect with other agonists (Figure 2). Prednisolone had no effect on platelet aggregation or platelet activation using flow cytometry (Figure 2 and Supplementary Figure S2). There was no difference in closure time with PFA-200 between samples with versus without prednisolone; 89.0 seconds ± 14.3 vs. 89.2 seconds ± 7.7, p = 0.96.

Tocilizumab

The middle and the highest concentration of tocilizumab inhibited ADP- and TRAP-induced platelet aggregation with 96-well LTA, while only the highest concentration of
tocilizumab inhibited collagen-induced platelet aggregation (Figure 3). No effect on platelet aggregation after stimulation with AA was found. With flow cytometry, no inhibition of platelet aggregation was observed after stimulation with agonists. With regard to platelet activation, tocilizumab significantly increased the percentage of platelets positive for CD63 after stimulation with TRAP; 63.9 % ± 13.4 for samples with tocilizumab vs. 61.3 % ± 13.5 for samples without tocilizumab, p = 0.04. There was no effect of tocilizumab on the percentage of platelets positive for GPIIb/IIIa or P-selectin and on the expression levels of GPIIb/IIIa, P-selectin or CD63 after stimulation with any agonists (Figure 3, and Supplementary Figure S2). Further, no significant difference in closure time with PFA-200 between samples with and without tocilizumab was found; 91.2 seconds ± 13.4 vs. 89.2 seconds ± 7.7, p = 0.54.

**Adalimumab**

With both 96-well LTA and flow cytometry, the highest concentration of adalimumab showed a significant increase in TRAP-induced platelet aggregation response compared to samples without adalimumab (Figure 4). Platelet aggregation after stimulation with TRAP tested with flow cytometry was for samples with and without adalimumab; 63.1 % ± 3.6 vs. 60.7 ± 4.3, p = 0.01. With regard to platelet activation tested with flow cytometry, adalimumab increased the percentage of platelets positive for P-selectin after stimulation with ADP compared to samples without adalimumab; 93.7 % ± 2.2 vs. 92.9 % ± 2.4, p = 0.02, and reduced the percentage of platelets positive for CD63 after stimulation with collagen-related peptide compared to samples without adalimumab; 53.5 % ± 16.8 vs. 57.3 % ± 18.8, p = 0.04. No effect on the percentage of platelets positive for GPIIb/IIIa was seen for any agonist. Furthermore, adalimumab significantly reduced the expression of P-selectin compared to samples without adalimumab after stimulation with collagen-related peptide; 23.4 ± 5.8 MFI vs. 26.4 ± 5.8 MFI, p = 0.01. No effect in the expression levels of GPIIb/IIIa or CD63 was seen (Supplementary Figure S2). There was no significant difference in closure time with PFA-200 for samples with adalimumab compared to samples without adalimumab; 85.2 seconds ± 10.9 vs. 89.2 seconds ± 7.7, p = 0.15.
Discussion

In this study, we evaluated the in vitro effect of 13 antirheumatic drugs on platelet function. We showed that teriflunomide, prednisolone and tocilizumab inhibited platelet aggregation, while adalimumab increased platelet aggregation.

Patients with RA have increased CVD risk and treatment with some antirheumatic drugs reduces this risk and inflammatory disease activity\textsuperscript{6,7}. Inflammation is central in the pathophysiology of RA and the advantageous effects of antirheumatic drugs has been shown related to an anti-inflammatory effect\textsuperscript{3}. However, increased levels of activated circulating platelets and platelet hyperresponsiveness are observed in RA and are associated with rheumatic disease activity\textsuperscript{4,17,18}. As platelets are pivotal in the formation of arterial thrombosis, platelet hyperresponsiveness could potentially contribute to the increased CVD risk observed in RA\textsuperscript{5}. Further, a high amount of platelet-derived microparticles has been detected in the synovial fluid of rheumatoid affected joints indicating that platelets have a pathophysiological role\textsuperscript{4}. As activated platelets release microparticles, inflammatory mediators and angiogenic factors, platelet-derived constituents could contribute to the joint disease and affect the vessel wall\textsuperscript{4}. Thus, platelets are potential targets for antirheumatic and antithrombotic therapy in RA. Knowledge of the pharmacodynamics of antirheumatic drugs is important when selecting treatment for RA patients at high CVD risk for and will contribute to the understanding of the mode of action of these drugs.

