Activation of the Acute-Phase Response in Hemophilia

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Thromb Haemost 2023;123:867–879.

Abstract

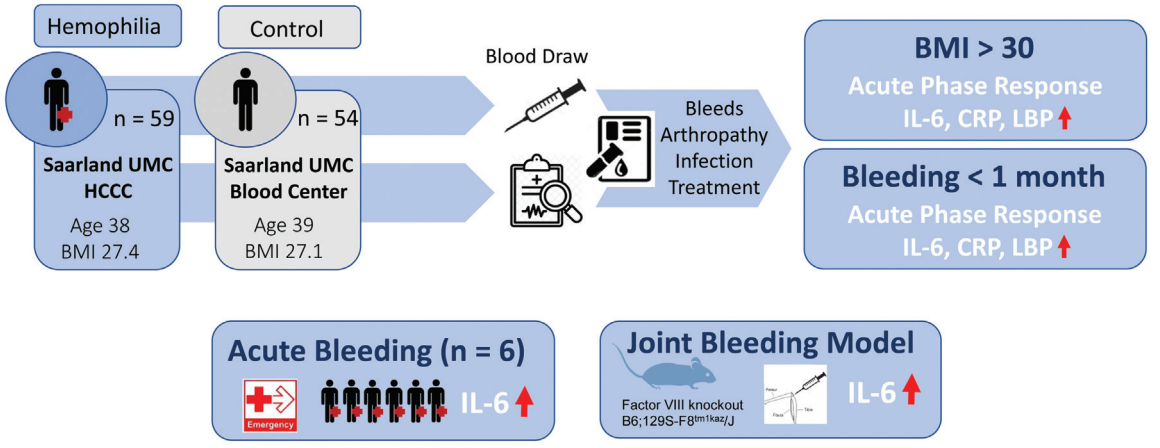
To identify recurrent inflammation in hemophilia, we assessed the acute-phase response in the blood of patients with hemophilia A and B. Compared to age- and weight-matched controls, blood levels of interleukin-6 (IL-6), C-reactive protein (CRP), and LPS-binding protein (LBP) were significantly elevated in the entire cohort of hemophilia patients but exhibited a particularly pronounced increase in obese hemophilia patients with a body mass index (BMI) $\geq$30. Subgroup analysis of the remaining nonobese hemophilia patients (BMI: 18–29.9) revealed a significant spike of IL-6, CRP, and LBP in connection with a de-novo increase of soluble IL-6 receptor α (sIL-6Rα) in patients with bleeding events within the last month. Hemophilia patients who did not experience recent bleeding had IL-6, CRP, and sIL-6Rα blood levels similar to healthy controls. We did not find increased IL-6 or acute-phase reactants in hemophilia patients with arthropathy or infectious disease. The role of IL-6 as a marker of bleeding in hemophilia was confirmed in hemophilia patients with acute bleeding events as well as in transgenic hemophilia mice after needle puncture of the knee, which exhibited an extensive hematoma and a 150-fold increase of IL-6 blood levels within 7 days of the injury compared to needle-punctured control mice. Notably, IL-6 blood levels shrunk to a fourfold elevation in hemophilia mice over controls after 28 days, when the hematoma was replaced by arthrofibrosis. These findings indicate that acute-phase reactants in combination with sIL-6Rα could be sensitive biomarkers for the detection of acute and recent bleeding events in hemophilia.

Keywords
- hemophilia
- interleukin-6
- bleeding
- wound healing
- inflammation

received
September 13, 2022
accepted after revision
March 22, 2023
accepted manuscript online
April 10, 2023
article published online
May 23, 2023

DOI https://doi.org/10.1055/a-2071-0477.
ISSN 0340-6245.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany



Visual Summary Activation of the acute-phase response is representative of acute and recent bleeding in hemophilia

Introduction

Hemophilia A and B are hereditary bleeding disorders that result from the inability to produce sufficient amounts of intact coagulation factor VIII or IX, respectively.[1] Severe forms of hemophilia are accompanied by spontaneous bleeding events that represent a major cause of morbidity and mortality in afflicted patients.[2,3] Consequently, the prophylactic application of coagulation factor concentrates in patients with severe hemophilia has become the standard of care for preventing bleeding events from occurring during everyday life.[4] The basic principal of coagulation factor prophylaxis is to convert severe hemophilia into a moderate or mild form by maintaining a coagulation factor activity with trough levels of 3 to 5% or more, which has become feasible with the advent of extended half-life coagulation factor VIII and IX concentrates and the approval of the bypassing-agent emicizumab.[5–7]

An important measure of any hemophilia therapy is its ability to prevent bleeding, which in absence of a functioning clotting cascade is increased and protracted.[2] Work with transgenic hemophilia mice has shown that even minor injuries can cause extensive hematomas that trigger the secretion of pro-inflammatory cytokines and recruitment of inflammatory cells.[8,9] While inflammation during normal wound healing speedily subsides, this does not seem to be the case in hemophilia, where a single joint bleed can suffice to induce hemophilic arthropathy.[8,10,11] An important role in this process is played by soluble IL-6 receptor α (sIL-6Rα), which perpetuates inflammation after binding to interleukin-6 (IL-6).[12–14] As such, it has been shown that anti-inflammatory treatment with a function blocking antibody against IL-6Rα in conjunction with clotting factor concentrates can mitigate hemophilic arthropathy after joint bleeds in transgenic hemophilia mice.[15]

Symptoms of inflammation in hemophilia patients such as deregulated innate immune cells and elevated C-reactive protein (CRP) suggest that the clinical and subclinical injuries sustained by hemophiliacs represent a recurrent inflammatory stimulus.[16,17] To systematically analyze the acute-phase response in this context, we assessed plasma levels of IL-6, sIL-6Rα, and their down-stream effectors CRP and LPS-binding protein (LBP) in hemophilia patients with or without recent bleeding compared to healthy blood donors. To test under controlled conditions if IL-6 correlates with acute joint bleeds in hemophilia, we induced a needle-puncture injury of the knee in transgenic mice with hemophilia A.

Materials and Methods

Human Subjects

To probe for markers of systemic inflammation, we drew blood randomly from 59 adult male patients with hemophilia A and B during routine visits to our Hemophilia Comprehensive Care Center. At enrollment, the patients were in average 38 years of age and had an average body mass index (BMI) of 27.4 kg/m^2 (►**Table 1**). For controls, we randomly recruited healthy male blood donors ($n = 54$; age, 39 years; BMI: 27.1) from our blood donation service at Saarland University Medical Center. Approval to conduct the study was received from the local ethics committee (Kenn-Nr. 73/14 and 135/19, Ethik-Kommission Ärztekammer Saarland). Comprehensive blood testing was performed routinely at the time of office visit (Saarland University Medical Center, Department of Laboratory Medicine). All patients were free of inhibitors against coagulation factor VIII or IX. As expected, patients with severe hemophilia A and B had residual coagulation factor VIII and IX activities of <1%, while the patients with moderate to mild hemophilia A and B combined had a mean residual factor VIII/IX activity of 12.2% ranging from 1.8 to 39.6% (►**Supplementary Table S1**, available in the online version). Activities of coagulation factor VIII/IX in patients with hemophilia A/B were comparably higher at the time of blood collection as a result

