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Autoimmunity

Janus kinase inhibitors enhance prostanoid biosynthesis in human whole blood *in vitro*: implications for cardiovascular side effects and prevention strategies

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ABSTRACT

Objectives: Janus kinase inhibitors (JAKis) effectively treat chronic inflammatory diseases but are associated with cardiovascular side effects through unknown mechanisms. This study aimed to investigate the impact of JAKis on prothrombotic thromboxane (TX)A_2 production in human whole blood (WB) as a possible mechanism.

Methods: We evaluated the effects of 4 JAKis— tofacitinib, baricitinib, filgotinib, and upadacitinib (0.04-20.0 μM)—on TXB_2 biosynthesis in clotting WB from healthy subjects, serving as a marker for platelet TXA_2 generation. Additionally, we assessed the impact of these JAKis on TXB_2 production in WB from healthy subjects, patients with systemic lupus erythematosus (SLE), and treatment-naïve patients with axial spondyloarthritis (axSpA) after 24-hour lipopolysaccharide (LPS) stimulation, as a marker of platelet and leukocyte prostanoid biosynthesis.

Results: All JAKis increased serum TXB_2 production in clotting WB, although not in a concentration-dependent manner. In LPS-stimulated WB, tofacitinib (1 μM) significantly increased TXB_2 production in healthy subjects (HSs) (42% ± 33%, n = 17), patients with SLE (57% ± 39%, n = 12), and patients with axSpA (31% ± 23%, n = 15). Baricitinib (1 μM) also increased TXB_2 in HSs (30% ± 22%, n = 10). Upadacitinib showed a trend towards increased TXB_2 (46% ±

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40%, n = 7), while filgotinib did not (21% ± 19%, n = 7). Aspirin (100 μM) almost completely reduced serum TXB_2 in the presence of all JAKis.
Conclusions: The enhanced biosynthesis of TXA_2 in platelets, with a minor contribution from leukocytes, may contribute to the increased cardiovascular risk associated with JAKis. Low-dose aspirin may offer a protective effect, warranting further investigations.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Tofacitinib, a Janus kinase inhibitor (JAKi), is associated with increased cardiovascular risk.
- Due to concerns about cardiovascular safety, the European Medicines Agency has restricted the use of JAKis in patients older than 65 years or those with other cardiovascular risk factors.
- Thromboxane (TX)A_2 is a vasoconstrictor and a lipid mediator with prothrombotic properties.

WHAT THIS STUDY ADDS

- This study demonstrates that JAKis increase the biosynthesis of prothrombotic TXA_2 in platelets, as well as TXA_2 and prostaglandin E_2 in leukocytes *in vitro*.
- Dysregulated TXA_2 biosynthesis induced by JAKis may contribute to an increased risk of cardiovascular side effects.
- Aspirin normalised tofacitinib-induced TXA_2 production *in vitro*.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Combined treatment with JAKis and low-dose aspirin might counteract thrombotic side effects.
- Reducing the cardiovascular risk profile may enable more patients to safely benefit from treatment with JAKis.

INTRODUCTION

Janus kinase inhibitors (JAKis) have immunosuppressive effects and are used to treat rheumatoid arthritis (RA) and other chronic inflammatory, immune-mediated diseases [1]. These agents belong to a new class of targeted synthetic disease modifying antirheumatic drugs [2]. JAKis are often well tolerated by patients with RA and provide significant symptom relief, joint preservation, and improved quality of life [2].

Tofacitinib is a first-generation oral drug that inhibits JAK1 and JAK3 with minor inhibition of JAK2 and tyrosine-protein kinase 2 (TYK2) [3], developed for the treatment of RA [4]. Other indications for tofacitinib include ankylosing spondylitis and psoriatic arthritis [5]. In a randomised, open-label safety-endpoint trial, patients with RA treated with tofacitinib were found to have an increased risk of major adverse cardiovascular events and cancer compared with patients treated with tumour necrosis factor inhibitors [6]. As a result, the European Medicines Agency [7] endorsed safety measures to minimise the risk of serious side effects that apply to all JAK inhibitors [8].

Baricitinib is a JAK1 and JAK2 inhibitor with moderate activity against TYK2 but significantly less inhibition of JAK3. It is approved for the treatment of RA [9]. Filgotinib and upadacitinib are both JAK1-selective inhibitors [10] and were developed as second-generation JAKis. They are approved in Europe for the treatment of patients with moderate to severe active RA who are intolerant to methotrexate [10,11]. Upadacitinib is also used for the treatment of psoriatic arthritis and axial spondylitis [12]. In systemic lupus erythematosus (SLE), JAKis, including tofacitinib and baricitinib, have shown beneficial effects [13] but have not been registered for this indication. Selectivity and pharmacologic properties of the JAKis have been summarised elsewhere [14], and an overview can be found in Supplementary Table S1.

In a recent screening, we unexpectedly observed that tofacitinib can increase the biosynthesis of prostaglandin (PG)E_2 and prothrombotic thromboxane (TX)B_2 in human whole blood (WB) stimulated with the bacterial endotoxin lipopolysaccharide (LPS) *in vitro* [15]. These prostanoids are generated by the release of arachidonic acid (AA) from the plasma membrane phospholipids in platelets and leucocytes. AA is first converted to PGH_2 by cyclooxygenase (COX)-1 and/or COX-2 and subsequently converted into the principal prostanoids, for example, PGE_2 and TXB_2, through the action of specific terminal synthases [16]. Platelets constitutively express COX-1 but not COX-2 [17]. They generate TXA_2, a major product of AA metabolism, which is rapidly converted to the stable but almost biologically inactive TXB_2 through a nonenzymatic hydrolysis reaction [18]. TXB_2 can be determined by using a validated immunoassay [19] or by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [15].

PGE_2 mediates signs of inflammation and is generated via the coordinated induction of the enzymes COX-2 and microsomal prostaglandin E synthase (mPGES)-1 [20]. The traditional non-steroidal anti-inflammatory drugs (NSAIDs) affect both COX isozymes, whereas coxibs act via the selective inhibition of COX-2 [16]. The inhibition of COX-dependent PGE_2 by these drugs translates into analgesic and anti-inflammatory effects [21]. During inflammation, enhanced biosynthesis of PGE_2 occurs via the induction of COX-2 and mPGES-1 in response to various proinflammatory stimuli, including cytokines and LPS (a major component of the outer cell membrane of gram-negative bacteria) [21].

TXA_2 is an unstable lipid mediator that plays a critical role in various pathophysiologic processes such as primary hemostasis, atherothrombosis, inflammation, and cancer [18]. TXA_2 is the primary AA derivative produced via the COX-1 pathway, mainly in human platelets [22], where its generation mediates different platelet responses, including platelet aggregation and granule release. Inhibition by the NSAID aspirin (acetylsalicylic acid [ASA]), when given at low doses, prevents cardiovascular disease and is commonly used worldwide as secondary prophylaxis against myocardial infarction or stroke [23].