Teriflunomide is the active metabolite of leflunomide and inhibits the pyrimidine synthesis\textsuperscript{19}. We found that teriflunomide inhibited ADP- and collagen-induced platelet aggregation. We used 96-well LTA, which is highly sensitive for reduced platelet function\textsuperscript{12,13} and is a modified version of the gold standard for testing platelet function suitable for multiple testing. We verified the findings with a flow cytometric approach, where platelet aggregation was measured by determination of aggregates of dyed platelets\textsuperscript{14}. We found no effect of teriflunomide on platelet activation. Platelet activation measurements comprised evaluation of P-selectin and CD63 as markers for the release of alpha granules and dense granules, and PAC-1 as a marker for the activated GPIIb/IIIa receptor\textsuperscript{15} – all are essential for platelet hemostatic potential. PFA-200 simulate platelet adhesion and aggregation triggered by collagen/ADP\textsuperscript{16}. Our findings may suggest that teriflunomide do not influence whether...
platelets can adhere to or activate at sites of injury, but as platelet aggregation is inhibited, the thrombus size could be reduced by teriflunomide.

Our finding is congruent with a recent study, which investigated the platelet aggregatory effect of leflunomide in 8 healthy dogs with LTA after 7 days treatment with leflunomide. Albeit leflunomide dose was high compared to doses used for RA, they demonstrated a significant inhibitory effect on ADP-induced platelet aggregation, and a tendency towards a lower collagen-induced platelet aggregation. Taken together the current evidence proposes that teriflunomide inhibits platelet aggregation.

In the present study, prednisolone inhibited ADP-induced platelet aggregation with 96-well LTA, but not with flow cytometry. Accordingly, others demonstrated an effect of prednisolone on ADP-induced platelet aggregation using Multiplate Analyzer on spiked whole blood samples from healthy volunteers. Previously, Moraes et al reported the existence of a functional glucocorticoid receptor in human platelets. They demonstrated an antiaggregatory effect of high dose prednisolone with ADP, thromboxane A2-receptor agonist U466619 and collagen with LTA on spiked samples of PRP from healthy individuals. However, Moraes and colleagues used high concentration of prednisolone (higher than normally used for RA), which may explain the discrepancy. Thus, it is not clear whether prednisolone have an inhibitory effect on both ADP- and collagen-induced platelet aggregation or only on ADP-induced platelet aggregation.

With tocilizumab, the initial screening demonstrated an effect on ADP-, TRAP- and collagen-induced platelet aggregation with 96-well LTA, but no effect on flow cytometric platelet aggregation. A previous study demonstrated no effect on TRAP-induced platelet aggregation in spiked samples from five healthy individuals. This study has some limitations as they used a lower concentration of tocilizumab than used for RA treatment. Further, they used high concentrations of TRAP-14 as agonist. Overall, it is uncertain whether tocilizumab affects platelet aggregation.

We observed that adalimumab increased TRAP-induced platelet aggregation with 96-well LTA and flow cytometry, while adalimumab had only minor effects on platelet activation. In contrast, previous studies showed that TNF-α inhibitors reduced platelet activation among RA patients receiving TNF-α inhibitors. In both studies, patients were treated with other antirheumatic drugs concomitantly with TNF-α inhibitors, and it cannot be excluded that the inhibitory effect on platelet activation may
be determined by the combination of TNF-α inhibitors and other DMARDs. Besides the effect of tocilizumab on platelet aggregation, Cognasse et al found that the TNF-α inhibitor etanercept, increased TRAP-induced platelet aggregation in spiked samples from healthy individuals\(^2^3\). This is in line with our result for adalimumab. However, the potential effect of TNF-α inhibitors on platelet function is unclear.

The mechanism by which the drugs interfere with platelet aggregation is uncertain. Classical antiplatelet drugs such as ADP-receptor inhibitors inhibit platelet receptors and lead to reduced platelet activation and aggregation. However, none of the observed effects seemed to be mediated through reduced platelet activation. This indicates that the examined drugs may instead physically interfere with the aggregation response. The divergent results may also be explained by methodological differences between the used methods. Pooled PRP was used for the 96-well LTA with focus on screening for antiplatelet effects, while other platelet function tests were conducted with whole blood which may influence the results. Further, the sample size for confirmatory tests was limited and therefore small effects on platelet aggregation may not be revealed. Finally, differences in agonists and agonist concentrations can influence the sensitivity and size of effect of antiplatelet therapy in platelet function tests\(^1^2\).