Table 1 Hemophilia patient cohort

BMI classification	All	$<25\ kg/m^2$	$25–29.9\ kg/m^2$	$\geq 30\ kg/m^2$
Patients (*n*)	59	21	20	18
BMI (IQR)	27.4 (23.0–31.0)	22.1 (21.3–23.1)	27.2 (26.0–28.0)	33.7 (31.1–36.7)
Age (IQR)	37.5 (25–50)	38.1 (22.5–48)	37.5 (25–55)	36.7 (25.8–46.8)
Baseline coagulation factor activity				
Severe (%)	39 (66)	11	15	13
Moderate (%)	5 (9)	2	1	2
Mild (%)	15 (25)	8	4	3
Last bleeding event				
>12 mo (%)	26 (44)	5	8	13
1–12 mo (%)	20 (34)	9	7	4
<1 mo (%)	13 (22)	7	5	1
Arthropathy (%)	32 (54)	9	12	11
Infection (%)	12 (20)	5	5	2
Therapy (%)	35 (59)	10	12	13
Controls (*n*)	54	14	33	7
BMI (IQR)	27.1 (24.9–29.0)	23.5 (22.8–24.8)	27.6 (26.7–28.6)	32.2 (30.6–33.4)
Age (IQR)	38.6 (26–50.3)	31.9 (24.3–35)	42.2 (27–53.5)	34.7 (28–44)

Abbreviations: BMI, body mass index; IQR, interquartile range; mo, month.

of clotting factor replacement or natural fluctuation and the activated partial thromboplastin time was prolonged accordingly. Quick prothrombin time (Quick PT), fibrinogen, and D-dimers as well as blood counts of leukocytes, platelets, and hemoglobin were within the normal range, with the exception of two patients with mild thrombocytopenia and one patient with transient hypofibrinogenemia/Quick PT reduction. Patients' history of bleeding, prophylaxis use, and chronic hepatitis B, chronic hepatitis C or human immunodeficiency virus were collected using the Smart Medication eDiary self-reporting app (Smart Medication eHealth Solution GmbH) or based on records after a visit with a board-certified physician in our outpatient clinic. Hemophilia patients who have been admitted to our hospital because of bleeding were diagnosed based on clinical appearance, patient history, and laboratory work-up. X-ray or computed tomography (CT) scans were routinely performed in cases of trauma. Magnetic resonance imaging (MRI) took place in order to specify joint bleeds and assess structural damage to the joint or in cases of head injury. Soft tissue hematomas underwent further imaging (ultrasound, CT scan) as clinically warranted. The diagnosis of arthropathy was based on orthopaedic physical examination, radiological diagnostics, the history of a target joint, or past orthopedic procedures (prosthetic joint replacement, synovectomy, etc.).

Plasma Isolation

After receiving informed consent, venous blood was drawn with a 20-gauge needle into S-Monovette citrate tubes (Sarstedt) and centrifuged at $2{,}000 \times g$ for 20 minutes at room temperature. Platelet-poor plasma was isolated, aliquoted, and stored at −80 °C until subsequent analysis.

Measurement of Inflammatory Markers

Human IL-6, tumor necrosis factor α (TNF)α, and IL-1β were examined using the Quantikine HS ELISA kits HS600C, HSTA00E, and HSLB00D from R&D Systems. IL-6Rα and CRP were measured using the Quantikine kits DR600 and DCRP00. LBP was analyzed with the Human LBP ELISA kit HK315 from Hycult Biotech. Platelet-poor plasma was diluted 100-fold for IL-6Rα, 200-fold for CRP, 500-fold for LBP, or used whole for IL-6 and TNFα measurements. ELISAs were performed according to manufacturer instructions. IL-6 measurements of six hemophilia patients with acute bleeding were performed using electrochemiluminescence immunoassay (ECLIA; Elecsys IL-6, Roche).

Animals

Factor VIII knockout B6;129S-F8^{tm1kaz}/J and wild-type control B6129SF2/J male mice were purchased from Jackson Laboratory at 6 weeks of age. Mice were housed in individual cages in a temperature-controlled environment under a 12 hour/12 hour light–dark cycle and fed a standard pellet diet (Altromin) and water *ad libitum*. All experiments were approved by the local governmental animal protection committee (Landesamt für Verbraucherschutz, Abteilung C Lebensmittel- und Veterinärwesen, Saarbrücken, Germany;

Permit Number: 14/2019) and were conducted in accordance with the European legislation on protection of animals (Guide line 2010/63/EU) and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/index.htm, 8th edition; 2011).

Joint Injury

Nine-week-old mice were anesthetized with 2 to 2.5% isoflurane (Piramal) and punctured for 10 seconds with a 30G hypodermic needle underneath the patella of the right knee joint. The left knee was left unpunctured and served as an internal control. For pain control, drinking water was supplemented with 1 mg/mL tramadol (Grünenthal) for the duration of the study. The joint diameter was measured over time using a digital caliper (Connex) and the joint ratio (punctured knee vs. nonpunctured knee) was calculated per mouse. Nonpunctured factor VIII knockout and wild-type control animals served as day 0 baseline controls. Mice were euthanized for blood and histopathologic analyses on day 0, 1, 7, and 28 ($n = 6$–10 mice per group per time point). Blood was collected from the inferior vena cava using a 1 mL syringe with a 26G needle and a Leica M651 surgical microscope, transferred to Microvette EDTA tubes (Sarstedt) and centrifuged for 20 minutes at 2,000 × g to isolate platelet-poor plasma. Plasma IL-6 and TNFα were examined using the Mouse Quantikine M6000B and Quantikine HS ELISA MHSTA50 kits from R&D Systems, respectively. Knee joints were collected by sectioning the femur and tibia/fibula 10 mm proximally and distally from the joint, fixed overnight in ice cold 4% paraformaldehyde, decalcified for 2 weeks in Decalcifier Soft solution (Carl Roth), and embedded in paraffin.

Histological Assessment

Midsagittal sections (1 µm thick) of the knee joints were deparaffinized using 100% xylene, rehydrated in isopropanol washes, rinsed in H_2O, and stained with hematoxylin and eosin (H&E) to assess bleeding/hematoma and synovitis. To assess fibrous formation, sections were stained with 0.1% Sirius red for 1 hour, rinsed with 3% acetic acid, and dehydrated with ethanol in order to visualize collagen formation, which appears in red. Red blood cells, on the other hand, appear in a greenish-yellow color after staining with Sirius red. Tissues were visualized microscopically on a Nikon Eclipse Ni microscope equipped with a camera and NIS Elements software (Nikon). Synovitis was scored using the established Valentino score based on a 0 to 10 point scale measuring synovial hyperplasia, neovascularization, hemosiderin staining, presence of blood, synovia villous formation, and cartilage degeneration in H&E-stained tissues.[18] Intraarticular bleeding severity was scored in 0 to 2 points, with 0 indicating no bleeding, 1 being some bleeding, and 2 indicating severe bleeding within the joint. Synovia thickness was measured according to image scale using Image J software.

Statistical Analysis

Data were evaluated according to specified subgroups with mean ± standard error of the mean. Significance was determined using Student's two-tailed *t*-tests, one-way ANOVA followed by the posthoc Tukey's multiple comparisons test or Pearson correlation, and linear regression analysis (GraphPad Prism 8). *p*-Values <0.05 were considered significant. To determine if the extent of bleeding correlates with the extent of inflammation in hemophilia, we performed Pearson correlation, pairing IL-6, CRP, and LBP plasma levels in hemophilia patients with the respective hemoglobin concentrations as a measure of potential blood loss (GraphPad Prism 8). In addition, we performed Pearson correlation analysis between IL-6, CRP, and LBP in the entire hemophilia cohort to assess interdependences of the three acute-phase reactants.

Results

Acute-phase reaction in hemophilia patients: direct comparison of the two cohorts with respect to inflammatory cytokines revealed that plasma concentrations of IL-6, CRP, and LBP were significantly higher in hemophilia patients compared to healthy controls (►**Fig. 1A–C**). IL-1β, on the other hand, was below the detection limit and sIL-6Rα as well as TNFα did not differ significantly (►**Table 2**). The increase in IL-6, CRP, and LBP was more pronounced in patients with severe hemophilia while those with moderate to mild hemophilia only showed a modest, nonsignificant increase (►**Fig. 1D–F**). IL-6 levels in hemophilia patients correlated positively and strongly with CRP and to a slightly lesser extent with LBP suggesting that the acute-phase response takes place in a specific and coordinated manner (►**Supplementary Fig. S1**, available in the online version). To assess the effect of age on the inflammatory profile of hemophiliacs, we stratified our cohort into groups ranging from 18 to 29, 30 to 49, and ≥50 years of age. In hemophilia, IL-6 levels were significantly elevated over controls at all age categories, whereas CRP and LBP levels were only elevated in younger to middle-aged hemophiliacs (►**Fig. 2A–C**). The acute-phase reaction in the hemophilia cohort was not influenced by chronic viral infections or treatment with coagulation factor concentrates (►**Table 2**). Together, these data demonstrate a significant increase of the acute-phase reactants IL-6, CRP, and LBP in hemophilia patients that correlates positively with the severity of the underlying coagulation defect.