We hypothesise that the altered production of TXA_2, primarily from platelets, and PGE_2 from leukocytes may contribute to an increased risk of thromboembolic events and diminished anti-inflammatory effects, respectively, associated with the administration of JAKis. In this study, we aimed to investigate the effects of tofacitinib on LPS-stimulated WB prostanoid biosynthesis to validate our previous preliminary findings and included 3 additional JAKis (baricitinib, filgotinib, and upadacitinib). In LPS-stimulated WB assay (24 hours of incubation), the biosynthesis of PGE_2 depends on the time-dependent induction of COX-2 and mPGES-1 in leukocytes [24]. TXB_2 can be derived from mainly platelet COX-1 with a minor contribution from

leukocyte COX-2. Prostanoid biosynthesis in LPS-stimulated WB was investigated in healthy subjects (HSs) and patients with SLE or treatment-naïve axial spondyloarthritis (axSpA). We also evaluated the impact of JAKis on platelet TXA_2 biosynthesis by measuring TXB_2 generated during WB clotting (serum TXB_2) of HSs [25] (the study's primary end point). Several lines of evidence suggest that serum TXB_2 is generated in platelets via COX-1 during clotting and in response to the endogenously generated thrombin in humans [19,25]. Moreover, the importance of COX-1 has been demonstrated *in vivo* in mice lacking platelet COX-1 [26]. Finally, we investigated the effect of ASA to verify whether it can counteract the increased prostanoid formation in WB by JAKis *in vitro*.

METHODS

Materials

Tofacitinib citrate (PZ0017-5MG) was purchased from Merck (Darmstadt). Baricitinib (16707-25MG), filgotinib (17669-5MG), and upadacitinib (29706-5MG) were purchased from Cayman Chemical. All inhibitors were reconstituted in 10 mM dimethyl sulfoxide (DMSO, D2650-5 × 5 mL; Sigma Aldrich). Further aliquots of JAKi were made at concentrations 0.1, 1, and 10 μM and stored at −80 °C. ASA (A2093-100G; Sigma Aldrich) was reconstituted in DMSO to 1 M, and aliquots were prepared at concentrations of 100 μM, 500 μM, and 1 mM and stored at −20 °C. LPS (L6529-1MG) was reconstituted in 1 mL of phosphate-buffered saline (PBS; D8537-500ML; Sigma Aldrich). These samples were aliquoted in Eppendorf tubes and stored at −20 °C. AA (Sigma Aldrich) was dissolved in saline solution (0.9% NaCl), and aliquots were stored at −80 °C.

Study population

Study population for WB clotting assay

Twelve HSs (8 females and 4 males) aged 31.10 ± 7.71 years (mean ± SD) were enrolled at the Center for Advanced Studies and Technology of G. d'Annunzio University, Chieti, Italy (Supplementary Table S2). The study was conducted in Italy after obtaining ethical approval (Approval no. 16, 2019). These subjects did not use NSAIDs, including ASA, for at least 2 weeks before donating blood for the WB clotting assay.

Study population for LPS-activated WB assay

The study enrolled 3 groups of individuals: 17 HSs; 12 patients with SLE; and 15 treatment-naïve patients with axSpA. Patients had stopped taking NSAIDs at least 1 week before blood donation (Supplementary Table S2). HSs were recruited from the Center for Blood Donors in Stockholm, Sweden, and had not experienced fever in the two weeks prior to donation. Individuals with rheumatic disease or major inflammation were also not allowed to donate blood. Patients with SLE were diagnosed according to the ACR SLE criteria [27] and recruited from the Rheumatology Clinic at Karolinska Hospital, Sweden. The median age was 37 years, ranging from 26 to 74 years. Ethical approval was granted by the Regional Ethical Review Board in Stockholm (Dnr: u2015/2001−32/2). Additionally, 6 female and 6 male treatment-naïve patients with axSpA with a median age of 31.5 years (ranging from 21 to 51 years) were recruited from the Rheumatology Clinic at Karolinska Hospital in Huddinge and the Center for Rheumatology (CFR) in Solna, Sweden, with ethical approval (Dnr: 2022-01789-01). All patients provided written informed consent.

Bioassays

WB clotting assay

Tofacitinib, baricitinib, filgotinib, and upadacitinib (0.002-1.0 mM) were dissolved in PBS and 10% DMSO; 20 μL of the solutions containing the compounds or vehicle were pipetted directly into glass tubes. The experiments were performed by a blinded researcher incubating 1 mL of WB aliquots drawn from healthy volunteers in glass tubes in the presence of vehicle or AA (Sigma Aldrich, Italy)—dissolved in saline solution (0.9% NaCl) at a final concentration of 20 μM—alone or with 4 JAKis (4 μM) and allowed to clot for 1 hour at 37 °C [25]. In addition, WB was incubated with vehicle or ASA (dissolved in DMSO to a final concentration of 100 μM) in the absence or presence of the 4 JAKis (4 μM) and allowed to clot for 1 hour at 37 °C. For all experiments, serum was separated via centrifugation (10 minutes at 1560 × *g* at 4 °C) and stored at −80 °C until it was assayed for TXB_2 by a validated enzyme immunoassay [19]. In serum, 8-iso-$PGF_{2\alpha}$ was also measured, as an index of nonenzymatic lipid peroxidation by a specific radioimmunoassay [28,29].

LPS-activated WB assay

WB in vitro stimulation

Fresh human blood was collected in heparinised tubes from male and female volunteers and patients. JAKis and the vehicle control (0.01% DMSO) were dissolved in PBS at room temperature. An aliquot of 200 μL of freshly drawn heparin blood (within 4 hours after sampling) was added to the wells of U-shaped 96-well plates (83.3925-TC Platte; Thermo Fisher Scientific) in triplicates; 25 μL aliquots of JAKis or vehicle control were added. The plate of 1 donor was incubated at 37 °C for 30 minutes, and then, 25 μL of 0.1 mg/mL LPS in PBS was added with a final concentration of LPS of 10 μg/mL, and the mixture was mixed with a pipette 3 times. The plate was incubated for 24 hours at 37 °C and centrifuged at 3000 × *g* for 10 minutes at 4 °C. An aliquot of 80 μL plasma was collected in a new deep well plate (2-mL Square Collection plate; Waters). The plate was stored at −80 °C until protein precipitation. The researcher performing the WB *in vitro* stimulation was not blinded to the different treatments. However, controls were investigated at the beginning and end of the plate to evaluate the risk of bias due to the small differences in time between treatments added to the plate at the beginning or end of the experiment.

Tofacitinib, baricitinib, upadacitinib, and filgotinib were investigated at different concentrations (0.01-1.0 μM). Moreover, an mPGES-1 inhibitor (118), the COX1/2 inhibitor diclofenac (Diclo), and a COX-2 inhibitor (NS-398) were also investigated at 1 μM to study the inhibition of the prostanoid pathway. ASA was studied at 3 different concentrations, with final concentrations in the wells of 10, 50, and 100 μM. The combination of 1 μM tofacitinib and ASA (10-100 μM) was also studied.

Protein precipitation

Plasma samples were spiked with 50 μL of the internal standard (IS) mixture of 100 μg/mL (PGE_2-d4, P/N 314010; PGD_2-d4, P/N 312010; TXB_2-d4, P/N 319030; 6-keto $PGF_{1\alpha}$-d4, P/N 315210; $PGF_{2\alpha}$-d4, P/N 316010; and 15d-PGJ_2-d4, P/N

318570; Cayman Chemical), before protein precipitation; 800 μL of 100% methanol (MeOH) was added to the samples in the deep well plate and mixed. The plate was subsequently centrifuged at 4 °C for 10 minutes at 3000 × *g*, after which the supernatants were collected in a new deep well plate. Using a speed vacuum (SPD120; ThermoScientific), the samples were evaporated for approximately 4 hours, and the plate was stored at −20 °C until solid-phase extraction.