For the remaining antirheumatic drugs we did not demonstrate any effect on platelet aggregation. In accordance with our findings, an in vitro study found no anti-aggregatory effect of methotrexate in concentrations corresponding to doses used among RA patients\(^2^6\), while high dose methotrexate was found to inhibit platelet aggregation\(^1^1,2^6\). For sulfasalazine and hydroxychloroquine, two in vivo studies demonstrated an effect on AA-induced platelet aggregation\(^9,1^0\). Patients with inflammatory arthritis were treated with sulfasalazine, and healthy individuals were treated with hydroxychloroquine. These positive in vivo results indicate that sulfasalazine and hydroxychloroquine may affect indirect platelet aggregation pathways, which cannot be tested in an in vitro study.

Studies show that methotrexate, hydroxychloroquine, sulfasalazine, leflunomide, tocilizumab and TNF-α inhibitors decrease the CVD risk\(^6-8,2^7\). According to our study, the inhibition of platelet aggregation by leflunomide and tocilizumab may contribute to this reduced CVD risk, while the reduced risk is likely not related to a direct effect on platelets for the other drugs. In a meta-analysis including 236,525 RA patients, it was demonstrated that treatment with glucocorticoids increased CVD risk\(^6\). This does not
seem to be related to an effect on platelet function as current evidence propose that prednisolone inhibits or has no effect on platelet function\textsuperscript{21,22}.

Overall, extensive clinical experience is available for all the included antirheumatic drugs and none have bleeding complications as frequent side effects. Thus, to put our findings into clinical context, the clinical experience implies that these drugs do not cause major impairment of platelet haemostatic properties.

A strength of our study is a thorough and specific testing of different aspects of platelet function with several different analyses and the use of various agonists stimulating different platelet activation pathways. The observed effect of various drugs on platelet function in healthy individuals suggests an independent effect on platelets. However, it is also a limitation that this in vitro study only used spiked samples from healthy individuals and not patients. A lack of effect does not exclude that antirheumatic drugs affect platelet function in patients with rheumatic disease.

Conclusion

In our in vitro study, we showed that teriflunomide, prednisolone and tocilizumab reduced platelet aggregation, indicating that these drugs may have direct antiplatelet properties. Further, it was demonstrated that adalimumab increased the platelet aggregation response, although this was not seen for the other TNF-\(\alpha\) inhibitors tested. The study results further propose that the positive effect on CVD risk, seen during treatment for the majority of antirheumatic drugs, is driven by indirect effects (e.g. reducing inflammation).
Acknowledgement
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Disclosure of interest
The authors report no conflict of interest.
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Table 1. Identified plasma concentrations for antirheumatic drugs

Figure 1. The effect of teriflunomide on platelet aggregation tested with 96-well LTA (a) and on platelet aggregation (b) and activation (c-e) tested with flow cytometry. Graph a are based on results from pooled samples from healthy subjects (n = 10), and all other graphs are based on results from individual samples from healthy subjects (n = 6). Samples were spiked with teriflunomide at concentration 0-3, where concentration 0 was without teriflunomide and corresponded to the vehicle control, concentration 1 = 27 mg/l, concentration 2 = 63 mg/l and concentration 3 = 99 mg/l. ADP: Adenosine diphosphate; TRAP: Thrombin receptor-activator peptide; AA: Arachidonic acid. Results are means ± standard deviations. In the experiment using 96-well LTA, comparisons were made with one-way analysis of variance and Tukey's posttest, and in experiments using flow cytometry, the comparisons were made with t-test. *p < 0.05.

Figure 2. The effect of prednisolone on platelet aggregation tested with 96-well LTA (a) and on platelet aggregation (b) and activation (c-e) tested with flow cytometry. Graph a are based on results from pooled samples from healthy subjects (n = 10), and all other graphs are based on results from individual samples from healthy subjects (n = 6). Samples were spiked with prednisolone at concentration 0-3, where concentration 0 was without prednisolone and corresponded to the vehicle control, concentration 1 = 0.21 mg/l, concentration 2 = 0.36 mg/l and concentration 3 = 0.51 mg/l. ADP: Adenosine diphosphate; TRAP: Thrombin receptor-activator peptide; AA: Arachidonic acid. Results are means ± standard deviations. In the experiment using 96-well LTA, comparisons were made with one-way analysis of variance and Tukey's posttest, and in experiments using flow cytometry, the comparisons were made with t-test. *p < 0.05.