The acute-phase reaction is amplified in obese hemophiliacs: to assess the effect of body weight on inflammation in hemophilia, we analyzed acute-phase reactants in hemophilia patients that were classified based on BMI as normal weight (BMI: 18.0–24.9), overweight (BMI: 25.0–29.9), or obese (BMI ≥30) (►**Table 1**). Comparison of IL-6, CRP, and LBP plasma concentrations between the three BMI groups revealed pronounced increases in obese hemophiliacs (►**Fig. 2D–F**). At the same time, we observed a robust and significant increase of the acute-phase markers in obese hemophiliacs compared to obese controls, whereas more modest elevations were documented in hemophiliacs with normal weight and, in the case of IL-6, in overweight hemophiliacs compared to their respective controls. Soluble

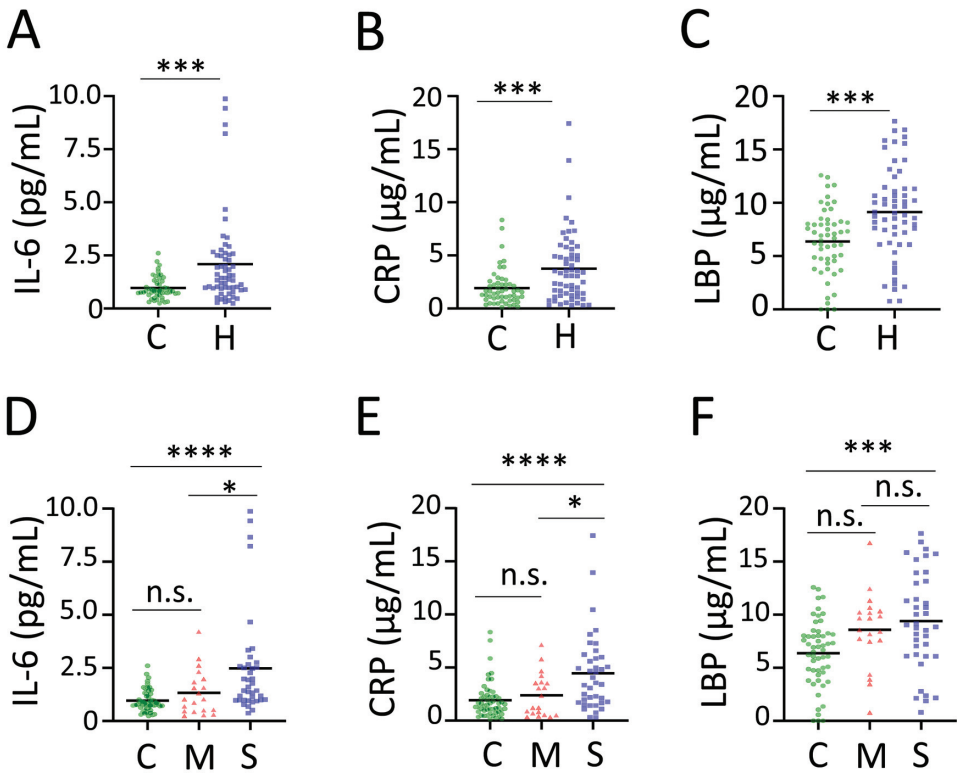


Fig. 1 Inflammatory signature in hemophilia. **(A–C)**, IL-6, CRP, and LBP concentrations in the plasma of hemophilia patients (H, hemophilia; $n = 59$) compared to healthy controls (C, control; $n = 54$). **(D–F)** IL-6, CRP, and LBP plasma levels in patients with severe (S; $n = 39$) and moderate-to-mild hemophilia (M; $n = 20$) compared to controls (C). $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Horizontal bars denote the mean. CRP, C-reactive protein; IL-6, interleukin-6; LBP, LPS-binding protein; n.s., nonsignificant.

IL-6Rα and TNFα, on the other hand, exhibited no relevant elevation in hemophilia patients over controls irrespective of the BMI classification (►**Table 2**). A distinctive characteristic of the group of obese hemophiliacs was that there was only one case of recent bleeding <1 month and that the majority of bleedings took place longer than 12 month ago (►**Table 1**). In addition, there was no significant difference in IL-6, CRP, or LBP in either of the three weight groups when we compared patients with or without arthropathy indicating that the increased acute-phase reaction in most obese hemophiliacs occurs independently of bleeding complications (►**Table 2**).

The acute-phase reaction in hemophilia patients correlates with recent bleeding: to determine the role of the bleeding disposition of hemophiliacs in triggering an acute-phase reaction, we categorized hemophilia patients according to their bleeding history in two subgroups, one with patients who had bleeding episodes within the last month and the other with patients who did not bleed in the last month or longer. To eliminate the confounding effects of obesity, 18 hemophilia patients and 7 controls with a BMI ≥30 were excluded from subsequent analysis (►**Table 2**). The most prevalent cause of hemorrhage in the bleeding <1 month group was contusions, which affected three patients with severe and three patients with moderate-to-mild hemophilia. In addition, one of the patients developed hematuria due to kidney stones and another one experienced a secondary bleed after genitourinary surgery. Joint bleeds, on the other hand, occurred spontaneously in three patients with severe and one patient with moderate-to-mild hemophilia. Subgroup analysis revealed that this diverse group of symptomatic hemophilia patients with bleeding <1 month exhibited significantly higher IL-6 and CRP plasma concentrations than the bleeding >1 month group, which had IL-6 and CRP levels almost as low as the controls (►**Fig. 3A, B**; ►**Table 2**). Paralleling these data, we found a significant up-regulation of sIL-6Rα in patients with recent bleeding that we did not observe in the complete hemophilia cohort or in patients with obesity (►**Fig. 3C**; ►**Table 2**). While LBP was elevated in hemophilia patients with bleeding history <1 month compared to controls, there was no significant difference in the bleeding <1 month compared to the bleeding >1 month group (►**Fig. 3D**). The presence of arthropathy, on the other hand, had no consistent effect on plasma levels of IL-6, sIL-6Rα, CRP, or LBP in hemophilia patients with or without recent bleeding (►**Table 2**). The potential role of IL-6, sIL-6Rα, and CRP as indicators of bleeding in hemophilia was further supported by a strong inverse correlation between blood hemoglobin levels and the acute-phase parameters in hemophilia patients with recent bleeding <1 month suggesting that patients with low hemoglobin (e.g., after bleeding) tend to present with high acute-phase reactants and vice versa (►**Supplementary Fig. S2**, available in the online version). The inverse relationship between hemoglobin