Solid-phase extraction

The evaporated samples were redissolved in 1 mL of 0.05% formic acid (FA) in water. A solid-phase extraction plate (Oasis HLB 96-well plate; Waters) was preconditioned with 1 mL of pure MeOH and 1 mL of 0.05% FA in water. After the samples were added to the cartridges, the plate was washed with a solution consisting of 1 mL of 10% MeOH in acidic water (0.05% FA). Subsequently, 1 mL of 100% MeOH was used for elution into new plates (2-mL Square Collection plate; Waters). The plates were set in SpeedVac apparatus overnight evaporation. On the following day, the plate was retrieved and preserved at −20 °C.

Prostanoid profiling by LC-MS/MS

Ultraperformance LC-MS/MS (UPLC-MS/MS (Waters) was used for the analysis of prostanoids. The samples and standards were reconstituted in 50 μL of 20% acetonitrile (MeCN) in water. The mobile phase consisted of a mixture of water (Milli-Q; Millipore) and MeCN, with 0.05% FA. A UPLC instrument (Acquity; Waters) connected to a triple quadrupole mass spectrometer (Waters) was used to analyse the samples in negative mode via multiple reaction monitoring as previously described (Supplementary Table S3) [15]. An ACQUITY UPLC BEH C18 analytical column measuring 50.0 mm × 2.1 mm × 1.7 μm was used, and the column temperature was maintained at 40 °C. Mobile phase A consisted of 0.05% FA in water, while mobile phase B contained 0.05% FA in MeCN. The analytes were retained in the stationary phase and eluted by increasing the linear gradient (20%-95%) of organic solvent in the mobile phase (Supplementary Table S4). The flow rate was set to 0.6 mL/min with an injection volume of 5 μL, and the samples were stored in the autosampler at 10 °C. The data quantification relied on the IS and standard curve. The external standards were injected at the beginning, middle, and end of the sample table from a 96-well plate, and quality control samples were injected before each batch. Peaks were integrated via MassLynx software, version 4.1. Although the WB *in vitro* stimulations were not blinded, the LC-MS/MS data analysis was conducted using automated software to integrate peaks, minimising human intervention. This automated approach ensured a high degree of consistency and objectivity in data handling, effectively reducing subjective influence, as the compound's locations were not known during the analysis. Additionally, for the analysis of 2 patients with axSpA, a second blinded researcher performed the data analysis in parallel with the other researcher, and the same results were obtained.

Statistical analysis

Data are presented as mean ± SD. The data were normalised to each individual's vehicle control to minimise intersubject variability. A P value <.05 was considered to indicate statistical significance (GraphPad Prism software, version Prism 10 for Windows or macOS). One-way analysis of variance (ANOVA) was used to investigate whether any JAKi at any concentration increased TXB_2 and PGE_2 compared with vehicle control, followed by the Dunnett's post hoc test. Reporting the actual values of TXB_2 generation in serum (nanograms per millilitre) is appropriate since some studies have found that it correlates with clinical outcomes and the platelet response to ASA [18,30−32]. In the case of LPS-stimulated WB, reporting the data as a percentage of the control value (vehicle) is appropriate because no evidence suggests that absolute prostanoid concentrations in this model are markers related to clinical conditions. The levels of prostanoids produced depend on the concentration of LPS used, which is arbitrary. The LPS-stimulated WB data reported as % of control were analysed by one-way ANOVA and Dunnett post hoc test. The absolute levels in nanograms per millilitre of PGE_2 and TXB_2 in HSs, SLE, and axSpA groups can be found in Supplementary Tables S5 and S6. Since our goal was to compare each JAKi (at different concentrations) with the vehicle control, that is, study a relative increase/decrease, we performed pairwise comparisons (t test) with Bonferroni correction for multiple testing, and the results are reported in Tables 1 and 2.

Sample size calculation

The impact of the JAKis on serum TXB_2 was the study's primary end point. Assuming a SD of serum TXB_2 in HS of 160 ng/mL as previously [19], the study would require a sample size of 12 individuals for each treatment to achieve a power of 80% and a level of significance of 5% (2-sided), for detecting a true difference in means between the baseline (vehicle) and treated samples of 210 ng/mL (ie, an increase of 50% from the vehicle). For the LPS-activated WB assay in HSs (secondary end point), based on the effect size of 1.35 (calculated by dividing the mean difference [1.9 ng/mL] by the SD of the difference [1.4 ng/mL]), according to Cohen d for paired samples t test) for TXB_2, 8 individuals were required to achieve a statistical power of 80% at a significance level of 0.05 [33].

In the ASA experiments, serum TXB_2 (primary end point) inhibition was anticipated to exceed 95% [19]. Therefore, a sample size of at least 7 individuals was suitable to detect an inhibition percentage of 70 or greater to achieve a power of 80% and a significance level of 5% (2-sided).

RESULTS

JAKis increase prostanoids in vitro

Effects of JAKis on prostanoids generation in WB clotting assay

The generation of serum TXB_2 (a marker of TXA_2 biosynthesis from platelets in response to thrombin, which is produced endogenously during blood clotting) [24] increased significantly in the presence of tofacitinib, baricitinib, filgotinib, and upadacitinib compared with the levels detected in the presence of vehicle (Fig 1). The response was not clearly concentration dependent. At 0.4 mM, a statistically significant increase of serum TXB_2 was detected for baricitinib and filgotinib. At 4 mM, the increase in serum TXB_2 was significant versus vehicle for tofacitinib and baricitinib (Fig 1). At higher concentrations, tofacitinib and upadacitinib resulted in a significant increase in serum TXB_2. We also studied whether exogenous AA could further enhance serum TXB_2 levels. As shown in Figure 2, serum TXB_2 increased significantly in the presence of exogenous AA but the 4 JAKis did not further increase serum TXB_2 in the presence of exogenous AA (Fig 2).

Table 1
The effect of JAKis on PGE_2 biosynthesis in LPS-stimulated WB from HS, SLE, and axSpA

JAKis	Concentration	HS[1]			SLE			AxSpA		
		n	Increase[2]	P[3]	n	Increase[2]	P[3]	n	Increase[2]	P[3]
Tofacitinib	1μM	n=17	48% ± 36%	**<u>≤0.001</u>**	n=12	89% ± 127%	**<0.01**	n=15	45% ± 53%	**<0.01**
	0.1μM	n=15	34% ± 28%	**<u>≤0.001</u>**	n=12	47% ± 66%	**<0.01**	n=14	18% ± 31%	ns
	0.01μM	n=15	16% ± 28%	**<0.05**	n=12	14% ± 16%	**<0.05**	n=11	-5% ± 11%	ns
Baricitinib	1μM	n=10	39% ± 52%	**<0.05**	n=7	5% ± 26%	ns	n=14	27% ± 46%	ns
	0.1μM	n=9	33% ± 31%	0.01	n=7	13% ± 21%	ns	n=11	7% ± 20%	ns
	0.01μM	n=9	21% ± 23%	**<0.05**	n=7	7% ± 12%	ns	n=11	-2% ± 19%	ns
Filgotinib	1μM	n=7	33% ± 18%	**<0.05**	n=7	24% ± 18%	ns	n=7	10% ± 12%	ns
	0.1μM	n=7	21% ± 20%	ns	n=6	6% ± 13%	ns	n=6	-6% ± 13%	ns
	0.01μM	n=7	18% ± 13%	ns	n=7	-1.6% ± 10%	ns	n=5	-13% ± 17%	ns
Upadacitinib	1μM	n=7	59% ± 84%	ns	n=7	25% ± 36%	ns	n=8	24% ± 28%	ns
	0.1μM	n=7	62% ± 44%	**<0.05**	n=7	21% ± 28%	ns	n=7	6% ± 23%	ns
	0.01μM	n=7	37% ± 27%	**<0.05**	n=7	5% ± 15%	ns	n=6	-9% ± 20%	ns