Figure 3. The effect of tocilizumab on platelet aggregation tested with 96-well LTA (a) and on platelet aggregation (b) and activation (c-e) tested with flow cytometry. Graph a are based on results from pooled samples from healthy subjects (n = 10), and all other graphs are based on results from individual samples from healthy subjects (n = 6). Samples were spiked with tocilizumab at concentration 0-3, where concentration 0 was without tocilizumab and corresponded to the vehicle control, concentration 1 = 116 mg/l, concentration 2 = 233 mg/l and concentration 3 = 350 mg/l. ADP: Adenosine diphosphate; TRAP: Thrombin receptor-activator peptide; AA: Arachidonic acid. Results are means ± standard deviations. In the experiment using 96-well LTA, comparisons were made with one-way analysis of variance and Tukey's posttest, and in experiments using flow cytometry, the comparisons were made with t-test. *p < 0.05.

Figure 4. The effect of adalimumab on platelet aggregation tested with 96-well LTA (a) and on platelet aggregation (b) and activation (c-e) tested with flow cytometry. Graph a are based on results from pooled samples from healthy subjects (n = 10), and all other graphs are based on results from individual samples from healthy subjects (n = 6). Samples were spiked with adalimumab at concentration 0-3, where concentration 0 was without adalimumab and corresponded to the vehicle control, concentration 1 = 3.4 mg/l, concentration 2 = 7.7 mg/l and concentration 3 = 11.1 mg/l. ADP: Adenosine diphosphate; TRAP: Thrombin receptor-activator peptide; AA: Arachidonic acid. Results are means ± standard deviations. In the experiment using 96-well LTA, comparisons were made with one-way analysis of variance and Tukey's posttest, and in experiments using flow cytometry, the comparisons were made with t-test. *p < 0.05.
Table 1