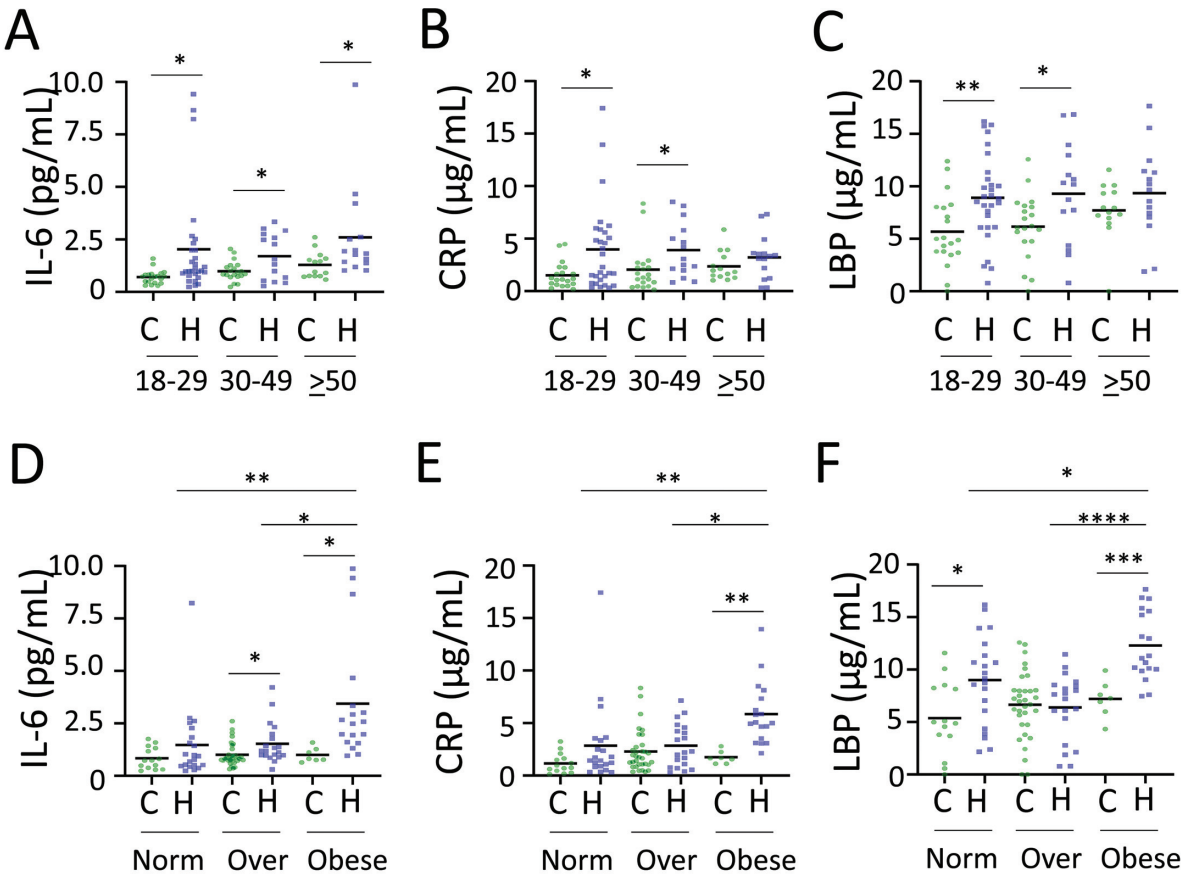


Fig. 2 The effect of age and BMI on the inflammatory signature in hemophilia. **(A–C)** IL-6, CRP, and LBP levels in hemophiliacs (H) with ages 18–29, 30–49, and ≥ 50 years compared to age-stratified controls (C). **(D–F)** Plasma concentrations of IL-6, CRP, and LBP in a BMI-stratified cohort of hemophiliacs (H) with normal weight (Norm), overweight (Over), or obese compared to weight-stratified controls (C). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{****}p < 0.0001$. Horizontal bars denote the mean. BMI, body mass index; CRP, C-reactive protein; IL-6, interleukin-6; LBP, LPS-binding protein.

and acute-phase reactants was considerably weaker in patients with bleeding >1 month and completely lost when we analyzed the entire hemophilia cohort. Together, the data indicate that IL-6, sIL-6Rα, and CRP are representative for patients with a history of recent bleeding.

IL-6 indicates acute bleeding events in hemophilia patients: to confirm that bleeding in hemophilia results in a systemic acute-phase reaction, we performed prospective, longitudinal measurements of IL-6 in six additional hemophilia patients who came to our hospital with acute bleeding episodes. Three of the patients presented with an acute traumatic hemarthrosis, two with prolonged bleeding after surgery, and one patient with a traumatic soft tissue hemorrhage. Patient 1 suffered from severe hemophilia B and incurred an extensive, traumatic hemarthrosis of the knee after a fall. He presented himself 3 days after the injury at our emergency department with plasma IL-6 levels that were 21-fold increased on day 3, 35-fold on day 5, 17-fold on day 10, and 7-fold on day 17 over baseline values that we determined from blood samples drawn twice in a 1-year interval prior to the injury (►**Fig. 4A**). Following the trauma, patient 1 received an intensified prophylaxis with an extended half-life factor IX product until the hematoma was completely absorbed after 3 weeks. Patients 2 and 3 have severe hemophilia A and suffered from traumatic bleeds of the ankle and the knee, respectively (►**Fig. 4B, C**). Like patient 1, patient 3 was repeatedly assessed with stable IL-6 values in absence of bleeding during an interval of 3 years prior to the current bleed. Increased baseline values in patients 2 and 3 can be explained by recent bleeding independent of the current bleeding event (patient 2) and obesity (patient 3, BMI: 37.6). Patients 4 and 5, both with severe hemophilia A, exhibited mild elevations of IL-6 due to prolonged bleeding after a tooth extraction and secondary bleeding after septoplasty 4 weeks earlier (►**Fig. 4D, E**). Lastly, patient 6 (severe hemophilia A) presented in our emergency room with an extensive traumatic bleed of the right latissimus dorsi muscle and a considerably increased IL-6 level that trended towards lower values at a control examination after 86 days when the muscle bleed was completely absorbed but new bruising occurred (►**Fig. 4F**). Together, these cases reinforce the connection between acute bleeding and systemic IL-6 increases in the plasma of hemophilia patients irrespective of the bleeding location.

IL-6 indicates acute joint bleeding in hemophilia mice: to test under controlled conditions if IL-6 correlates with acute