axSpA, axial spondyloarthritis; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; ns, nonsignificant; PG, prostaglandin; SLE, systemic lupus erythematosus; TX, thromboxane; WB, whole blood.
[a]The data for HSs have already been visualised in Figure 3 (using ANOVA) but are shown here for comparison with patient data, using paired *t* test to assess significance.
[b]Increased level of PGE_2 compared with the vehicle control. All vehicle solutions were 0.01% dimethyl sulfoxide. An increase>20% is highlighted in gray.
[c]Paired *t* test. $P < .05$ is highlighted in bold. $P < .01$ is shaded in light orange and $P < .001$ is underlined. Compensating for multiple testing (36 comparisons) using Bonferroni correction corresponds to a cutoff of the nonadjusted *P* value at .0013 to maintain a significance level of .05. However, it should be noted that the Bonferroni correction was highly stringent, and the comparisons were not fully independent due to the inclusion of multiple concentrations of the same JAKi and the investigation of the same JAKi in 3 different cohorts. This suggests that unadjusted *P* values at thresholds of .01 and .05 could also be considered not to be too conservative, depending on the level of certainty required for interpretation. Furthermore, it is important to emphasise that the number of individuals analysed was higher in the tofacitinib and HS groups, which may increase the likelihood of achieving statistical significance in these groups.

Effects of JAKis on prostanoids generation in LPS-stimulated WB of HS

Tofacitinib (1 μM) significantly increased both PGE_2 (48% ± 36%, n = 17) (Fig 3) and TXB_2 (42% ± 33%, n = 17) (Fig 4). Non-normalised data can be found in Supplementary Table S5 and S6 and Supplementary Figure S1. In addition, tofacitinib at 0.1 μM also significantly ($P_{adj} < .05$) increased PGE_2 (34% ± 28%, n = 15) (Fig 3) and TXB_2 (23% ± 15%, n = 15) (Fig 4). These concentrations can be obtained after dosing with the therapeutic dose of tofacitinib of 10 mg (Supplementary Table S1). Baricitinib significantly increased TXB_2 at 1 μM (30% ± 22%, n = 10) (Fig 4). Filgotinib at 1 μM significantly increased PGE_2 (33% ± 18%, n = 7) but not TXB_2 (Figs 3 and 4). Upadacitinib at 0.01 μM significantly enhanced PGE_2 but did not reach significance at higher concentrations. Upadacitinib at 1 μM showed a trend towards an increase in TXB_2 (46% ± 40%, n = 7) but did not reach significance using very stringent Bonferroni correction for multiple tests.

Effects of JAKis on prostanoid generation in LPS-stimulated WB of patients

The effect of JAKis on WB of patients stimulated with LPS was investigated. In patients with SLE, tofacitinib (1 mM) significantly increased both PGE_2 (89% ± 127%, n = 12) (Table 1) and TXB_2 (57% ± 39%, n = 12) (Table 2). In patients with axSpA, tofacitinib (1 mM) significantly increased PGE_2 (45% ± 53%, n = 15) (Table 1) and TXB_2 (31% ± 23%, n = 15) (Table 2). Furthermore, tofacitinib at 0.1 μM also significantly increased PGE_2 (47% ± 66%, n = 12) (Table 1) and TXB_2 (28% ± 35%, n = 12) (Table 2) in SLE, but not in axSpA.

In SLE and axSpA groups, baricitinib significantly increased TXB_2 at 1 μM (24% ± 16%, n = 7, and 43% ± 31%, n = 14, respectively), but not PGE_2 (Tables 1 and 2). Upadacitinib at 1 μM showed a trend towards increased TXB_2 (43% ± 41%, n = 7). Filgotinib showed no significant effect on PGE_2 and TXB_2 in either patients with SLE or patients with axSpA.

Investigation of prevention strategies

Effects of ASA on serum TXB_2 of HS in the presence of JAKis in the WB clotting assay

We verified whether the antiplatelet agent ASA can inhibit serum TXB_2 generation in the presence of JAKis. As shown in Figure 5, ASA almost completely inhibited the production of serum TXB_2 (96% ± 2%) and was not significantly affected by the presence of JAKis.

Effects of NSAIDs and a selective mPGES-1 inhibitor on prostanoids generated by LPS-activated WB assay of HS in the absence or presence of JAKis

Inhibitors of the AA cascade were investigated. Figures 3 and 4 show that production of PGE_2 and TXB_2 was inhibited by the addition of diclofenac (Diclo), a nonselective NSAID, or NS-398, a selective COX-2 inhibitor, supporting that prostanoids originate from COX-2. A selective mPGES-1 inhibitor, compound 118 [34], reduced PGE_2 but not TXB_2 as expected (Figs. 3 and 4).

Table 2
The effect of JAKis on TXB_2 biosynthesis in LPS-stimulated WB from HS, SLE, and axSpA

JAKis	Concentration	HS[1]			SLE			AxSpA		
		n	Increase[2]	P[3]	n	Increase[2]	P[3]	n	Increase[2]	P[3]
Tofacitinib	1μM	n=17	42% ± 33%	**≤0.001**	n=12	57% ± 39%	**<0.01**	n=15	31% ± 23%	**≤0.001**
	0.1μM	n=15	23% ± 15%	**≤0.001**	n=12	28% ± 35%	**<0.01**	n=14	13% ± 19%	ns
	0.01μM	n=15	5% ± 19%	ns	n=12	14% ± 21%	ns	n=11	5% ± 21%	ns
Baricitinib	1μM	n=10	30% ± 22%	**≤0.001**	n=7	24% ± 16%	**<0.01**	n=14	43% ± 31%	**≤0.001**
	0.1μM	n=9	19% ± 19%	ns	n=7	7% ± 12%	ns	n=13	12% ± 23%	ns
	0.01μM	n=9	17% ± 15%	**<0.05**	n=7	12% ± 15%	ns	n=11	13% ± 30%	ns
Filgotinib	1μM	n=7	21% ± 19%	ns	n=7	15% ± 19%	ns	n=7	39% ± 47%	ns
	0.1μM	n=7	21% ± 21%	ns	n=7	0.5% ± 23%	ns	n=6	7% ± 24%	ns
	0.01μM	n=7	19% ± 16%	**<0.05**	n=6	0.5% ± 31%	ns	n=6	9% ± 53%	ns
Upadacitinib	1μM	n=7	46% ± 40%	**<0.05**	n=7	43% ± 41%	**<0.05**	n=8	20% ± 31%	ns
	0.1μM	n=7	33% ± 32%	ns	n=7	18% ± 29%	ns	n=7	15% ± 21%	ns
	0.01μM	n=7	41% ± 65%	ns	n=7	15% ± 20%	ns	n=6	12% ± 24%	ns