Table 1. Identified plasma concentrations for antirheumatic drugs

<table>
<thead>
<tr>
<th>Drug and recommended dosage by EMA</th>
<th>Study</th>
<th>Study population</th>
<th>Administration and dose</th>
<th>Cmax (± SD)</th>
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<tr>
<td>Methotrexate, 5-25 mg/day PO</td>
<td>Schiff\textsuperscript{28}</td>
<td>RA, n = 47</td>
<td>25 mg PO once a week</td>
<td>567 ng/mL (187)\textsuperscript{a}</td>
</tr>
<tr>
<td>Sulfasalazine, 2 g/day PO</td>
<td>Lee\textsuperscript{29}</td>
<td>RA, n = 12</td>
<td>29 (± 7) mg/kg/day</td>
<td>6 µg/mL (4)\textsuperscript{b}</td>
</tr>
<tr>
<td>Hydroxychloroquine, 200-400 mg/day</td>
<td>Miller\textsuperscript{30}</td>
<td>RA, n = 28</td>
<td>400 mg/day PO</td>
<td>213 ng/mL (18.7)\textsuperscript{b}</td>
</tr>
<tr>
<td>Leflunomide, 20 mg/day PO</td>
<td>Rozman\textsuperscript{31}</td>
<td>RA, n = 54</td>
<td>25 mg/day PO</td>
<td>63 mg/L (36)\textsuperscript{b}</td>
</tr>
<tr>
<td>Prednisolone, 7.5 mg/day PO</td>
<td>Taggart\textsuperscript{32}</td>
<td>RA, n = NA</td>
<td>10 mg/day PO</td>
<td>0.36 mg/L (0.15)</td>
</tr>
<tr>
<td>Tocilizumab, 162 mg SC once a week</td>
<td>Burmester\textsuperscript{33}</td>
<td>RA, n = 13</td>
<td>162 mg/kg SC once a week</td>
<td>52.7 µg/mL (27.3)</td>
</tr>
<tr>
<td>or 8 mg/kg IV every 4 weeks</td>
<td></td>
<td>RA, n = 13</td>
<td>8 mg/kg IV every 4 weeks</td>
<td>233 µg/mL (117)</td>
</tr>
<tr>
<td>Abatacept, 10 mg/kg IV every 4 weeks</td>
<td>Iwahashi\textsuperscript{34}</td>
<td>RA, n = 59</td>
<td>125 mg SC once a week</td>
<td>42.6 (CV: 28 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RA, n = 59</td>
<td>10 mg/kg IV every 4 weeks</td>
<td>277.4 µg/mL (CV: 35 %)</td>
</tr>
<tr>
<td>Rituximab, 2 doses of 1000 mg IV on day 1 and 15</td>
<td>Breedveld\textsuperscript{35}</td>
<td>RA, n = 107</td>
<td>1000 mg IV on day 1 and 15</td>
<td>453 µg/mL (209)</td>
</tr>
<tr>
<td>Infliximab, 3 mg/kg IV every 8 weeks</td>
<td>St Clair\textsuperscript{36}</td>
<td>RA, n = 75</td>
<td>3 mg/kg IV every 8 weeks</td>
<td>69.7 µg/mL (55.1-84.6)\textsuperscript{c}</td>
</tr>
<tr>
<td>Etanercept,</td>
<td>Takeuchi\textsuperscript{37}</td>
<td>RA, n = 18</td>
<td>25 mg SC twice a week</td>
<td>3.5 µg/mL (1.1)</td>
</tr>
<tr>
<td>Dose and Agent</td>
<td>Study</td>
<td>Population</td>
<td>Route and Frequency</td>
<td>C_{\text{max}}</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
<td>------------</td>
<td>---------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>25 mg SC twice a week</td>
<td>Adalimumab, 40 mg SC every 2 weeks</td>
<td>Nestorov\textsuperscript{38} RA, n = NA</td>
<td>40 mg SC every 2 week</td>
<td>7.7 mg/L</td>
</tr>
<tr>
<td>50 mg SC once a month</td>
<td>Golimumab, 50 mg SC (single dose)</td>
<td>ZHuang\textsuperscript{39} Healthy, n = 12</td>
<td></td>
<td>3.6 \mu g/mL</td>
</tr>
<tr>
<td>200 mg SC every 2 weeks</td>
<td>Certolizumab, 400 mg SC every 4 weeks</td>
<td>Colombel\textsuperscript{40} CD, n = 9</td>
<td></td>
<td>38.1 \mu g/mL</td>
</tr>
</tbody>
</table>

EMA = European Medicines Agency, \( C_{\text{max}} \) = maximum plasma concentration, SD = standard deviation, RA = patients with rheumatoid arthritis, CD = patients with Crohn’s disease, PO = per os, SC = subcutaneously, IV = intravenously

\textsuperscript{a} \( C_{\text{max}} \) was estimated for a dose of 25 mg. Schiff et al reported a dose-normalised \( C_{\text{max}} \) on 22.7 ng/mL (SD: 7.5; CV: 33 %).

\textsuperscript{b} Mean plasma concentration

\textsuperscript{c} Median and interquartile range

Figure 1
Figure 2
Figure 3
Figure 4
Supplementary Figure 1
Supplementary Figure S1. The effect of anti-rheumatic drugs on platelet aggregation tested with 96-well LTA. Results are based on pooled samples from healthy subjects spiked with drug or vehicle (n = 10). Concentration 0 is without drug, concentration 1 = Cmax – 1 SD, concentration 2 = Cmax and concentration 3 = Cmax + 1 SD. Cmax for each drug was identified in Table 1. ADP: Adenosine diphosphate, TRAP: Thrombin receptor-activator peptide, AA: Arachidonic acid. Results are mean ± SD. Comparisons were made with one-way analysis of variance and Tukey’s post-test. *p < 0.05.
Supplementary Figure S2. The effect of fenfluramine, prednisolone, ticlopidine, and adalimumab on platelet activation tested with flow cytometry. The concentration of fenfluramine was 99 ng/ml, of prednisolone 0.51 mg/l, of ticlopidine 250 mg/l, and of adalimumab 111.1 mg/l. Graphs show the expression of activated GPIb/IIIa, P-selectin, CD63, ADP, Adenosine diphosphate, TRAP, Thrombin receptor-activator peptide. Results are mean ± SD. Comparisons were made with t test, *p < 0.05.