Table 2 Acute-phase reactants in hemophilia

Acute phase reactant	# patients	BMI	IL-6 (pg/mL)	CRP (μg/mL)	LBP (μg/mL)	TNFα (pg/mL)	sIL-6Rα (ng/mL)
Controls	54	27.1 (24.9–29.0)	0.96 (0.69–1.27)	1.93 (0.84–2.32)	6.38 (4.49–8.16)	0.61 (0.46–0.71)	29.48 (24.06–33.04)
Age							
18–29	20	26.5 (24.3–28.0)	0.71 (0.45–0.84)	1.51 (0.63–2.13)	5.67 (3.71–8.00)	0.56 (0.42–0.69)	28.56 (24.55–31.89)
30–49	20	27.9 (25.8–30.0)	0.99 (0.74–1.25)	2.05 (0.56–2.49)	6.15 (4.75–8.11)	0.59 (0.48–0.64)	30.17 (23.70–35.07)
≥ 50	14	27.0 (25.6–28.5)	1.28 (0.75–1.63)	2.37 (1.36–3.41)	7.71 (6.85–9.58)	0.69 (0.49–0.78)	39.78 (22.87–33.73)
BMI							
$< 25\,kg/m^2$	14	23.5 (22.8–24.8)	0.84 (0.36–1.35)	1.16 (041–1.74)	5.37 (3.02–8.31)	0.52 (0.42–0.61)	30.40 (24.06–35.06)
$25–29.9\,kg/m^2$	33	27.6 (26.7–28.6)	1.00 (0.73–1.14)	2.30 (1.04–2.81)	6.63 (4.75–8.02)	0.63 (0.49–0.72)	28.70 (24.27–32.37)
$\geq 30\,kg/m^2$	7	32.2 (30.6–33.4)	0.99 (0.75–1.27)	1.74 (1.13–2.79)	7.20 (5.99–8.49)	0.67 (0.57–0.82)	31.28 (22.68–36.39)
Patients *vs Controls*	59	27.4 (23.0–31.0)	2.09 (0.94–2.52) *p = 0.0002*	3.76 (1.22–5.07) *p = 0.0004*	9.12 (6.24–11.32) *p = 0.0002*	0.70 (0.50–0.82) *p = 0.052*	31.88 (26.33–38.02) *p = 0.078*
Age							
18–29 *vs Controls*	29	26.7 (21.9–29.8)	2.03 (0.82–2.15) *p = 0.021*	3.97 (0.91–5.73) *p = 0.013*	8.91 (6.08–11.00) *p = 0.005*	0.62 (0.49–0.77) *p = 0.342*	30.99 (24.43–37.33) *p = 0.203*
30–49 *vs Controls*	15	28.6 (22.4–33.6)	1.71 (0.53–2.75) *p = 0.013*	3.90 (1.97–5.80) *p = 0.028*	9.28 (4.36–12.95) *p = 0.025*	0.57 (0.46–0.67) *p = 0.690*	32.46 (28.28–35.93) *p = 0.328*
≥ 50 *vs Controls*	15	27.4 (24.9–31.1)	2.60 (1.37–2.61) *p = 0.046*	3.21 (1.21–3.62) *p = 0.217*	9.35 (7.04–11.45) *p = 0.234*	0.98 (0.69–1.18) p = 0.009	33.00 (25.89–40.46) *p = 0.341*
BMI							
$< 25\,kg/m^2$ *vs Controls*	21	22.1 (21.3–23.1)	1.47 (0.48–2.05) *p = 0.198*	2.84 (0.86–3.44) *p = 0.119*	8.99 (5.22–11.89) *p = 0.012*	0.61 (0.46–0.71) *p = 0.257*	32.27 (28.07–39.51) *p = 0.529*
$25–29.9\,kg/m^2$ *vs Controls*	20	27.2 (26.0–28.0)	1.53 (0.98–1.94) *p = 0.014*	2.84 (0.84–4.50) *p = 0.344*	6.39 (3.42–8.49) *p = 0.790*	0.72 (0.51–0.95) *p = 0.163*	31.03 (23.31–37.26) *p = 0.202*
$\geq 30\,kg/m^2$ *vs Controls*	18	33.7 (31.1–36.7)	3.44 (1.59–3.67) *p = 0.036*	5.86 (3.42–7.52) *p = 0.002*	12.29 (9.98–15.65) *p = 0.0008*	0.78 (0.52–0.99) *p = 0.438*	32.36 (26.26–37.85) *p = 0.738*
Disease severity							
Moderate–mild *vs Controls*	20	26.4 (22.3–29.8)	1.33 (0.47–1.94) *p = 0.628*	2.40 (0.58–3.60) *p = 0.768*	8.57 (7.50–10.29) *p = 0.073*	0.71 (0.49–0.78) *p = 0.298*	31.16 (25.81–38.01) *p = 0.645*
Severe *vs Controls*	39	27.9 (24.3–31.1)	2.48 (0.99–2.66) *p < 0.0001*	4.46 (1.71–5.98) *p < 0.0001*	9.40 (6.09–13.14) *p = 0.0007*	0.70 (0.50–0.87) *p = 0.209*	32.24 (26.54–38.02) *p = 0.164*

(*Continued*)

Table 2 (*Continued*)

Acute phase reactant	# patients	BMI	IL-6 (pg/mL)	CRP (μg/mL)	LBP (μg/mL)	TNFα (pg/mL)	sIL-6Rα (ng/mL)
Infection *vs No infection*							
No	47	27.6 (23.0–31.6)	2.19 (0.94–2.52)	3.98 (1.42–5.07)	9.30 (7.04–11.32)	0.68 (0.50–0.78)	31.79 (25.89–38.05)
Yes *vs No infection*	12	26.5 (22.8–27.8)	1.72 (0.93–2.69) *p = 0.510*	2.93 (0.84–5.09) *p = 0.333*	8.41 (3.68–11.25) *p = 0.526*	0.80 (0.62–0.99) *p = 0.217*	32.20 (28.12–37.02) *p = 0.875*
Therapy							
Prophylaxis vs On-Demand							
On-Demand	24	26.2 (22.2–28.9)	1.75 (0.57–2.20)	3.23 (0.58–4.07)	8.65 (7.49–10.57)	0.77 (0.52–0.99)	32.13 (25.81–38.48)
Prophylaxis *vs On-Demand*	35	28.2 (24.3–32.3)	2.33 (0.97–2.66) *p = 0.313*	4.13 (1.68–5.86) *p = 0.312*	9.44 (6.09–12.95) *p = 0.487*	0.65 (0.50–0.79) *p = 0.134*	31.70 (26.54–37.79) *p = 0.840*
Plasmatic vs *Recombinant*[a]							
Recombinant	34	27.1 (22.2–30.4)	2.11 (0.84–2.54)	4.36 (1.37–6.04)	9.22 (6.09–11.78)	0.61 (0.50–0.76)	31.58 (26.26–36.10)
Plasmatic vs *Recombinant*	17	27.2 (22.9–31.6)	2.29 (0.99–2.63) *p = 0.794*	2.93 (1.16–4.24) *p = 0.174*	8.68 (2.91–12.20) *p = 0.695*	0.80 (0.57–1.04) *p = 0.134*	33.00 (28.17–39.92) *p = 0.840*
Arthropathy vs No arthropathy							
No arthropathy							
$< 25\,kg/m^2$	12	21.8 (20.3–23.1)	1.82 (0.47–2.59)	3.28 (0.58–3.60)	8.92 (4.79–12.17)	0.66 (0.50–0.77)	32.23 (22.08–40.11)
$25–29.9\,kg/m^2$	8	27.8 (26.5–29.0)	1.45 (0.73–2.07)	2.82 (0.58–5.25)	7.45 (6.50–9.38)	0.67 (0.50–0.76)	31.66 (25.86–38.71)
$\geq 30\,kg/m^2$	7	33.5 (31.0–36.6)	2.97 (1.60–2.92)	5.66 (3.53–6.23)	12.61 (9.85–15.84)	0.92 (0.59–1.01)	32.33 (28.07–38.05)
Plus arthropathy							
$< 25\,kg/m^2$ *vs No Arthropathy*	9	22.5 (21.3–24.0)	1.01 (0.45–1.50) *p = 0.307*	2.25 (1.21–2.85) *p = 0.557*	9.10 (5.47–12.30) *p = 0.925*	0.54 (0.43–0.68) *p = 0.290*	32.32 (28.21–36.91) *p = 0.982*
$25–29.9\,kg/m^2$ *vs No Arthropathy*	12	26.8 (25.7–27.7)	1.58 (1.04–1.94) *p = 0.769*	2.86 (1.20–4.45) *p = 0.972*	5.69 (2.29–7.90) *p = 0.236*	0.76 (0.50–0.99) *p = 0.480*	30.61 (21.99–37.23) *p = 0.779*
$\geq 30\,kg/m^2$ *vs No Arthropathy*	11	33.8 (31.1–37.1)	3.74 (1.55–4.66) *p = 0.595*	5.98 (3.08–8.13) *p = 0.829*	12.09 (10.12–15.56) *p = 0.754*	0.70 (0.46–0.89) *p = 0.220*	32.38 (26.06–37.79) *p = 0.988*
Last Bleeding Event (BMI < 30.0)							
Controls	47	26.4 (24.8–28.1)	0.96 (0.62–1.28)	1.96 (0.74–2.40)	6.26 (3.80–8.13)	0.60 (0.43–0.71)	29.21 (24.10–32.37)
< 1 mo *vs Controls*	12	24.2 (21.9–27.3)	2.57 (1.44–2.69) *p < 0.0001*	4.57 (1.49–7.01) *p = 0.003*	9.03 (4.45–13.32) *p = 0.048*	0.72 (0.53–0.93) *p = 0.256*	38.62 (33.16–45.53) *p < 0.0001*
> 1 mo *vs Controls*	29	24.7 (21.8–27.4)	1.11 (0.69–1.40) *p = 0.757*	2.13 (0.79–3.58) *p = 0.951*	7.19 (4.85–8.99) *p = 0.514*	0.64 (0.49–0.75) *p = 0.677*	28.78 (21.97–34.72) *p = 0.960*