axSpA, axial spondyloarthritis; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; ns, nonsignificant; PG, prostaglandin; SLE, systemic lupus erythematosus; TX, thromboxane; WB, whole blood.
[a]The data for HSs have already been visualised in Figure 4 (using ANOVA) but are shown here for comparison with patient data, using paired *t* test to assess significance.
[b]Increased level of PGE_2 compared with the vehicle control. All vehicle solutions were 0.01% dimethyl sulfoxide. An increase>20% is highlighted in gray.
[c]Paired *t* test. $P < .05$ is highlighted in bold. JAKi *vs* vehicle control. $P < .01$ is shaded in light orange and $P < .001$ is underlined. Compensating for multiple testing (36 comparisons) using Bonferroni correction corresponds to a cutoff of the nonadjusted *P* value at .0013 to maintain a significance level of .05. However, it should be noted that the Bonferroni correction was highly stringent, and the comparisons were not fully independent due to the inclusion of multiple concentrations of the same JAKi and the investigation of the same JAKi in 3 different cohorts. This suggests that unadjusted *P* values at thresholds of .01 and .05 could also be considered not to be too conservative, depending on the level of certainty required for interpretation. Furthermore, it is important to emphasise that the number of individuals analysed was higher in the tofacitinib and HS groups, which may increase the likelihood of achieving statistical significance in these groups.

Part of the TXB_2 production can derive from platelet COX-1 activity, as shown by the finding that the NS-398 inhibited PGE_2 more profoundly than TXB_2. A combination of the mPGES-1 inhibitor and tofacitinib at 1 μM normalised the production of PGE_2 caused by JAKis (Fig. 3).

ASA inhibited TXB_2 in a concentration-dependent manner and reached 45% inhibition at 100 μM, while the inhibition of PGE_2 was only significant at the highest concentration (100 μM) (Fig 6). These data suggest that ASA reduced the contribution of platelets to TXB_2 generation in LPS-stimulated WB. Coincubation with ASA (100 μM) attenuated the enhanced generation of the 2 prostanoids induced by 1 μM tofacitinib.

DISCUSSION

We hypothesised that tofacitinib and other JAKis increase the biosynthesis of TXA_2 and that this may contribute to the increased risk of cardiovascular events associated with their use. To address this hypothesis, we examined the effects of 4 JAKis—tofacitinib, baricitinib, filgotinib, and upadacitinib—on the generation of TXB_2 in WB that was allowed to clot for 1 hour at 37 °C (serum TXB_2). Under these experimental conditions, thrombin is produced, which causes the release of AA from platelet membrane phospholipids [18,25]. AA is then converted to TXA_2 by platelet COX-1, and TXB_2 is generated through nonenzymatic hydrolysis of TXA_2 [18,25]. TXB_2 is a validated marker of TXA_2 formation; it is stable and can be readily measured [18]. We found that the 4 JAKis increased the production of serum TXB_2. Serum TXB_2 is an easily measurable and highly reproducible capacity index primarily linked to platelet COX-1 and TXA-synthase activity. It can be used to monitor drug-induced or disease-induced changes in these enzyme activities [35]. For instance, it has been shown that serum TXB_2 production is significantly reduced by 60% in uremic patients compared with that in age-matched and sex-matched controls. This reduction contributes to the impairment of platelet function observed in uraemia [30]. Additionally, measuring serum TXB_2 levels a reduced platelet synthesis of proaggregatory TXA_2 was detected in cirrhotic patients, which may help explain the bleeding tendency seen in cirrhosis [31]. Serum TXB_2 is derived exclusively from platelet TXA_2, serves as a direct indicator of the platelets' ability to produce TXA_2, and clearly reflects pharmacologic effects of ASA on platelets [19]. Furthermore, in a prospective study involving 700 ASA-treated patients who underwent angiographic evaluation for coronary artery disease, residual platelet COX-1 function, as measured by serum TXB_2, was found to correlate with subsequent major adverse cardiovascular events [32]. The evidence for a central role of TXA_2 in thrombosis and vascular occlusive events has come from the findings that TXA_2 biosynthesis is enhanced in cardiovascular diseases and that low-dose ASA, which reduces TXA_2 biosynthesis, is associated with a reduced risk [23]. The saturation of the clinical response at low doses of ASA correlates with the maximal inhibition of platelet TXA_2 biosynthesis (serum TXB_2) [19,36].

Although the effect of the JAKis was not clearly concentration dependent, we detected higher average levels of serum TXB_2 with all JAKis. However, upadacitinib was associated with

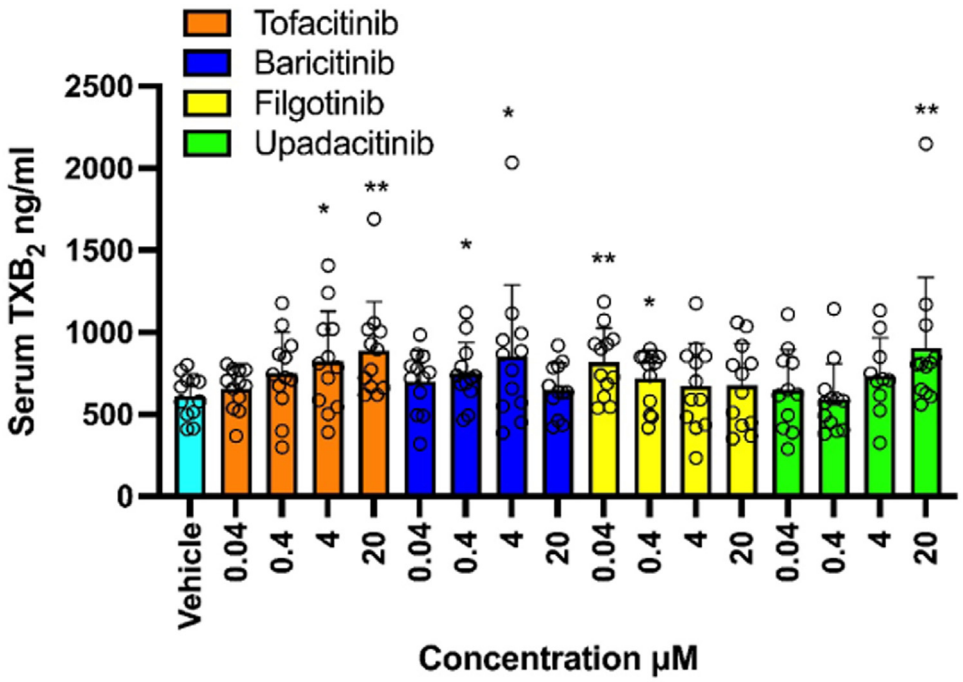

Figure 1. Effects of JAKis on the generation of TXB_2 using the WB clotting assay. Tofacitinib, baricitinib, filgotinib, and upadacitinib (dissolved in phosphate-buffered saline [10% dimethyl sulfoxide, final concentrations 0.04-20 mM]) or their vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560*g* at 4 °C) and kept at −80 °C until assayed for TXB_2. Values (ng/mL of serum) are mean ± SD (n = 12). $^*P < .05$, $^{**}P < .01$ vs vehicle using one-way ANOVA, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for macOS). ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