Table 2 (*Continued*)

Acute phase reactant	# patients	BMI	IL-6 (pg/mL)	CRP (µg/mL)	LBP (µg/mL)	TNFα (pg/mL)	sIL-6Rα (ng/mL)
Arthropathy—Last Bleeding Event vs No arthropathy (BMI < 30.0)							
No arthropathy							
< 1 mo	7	24.3 (20.8–28.1)	3.36 (2.13–4.21)	5.28 (0.54–7.14)	9.55 (6.09–14.01)	0.74 (0.52–0.87)	38.94 (34.99–42.39)
> 1 mo	13	24.0 (21.6–27.7)	0.89 (0.45–0.98)	1.92 (0.54–3.58)	7.67 (5.22–9.66)	0.63 (0.49–0.71)	28.27 (21.07–34.98)
< 1 mo + > 1 mo	20	24.1 (21.6–27.7)	1.75 (0.56–2.47)	3.10 (0.58–4.07)	8.33 (6.08–10.56)	0.66 (0.50–0.77)	32.00 (25.86–39.87)
Plus arthropathy							
< 1 mo *vs No arthropathy*	5	24.1 (22.0–26.7)	1.47 (0.41–2.39) $p = 0.122$	3.57 (1.79–5.42) $p = 0.560$	8.30 (2.88–12.70) $p = 0.683$	0.69 (0.40–1.01) $p = 0.789$	38.18 (30.56–46.94) $p = 0.858$
> 1 mo *vs No arthropathy*	16	25.2 (23.3–27.2)	1.29 (0.98–1.43) $p = 0.116$	2.29 (1.12–3.88) $p = 0.570$	6.80 (3.42–8.77) $p = 0.500$	0.66 (0.48–0.80) $p = 0.770$	29.21 (21.99–34.17) $p = 0.737$
< 1 mo + > 1 mo *vs No arthropathy*	21	25.0 (22.5–27.1)	1.34 (0.96–1.67) $p = 0.347$	2.60 (1.21–3.81) $p = 0.608$	7.15 (3.34–9.73) $p = 0.344$	0.66 (0.49–0.88) $p = 0.994$	31.34 (25.20–36.91) $p = 0.805$

Abbreviations: BMI, body mass index; CRP, C-reactive protein; IL-6, interleukin-6; LBP, LPS-binding protein; SIL-6Rα, soluble IL-6 receptor α; TNFα, tumor necrosis factor α mo, month.
Note: IQR is indicated in parentheses.
[a]Eight hemophilia patients who did not receive plasmatic and recombinant clotting factor concentrates were not included in the analysis.

joint bleeding in hemophilia, we induced needle-puncture injuries of the knee in transgenic mice with hemophilia A. The needle puncture caused an extensive hematoma on day 1 that lasted well over a week and was documented by gross examination as well as by measurements of the mediolateral diameter of the knee to assess swelling (►**Fig. 5A, E**). Histological examination of H&E and Sirius Red-stained mouse joints demonstrated the presence of free blood in the synovia of the knee and the surrounding soft tissue on day 1 as well as day 7 after needle puncture and confirmed complete resorption of the hematoma by day 28 (►**Fig. 5B, C, F**). The bleeding injury was accompanied by a significant cell expansion of the synovia that ultimately replaced the hematoma with fibrotic tissue (►**Fig. 5B–D, G**). Systematic scoring of H&E-stained joint tissues demonstrated a significantly increased Valentino-Score (►**Fig. 5H**; ►**Supplementary Fig. S3** [available in the online version]). The knee puncture of healthy control mice, in contrast, did not result in any measurable hematoma or otherwise relevant trauma recognizable by gross or histological examination (►**Supplementary Fig. S4**, available in the online version). Paralleling these data, the blood analysis of punctured hemophilia mice yielded a more than 10-fold elevation of IL-6 on day 1 and a 150-fold elevation on day 7 over punctured control mice, which produced only a minor IL-6 peak on day 1 (►**Fig. 5I**). On day 28 after knee puncture, IL-6 levels in hemophilia mice returned to fourfold over controls indicating that the course of IL-6 in hemophilia is closely aligned with the bleeding event. Plasma concentrations of TNFα, on the other hand, were not significantly different in punctured hemophilia mice compared to punctured controls (►**Fig. 5J**). Together, these data demonstrate that IL-6 is strongly and specifically elevated during bleeding events in hemophilia mice that result in the fibrotic remodeling of the afflicted joint.

Discussion

We recently demonstrated that the diminished clotting activity in hemophilia patients is associated with activation of the innate immune system.[16] Here we show that the acute-phase reactants IL-6, CRP, and LBP are elevated in the blood of hemophiliacs and that this increase is mainly associated with recent bleeding and high BMI. The pro-inflammatory effect of acute bleeding in hemophilia was confirmed by longitudinal measurements of IL-6 in patients as well as transgenic mice.

Acute bleeding events in hemophilia have been shown to be associated with local expression of IL-1β, TNFα, and IL-6.[9] Among these inflammatory markers, we detected a significant systemic elevation of IL-6 in conjunction with CRP and LBP in our hemophilia cohort that occurred independent of chronic viral infections or clotting factor treatment modalities. The acute-phase reaction was proportional to the severity of the underlying clotting disorder and proved to be particularly pronounced in the subgroup of obese hemophilia patients with BMI ≥30. To account for the confounding effect of obesity on the acute-phase reaction, we subsequently excluded obese

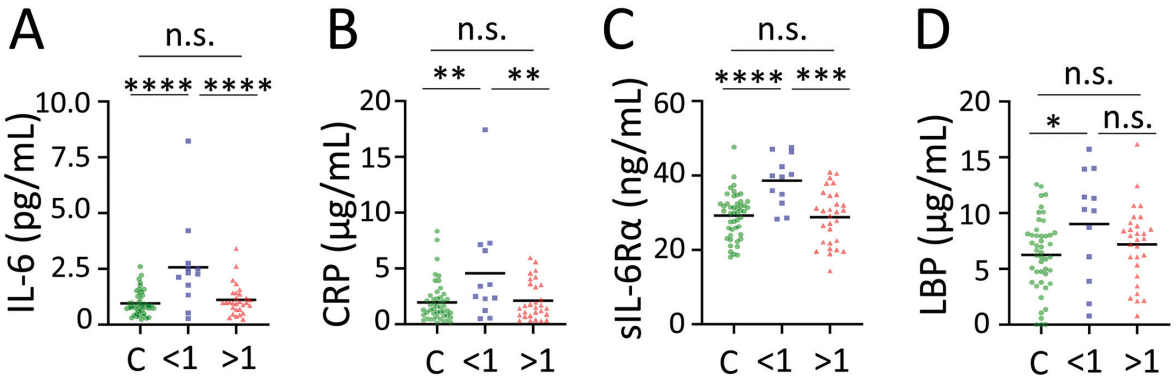


Fig. 3 Bleeding affects the inflammatory signature. Plasma IL-6 **(A)**, CRP **(B)**, IL-6Rα **(C)**, and LBP levels **(D)** in hemophilia patients with BMI <30 and recent bleeding events occurring <1 month (<1) compared to hemophilia patients with BMI <30 and bleeding episodes >1 month (>1) and weight-stratified controls (C). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{****}p < 0.0001$. Horizontal bars denote the mean. CRP, C-reactive protein; IL-6, interleukin-6; LBP, LPS-binding protein; n.s., nonsignificant; sIL-6Rα, soluble IL-6 receptor α.