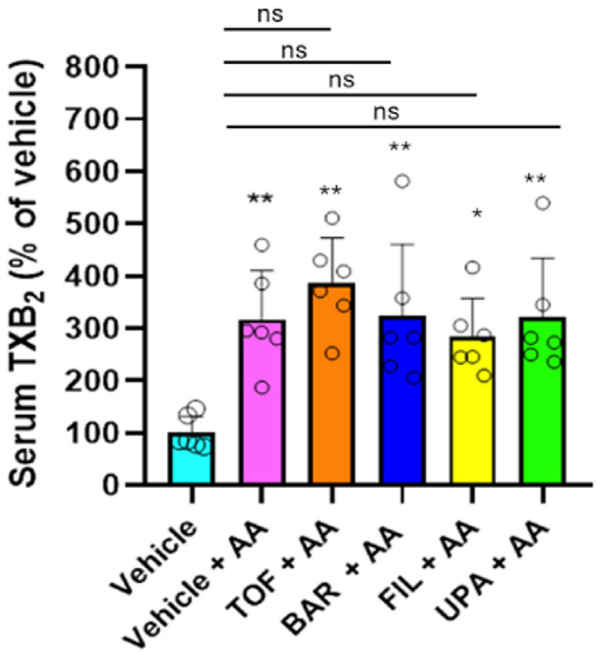

Figure 2. Effect of exogenous AA on serum TXB_2 generation in the absence and presence of JAKis. Tofacitinib (TOF), baricitinib (BAR), filgotinib (FIL), and upadacitinib (UPA; dissolved in phosphate-buffered saline, and 10% dimethyl sulfoxide, final concentration 4 μM) and AA (dissolved in saline solution at the final concentration of 20 μM) or their vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560*g* at 4 °C) and stored at −80 °C until assayed for TXB_2. The results are depicted as serum TXB_2 (% of control, ie, vehicle), mean ± SD (n = 6), and individual values. Addition of AA resulted in a significant increase in serum TXB_2 compared with that in vehicle control ($^*P < .001$ by one-way ANOVA and multiple comparisons with Tukey test). The 4 JAKis did not further increase serum TXB_2 in response to exogenous AA (ns = no significant difference between vehicle + AA and TOF + AA, BAR + AA, FIL + AA and UPA + AA). AA, arachidonic acid; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

a statistically significant increase in serum TXB_2 only at the supratherapeutic concentration of 20 μM. The most plausible explanation for the increase in TXB_2 is that the JAKis enhance the availability of free AA for the platelet COX-1 and thereby increase serum TXB_2 levels. As expected, the addition of exogenous AA increased serum TXB_2 levels, indicating the system's responsiveness to higher concentrations of the COX substrate. The 4 JAKis did not further increase serum TXB_2 levels in response to exogenous AA. This suggests that the effect of JAK inhibitors on TXB_2 production is probably not downstream of AA and supports the hypothesis that JAK inhibitors promote the release of AA from platelet phospholipids. JAKis may cause off-target effects via enhanced oxidative stress [37], thus contributing to AA release from platelet membrane phospholipids. To address this hypothesis, we verified whether the JAKis promoted the increased generation of 8-iso-$PGF_{2\alpha}$, in blood allowed to clot at 37 °C. Moreover, 8-iso-PGF_{2a} is an abundant F_2-isoprostane formed *in vivo* nonenzymatically through a free radical catalysed attack on esterified arachidonate and provides a reliable tool for detecting enhanced rates of lipid peroxidation [28]. However, in this study, we were only able to analyse a limited number of serum samples that remained after the assessment of TXB_2 levels. A comprehensive assessment of F_2-isoprostanes can be undertaken in a specific study focused on elucidating the molecular mechanisms driving the increased production of prostanoids by JAKis. The preliminary data show that JAKis enhanced serum 8-iso-PGF_{2a} levels, supporting our hypothesis that these drugs, as a class, caused an off-target effect by

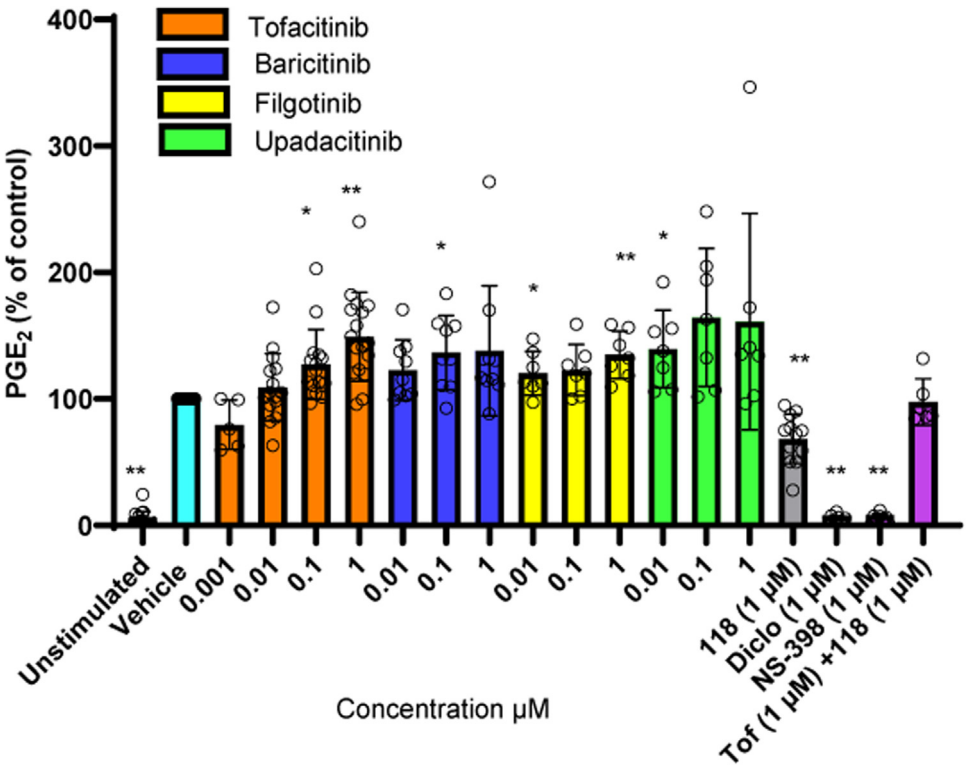

Figure 3. Effect of JAKis on PGE_2 biosynthesis in the LPS WB assay with blood from HS. Different concentrations of tofacitinib (Tof) (n = 15-17), baricitinib (Bar) (n = 9-10), filgotinib (Fil) (n = 7), upadacitinib (Upa) (n = 7) or vehicle (0.01% dimethyl sulfoxide in phosphate-buffered saline) were used. Unstimulated conditions were investigated without the addition of LPS. Diclofenac (Diclo), a nonselective nonsteroidal anti-inflammatory drug; NS-398, a selective COX-2 inhibitor; and 118, an mPGES-1 inhibitor. The data were normalised to the vehicle control. $^*P < .05$, $^{**}P < .01$ vs vehicle, using one-way ANOVA on non-normalized data, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for Windows). The nonnormalised data can be found in Supplementary Table S5. ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; PG, prostaglandin; TX, thromboxane; WB, whole blood.

inducing oxidative stress (Supplementary Fig S2). It is proposed that the JAKis induce oxidative stress at low or clinical concentrations through an off-target effect and that, at higher concentrations, other signalling pathways can inhibit reactive oxygen species (ROS) formation. This could explain why the increased TXB_2 production by the JAKi did not follow a concentration-response relationship but instead exhibited a Gaussian model (a bell-shaped curve). In addition to triggering TXA_2 generation, the role of ROS in platelet function has recently emerged. They regulate platelet activation, aggregation, and recruitment, fine-tuning several cellular signalling pathways [38]. Excessive ROS can increase TXA_2 production by potentiating PLCγ2/PKC/p38α MAPK signalling, thus providing more AA substrate for COX-1 and in line more substrate (PGH_2) for the TXA synthase to produce more TXA_2, leading to platelet hyperactivity [39]. Oxidative stress can also lead to enhanced surface expression of the TXA_2 receptor, which contributes to enhanced platelet activation in response to TXA_2 [39].