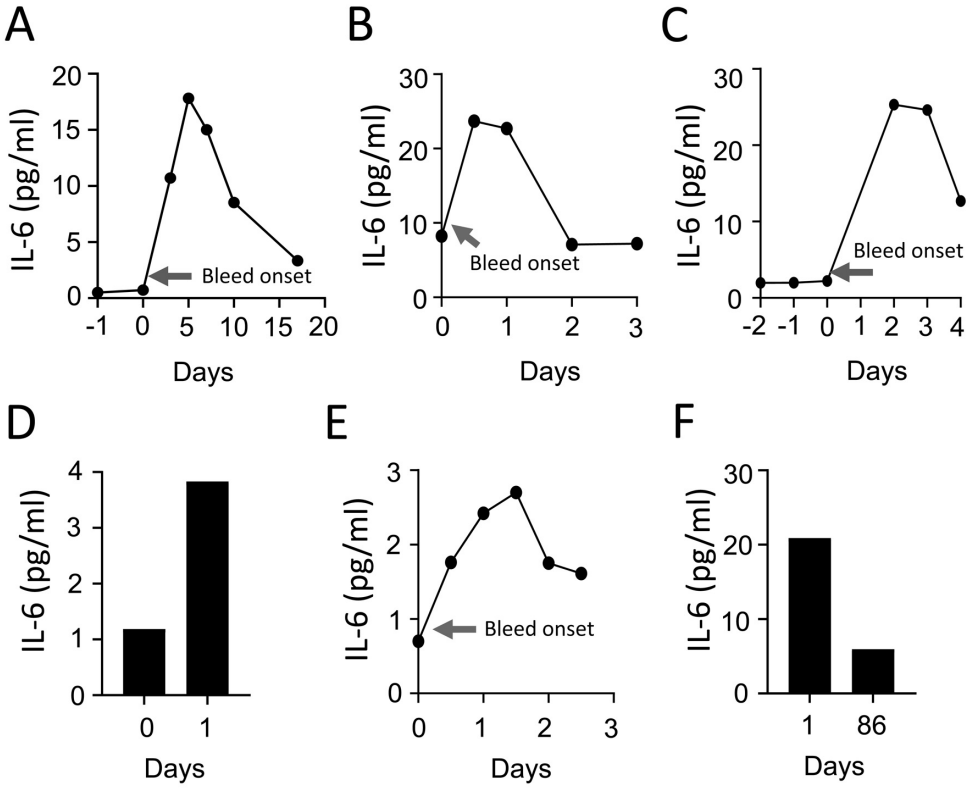


Fig. 4 IL-6 as a marker of acute bleeding in hemophilia patients. Plasma IL-6 was measured longitudinally in hemophilia patients following an acute bleeding episode until the bleeding was resolved with coagulation factor replacement therapy. Courses of plasma IL-6 levels are shown in a patient with severe hemophilia B and a traumatic hemarthrosis of the knee **(A)** and five patients with severe hemophilia A, two presenting with traumatic joint bleeds **(B, C)**, one with a bleed following a tooth extraction **(D)**, one with a minor bleed 4 weeks after septoplasty surgery **(E)**, and one with an extensive traumatic bleed of the right latissimus dorsi muscle **(F)**. IL-6 values at time points 0, −1, and −2 are based on blood testing during routine visits prior to bleeding. Grey arrows symbolize bleeding onset. IL-6, interleukin-6.

hemophiliacs from further analysis and found that the expression of acute-phase markers in nonobese hemophilia patients (BMI 18–29.9) is on par with plasma levels in healthy controls unless a bleeding episode occurred within the last month. In patients with recent bleeding, we detected a significant increase of IL-6, sIL-6Rα, and CRP plasma levels in comparison to healthy controls and patients without bleeding. As we counted only one case of bleeding <1 month among the obese hemophilia patients, we concluded that activation of the acute-phase response in our hemophilia cohort occurred either in the context of acute bleeding or obesity.