It is noteworthy that JAKis did not affect the capacity of ASA (100 μM) to almost completely inhibit serum TXB_2 production in clotting WB, a necessary action to translate into an antiplatelet effect *in vivo* [18]. Our findings suggest that coadministration of low-dose ASA could counteract the increase of platelet TXA_2 and, therefore, mitigate the risk of thromboembolic events caused by JAKis.

LPS-stimulated WB was used to evaluate the effect of JAKis on the biosynthesis of both PGE_2 and TXB_2 during an inflammatory response triggered by LPS in WB from HSs and patients with SLE or axSpA. Under these experimental conditions, leucocytes generate mainly PGE_2 in response to the induction of COX-2/mPGES-1 by LPS stimulation [24]. However, a significant amount of TXB_2 can also be produced by platelet COX-1/TXA_2 synthase [24]. Tofacitinib and baricitinib significantly increased both PGE_2 and TXB_2 in HSs. Filgotinib and upadacitinib significantly increased PGE_2, while only a trend towards increased TXB_2 was observed. This effect potentially mitigates these drugs' anti-inflammatory benefits, and it is crucial to note that the enhanced TXA_2 biosynthesis during inflammation is a hallmark of increased cardiovascular risk [40].

In patients with SLE, all JAKis, except for filgotinib, increased LPS-stimulated WB TXB_2 production at a concentration of 1 μM. The concentrations used in this study are within the range of the average maximum concentration (C_{max}) observed after therapeutic doses for all JAKis, except for filgotinib, for which a C_{max} of 5 µM is reported following a 200 mg/day dose (Supplementary Table S1).

Notably, filgotinib was associated with enhanced serum TXB_2 levels at 0.04 and 0.4 μM. A limitation of this study is that a smaller number of individuals were evaluated for filgotinib and upadacitinib in the LPS-activated WB assay compared with those for tofacitinib. However, assessing prostanoids generated in LPS-stimulated WB was a secondary end point. Serum TXB_2, which was a primary end point, was assessed with a comparable sample size for all JAKis. It is also important to note that this study was conducted *in vitro*, and its translational relevance requires validation through *in vivo* studies. Furthermore, after *in vivo* confirmation of the ability of JAKis to enhance platelet TXA_2 generation, their use should be approached with caution in patients carrying the JAK2V617F mutation.

Since we propose that the mechanism of enhanced platelet TXA_2 generation involves oxidative stress resulting from JAK inhibitors, we predict that the response might be greater in older

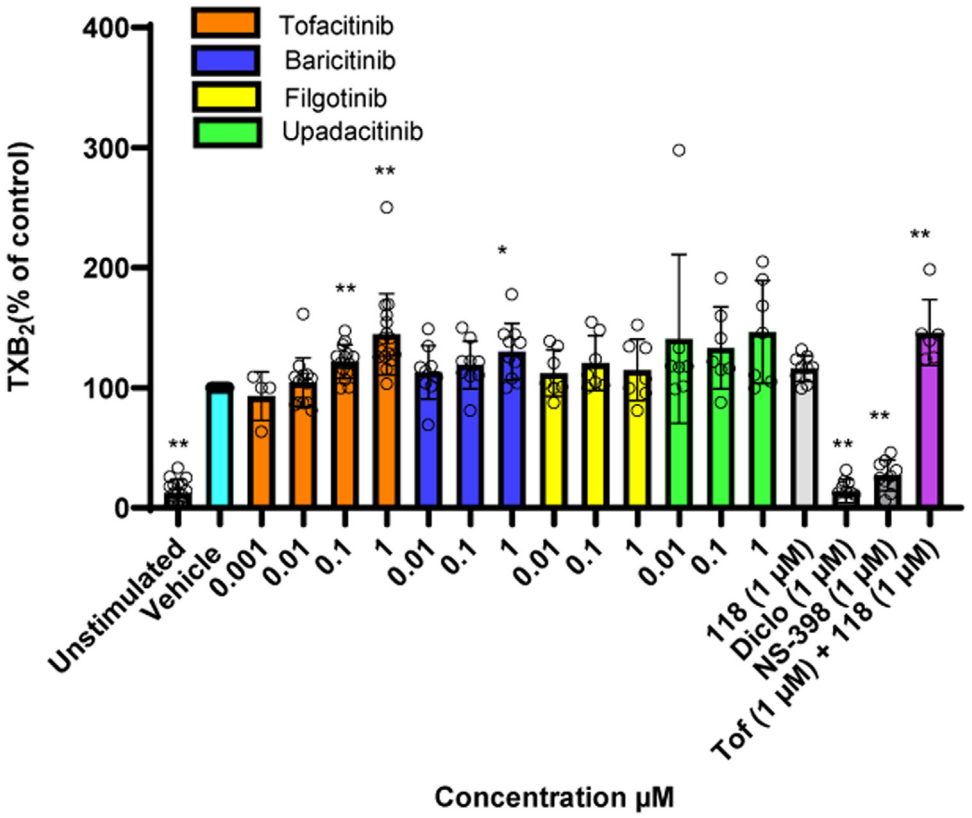

Figure 4. Effect of JAKis on TXB_2 biosynthesis in the LPS-activated WB assay. Different concentrations of tofacitinib (Tof) (n = 15-17), baricitinib (Bar) (n = 9-10), filgotinib (Fil) (n = 7), upadacitinib (Upa; n = 7), or vehicle (phosphate-buffered saline/dimethyl sulfoxide) were used. Unstimulated conditions were investigated without the addition of LPS. Diclofenac (Diclo), a nonselective NSAID, NS-398, a selective COX-2 inhibitor, and 118, an mPGES-1 inhibitor, were investigated at a concentration of 1 μM. The data were normalised to the vehicle control. $^*P < .05$, $^{**}P < .01$ vs vehicle, using one-way ANOVA on non-normalized data, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for Windows). The nonnormalised data can be found in Supplementary Table S6 and Supplementary Figure S2. ANOVA, analysis of variance; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; TX, thromboxane; WB, whole blood.

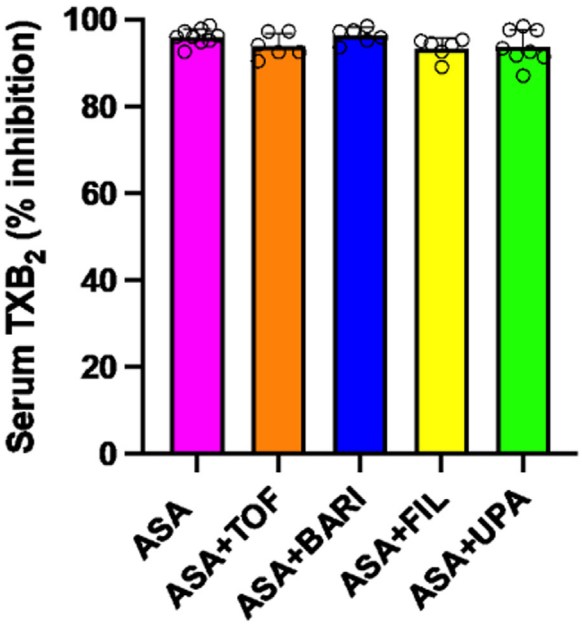

Figure 5. Aspirin profoundly affected serum TXB_2 generation in the absence and presence of JAKis. Tofacitinib (TOF), baricitinib (BARI), filgotinib (FIL), and upadacitinib (UPA) (dissolved in phosphate-buffered saline and 10% dimethyl sulfoxide [DMSO], final concentration 4 mM) and aspirin (acetylsalicylic acid [ASA] dissolved in DMSO at a final concentration of 100 μM) or vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560*g* at 4 °C) and kept at −80 °C until assayed for TXB_2. Results are presented as serum TXB_2 (% inhibition of baseline vehicle values), mean ± SD (n = 6-9), and individual values are shown. There was no significant difference between ASA and ASA + JAKi ($P > .05$) using one-way ANOVA and multiple comparisons with the Tukey test (GraphPad Prism software version Prism 10 for macOS). ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

populations or those with a history of cardiovascular risk. A specific study needs to be conducted to investigate this hypothesis. We show that enhanced platelet TXB_2 generation by JAKis can be prevented by the coincubation with ASA. The antiplatelet agent ASA is recommended for secondary prevention of atherothrombotic events in most patients with established atherosclerotic cardiovascular disease [41]. It exerts its antithrombotic effect by inhibiting platelet TXA_2 via the irreversible inhibition of the COX-1 enzyme [18,22,42]. ASA can raise the risk of bleeding. While its benefits for secondary prevention of cardiovascular disease outweigh these risks, the use of low-dose ASA for primary prevention is still controversial [43]. The challenge in assessing Asa's net benefits stems from the strong correlation between its benefits and risks, complicating the identification of individuals who are at high risk for vascular ischaemia but have a low risk of bleeding. New strategies, such as using coronary imaging to identify high-risk individuals and providing gastroprotective therapy, may help address this issue [43].

In conclusion, the enhanced biosynthesis of TXA_2 from platelets and leukocytes may increase the risk of atherothrombosis and venous thromboembolism associated with JAKis. This effect is

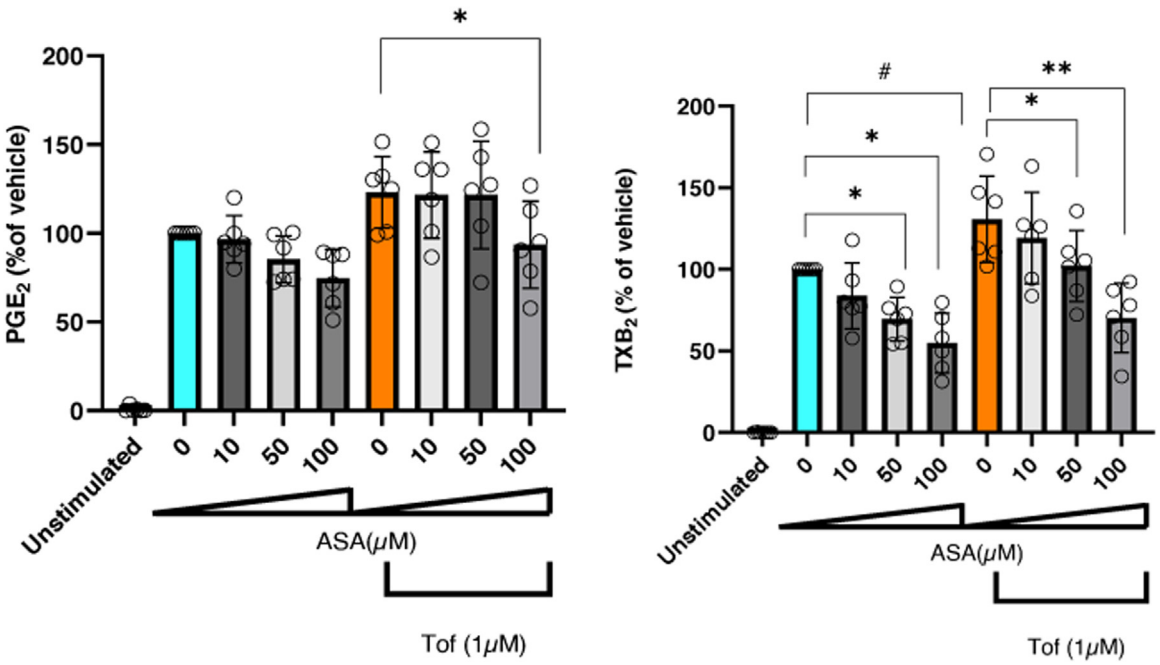

Figure 6. Aspirin inhibits the biosynthesis of PGE_2 and TXB_2 in LPS-stimulated WB *in vitro*. The effect of aspirin (acetylsalicylic acid [ASA]) was investigated in HSs (n = 6) to study its ability to inhibit the production of PGE_2 (left panel) and TXB_2 (right panel). ASA significantly reduced the production of prostanoids in a concentration-dependent manner in both the presence and the absence of tofacitinib 1 μM (*$P < .05$, **$P < .01$; paired *t* test compared with 0 μM ASA). Tofacitinib 1 μM significantly increased TXB_2 (#$P < .05$, paired *t* test compared with vehicle). HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; PG, prostaglandin; TX, thromboxane; WB, whole blood.

particularly pronounced with tofacitinib; however, all 4 JAKis examined influenced TXA_2 production. The findings suggest that combining JAKis with low-dose ASA may reduce cardiovascular side effects. To validate this hypothesis, it is crucial to conduct registry-based epidemiologic studies or randomised clinical trials involving patients with inflammatory diseases who need JAKis for treatment. If the risk of cardiovascular side effects can be effectively lowered, more patients may benefit from JAK inhibitor therapy.

Competing interests

Part of this work was conducted on behalf of the Aspirin for Cancer Prevention Group, Wolfson Institute of Preventive Medicine, Queen Mary School of Medicine and Dentistry, University of London (UK) to PP.

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Authors contributions

P-JJ, PP, and HI contributed to the ideas, formulation, or development. SA, HI, PP, and P-JJ drafted the manuscript. SA and HI set up the analysis of LPS-WB assay. HI was involved in the development of the methodology and the creation of models. SA performed experiments and analysed the data for HSs and patients in the LPS-activated WB. ST, ADM, and PDG performed WB experiments on clotting WB. PP performed the statistical analysis for the clotting WB. MV helped with patient samples with axial spondyloarthritis (axSpa). IV helped with patients with SLE at Karolinska University Hospital. VO and MK helped to collect samples from patients with axSpa at CFR. All authors have revised and approved the manuscript.

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Patients consent for publication

Not applicable.

Ethics approval

The Regional Ethical Review Board in Stockholm: Dnr: u2015/2001−32/2 regarding healthy individuals and Dnr: 2022-01789-01 regarding patients with axSpA. The Regional Ethical Review Board in Italy: Approval no. 16, 2019, regarding samples from healthy individuals in Italy

Provenance and peer review

Not commissioned; externally peer reviewed.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT4 developed by OpenAI, in order to improve language and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Patients and public involvement

Patients or the public were not involved in the design, conduct, reporting, or dissemination plans of this research.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.03.014.

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