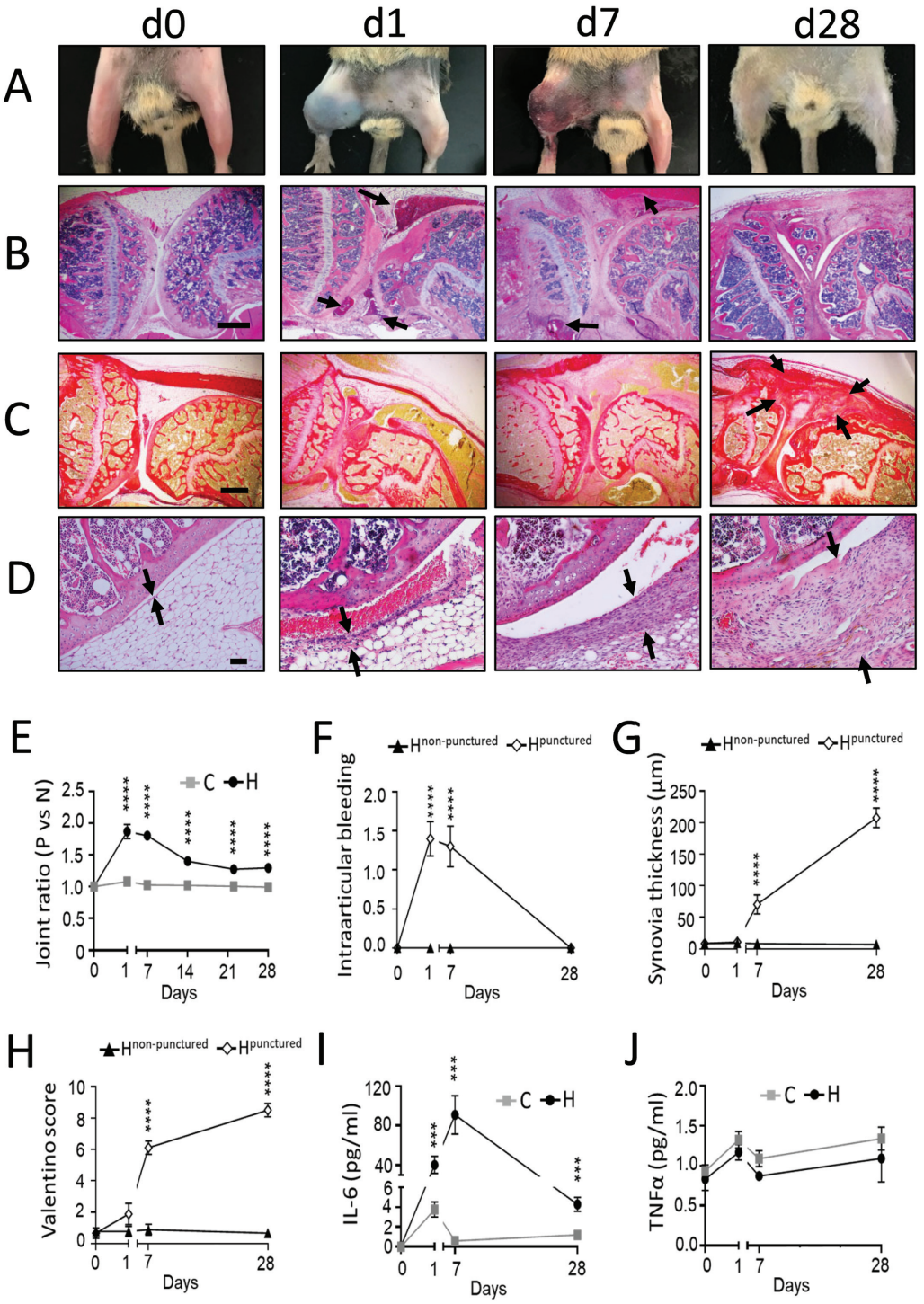


Fig. 5 Plasma IL-6 correlates with intraarticular bleeding in transgenic hemophilia mice. **(A)** Representative images of transgenic factor VIII knockout B6;129S-F8^{tm1kaz}/J mice before (day 0) and day 1, 7, and 28 after needle puncture of the right knee joint compared to the nonpunctured left knee. **(B, C)** Panels depict whole mounts of the right knee joint before and after needle puncture injury (left, tibial plateau; right, femoral head) stained with H&E (B) or Sirius red (C). Black arrows indicate bleeding/hematoma (B, d1, and d7) and arthrofibrosis (C, d28; red). Scale bar: 500 µm. **(D)** High magnification images of the synovia. Black arrows show synovia hyperplasia and the thickening of the synovia layer over time. Scale bar: 50 µm. **(E)** Joint ratio (diameter of the punctured knee divided by the nonpunctured knee) presented as an average per treatment group and time point (H, hemophilia, black circles; C, control, grey squares; ea. $n = 6–10$). **(F)** Intraarticular bleeding score (0, no bleeding; 1, some bleeding; 2, severe bleeding) in the punctured knee of hemophilia mice (H^{punctured}, white diamonds) compared to the nonpunctured knee (H^{nonpunctured}, black triangles). **(G)** Thickness of the synovia layer was measured according to the image scale in the joints of hemophilia mice with knee puncture (H^{punctured}, white diamonds) or without knee puncture (H^{nonpunctured}, black triangles). **(H)** Hemophilic synovitis was calculated using the Valentino scoring system (0–10 points). With knee puncture (H^{punctured}, white diamonds) and without knee puncture (H^{nonpunctured}, black triangles). **(I, J)** IL-6 and TNFα were measured over time in blood isolated from hemophilia (H) and control (C) mice. $^{***}p < 0.001$, $^{****}p < 0.0001$. IL-6, interleukin-6; TNFα, tumor necrosis factor α.

To follow up on our finding that the acute-phase reaction was activated in hemophilia patients with recent bleeding, we performed longitudinal measurements of plasma IL-6 in six hemophilia patients who visited our hospital with acute bleeding episodes. In these patients, we were able to document a pronounced increase of IL-6 proportional to the degree of bleeding followed by a subsequent decline after bleeding subsided. As was the case in the hemophilia patients with recent bleeding, the acute-phase reaction in hemophilia patients with acute bleeds was not limited to joint bleeds but also occurred in response to blunt muscle trauma or secondary to surgery. This is relevant as it suggests that the acute-phase response represents a uniformly conserved inflammatory reaction to bleeds in general rather than a reaction that is specific to joint injuries. Accordingly, we did not observe a connection between the levels of acute-phase reactants and the diagnosis of arthropathy in our hemophilia patients even though joint degeneration is a common complication of joint bleeds and has been reported to be prevalent in obese hemophiliacs.[19,20] These results suggest that chronic synovitis per se is not accompanied by a systemic inflammatory reaction, which is in line with previously published data showing no difference of CRP levels in hemophilia patients with or without advanced joint damage in absence of bleeding.[21] However, the same study demonstrated a close alignment of the acute-phase response with joint bleeds that we were able to reiterate in our case studies and in transgenic hemophilia mice with induced joint bleeds.

The acute-phase reaction after bleeding is associated with red blood cell lysis and the subsequent release of danger signals such as heme, which has strong proinflammatory effects.[22,23] The resulting secretion of IL-1β, IL-6, and TNFα has been strongly implicated in causing typical complications of joint bleeds such as synovitis, cartilage erosion, and bone loss.[15,24–26] The association between blood-induced inflammation and arthropathy was reiterated in our mouse model of hemarthrosis, where the fibrous remodeling of the punctured knee joint was preceded by a large increase of plasma IL-6. TNFα, on the other hand, remained unchanged in the plasma of mice with hemarthrosis and increased only insignificantly in hemophilia patients, which is in line with previous reports.[24,25] Previous data also reinforce a role of IL-6 in the natural history of hemophilic arthropathy as the IL-6/sIL-6Rα complex has been shown to trigger proliferation in synoviocytes and subsequent fibrotic remodeling of the synovia in hemophilia mice.[15,27] Taken together, these findings could be relevant for assessing the effect of bleeding on joint health in hemophilia patients as we found sIL-6Rα to be specifically upregulated in hemophilia patients with recent bleeding in conjunction with IL-6. As such, our data suggest that IL-6 and sIL-6Rα could represent a useful set of plasma markers for blood-induced inflammation.

IL-6 is a pleiotropic cytokine that responds to local tissue damage caused by infection, inflammation, or injury with the release of acute-phase reactants such as CRP and LBP from the liver into the blood circulation.[28,29] This interdependence between IL-6, CRP, and LBP was also evident in hemophilia patients, which exhibit a positive correlation between blood levels of IL-6, CRP, and LBP, indicating that the increases of the acute-phase reactants that we measured are not random phenomena but took place within the regulatory axis of the acute-phase response. The activation of the acute-phase response through IL-6 occurred reproducibly in hemophilia patients with acute and recent bleeding but also under the controlled conditions of an inducible hemarthrosis model in transgenic hemophilia mice. Reliable detection of IL-6 in this context was made possible by a highly sensitive ELISA or in the case of hemophilia patients with acute hemorrhage through ECLIA. We, therefore, postulate that IL-6 is a sensitive marker of hemorrhage in hemophilia patients that can complement diagnostic imaging such as ultrasound, CT scans, or MRI in complex cases such as swollen or painful joints.[4,30] A limitation of our study is the lack of specificity of IL-6 as was seen in obese hemophilia patients, which exhibit increased IL-6 values without having experienced bleeding episodes recently. In absence of bleeding, nonobese hemophiliacs were virtually indistinguishable from healthy, weight-matched controls, suggesting that the induction of IL-6 is not completely indiscriminate. Moreover, our data from patients with acute bleeding show that bleeding-induced IL-6 increases can occur in addition to elevated baseline IL-6, highlighting the need for repeated and, in the case of bleeding, longitudinal IL-6 measurements to define individual baseline levels, intraindividual fluctuations, and the effect of hemostyptic therapy. Overall, our data suggest that IL-6, CRP, LBP, and sIL-6Rα could represent a useful set of plasma markers for blood-induced inflammation. We, therefore, propose to systematically assess if these acute-phase reactants are suitable indicators of bleeding episodes in hemophilia in general and joint bleeds in particular and whether they may give guidance in choosing the most appropriate treatment option for hemophilia patients with acute and recurring bleeding episodes.

What is known about this topic?

- IL-6 mediates the acute-phase response as it induces secretion of acute-phase proteins such as CRP and LBP.
- Anti-inflammatory therapy targeting the interaction between IL-6 and IL-6Rα can mitigate hemophilic arthropathy in transgenic mice with hemophilia.

What does this paper add?

- The acute-phase reactants IL-6, CRP, and LBP are elevated in the blood of hemophiliacs as a sign of an activated acute-phase response.
- The acute-phase response indicates recent bleeding events in nonobese hemophiliacs (BMI 18–29.9) in conjunction with elevated sIL-6Rα but can also be upregulated in obese hemophilia patients independently of bleeding.
- The acute-phase response in hemophilia occurs independently of arthropathy, chronic viral infection, and clotting factor replacement therapy suggesting that the acute-phase markers could be sensitive biomarkers for the detection of acute and recent bleeding events.

Authors' Contribution

L. M. K. and J. P. designed the research; J. P., H. E., and U. G. evaluated patients and blood donors; L. M. K., C. W., L. B., and J. P. performed the experiments and analyzed the data; M. D. M. and M. W. L. provided expertise and reagents for the histological analysis and critically reviewed the data; L. M. K. and J. P. wrote the manuscript. All authors approved the final manuscript prior to submission.

Funding

This work was supported by the Bayer Joint Health Award (J. P.), CSL Behring GmbH (J. P. and H. E.), the Pfizer ASPIRE Award (J. P.), and the Hans Egli Stipend of the Gesellschaft für Thrombose-und Hämostaseforschung e.V. (L. M. K.).

Conflict of Interest

None declared.

Acknowledgment

The authors thank the staff of the hemophilia clinic and the blood donation center for their support and care in drawing blood from our hemophilia patients and healthy blood donors. We also like to thank Janine Becker for providing expert help with the histologies.

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