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Impact of a Vancomycin-Induced Shift of the Gut Microbiome in a Gram-Negative Direction on Plasma Factor VIII:C Levels: Results from a Randomized Controlled Trial

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Abstract

Rationale   Inflammation is present in several conditions associated with risk of venous thromboembolism. The gut microbiome might be a source of systemic inflammation and activation of coagulation, by translocation of lipopolysaccharides from gram-negative bacteria to the systemic circulation.

Objective   To investigate whether a vancomycin-induced shift of the gut microbiome in a gram-negative direction influences systemic inflammation and plasma factor (F) VIII procoagulant activity (FVIII:C).

Methods and Results   We performed a randomized controlled trial including 43 healthy volunteers aged 19 to 37 years. Twenty-one were randomized to 7 days of oral vancomycin intake and 22 served as controls. Feces and blood were sampled at baseline, the day after the end of intervention, and 3 weeks after intervention. Gut microbiome composition was assessed by amplicon sequencing. FVIII:C was measured using an activated partial thromboplastin time-based assay, cytokines were measured using multiplex technology, complement activation was measured using the enzyme-linked immunosorbent assay, and high-sensitivity C-reactive protein (CRP) was measured by an immunoturbidimetric assay. Vancomycin intake reduced gut microbiome diversity and increased the abundance of gram-negative bacteria. Change in FVIII:C in the intervention group was +4 IU/dL versus −6 IU/dL ($p = 0.01$) in the control group. A

Keywords
► microbiome
► inflammation
► coagulation
► randomized controlled trial

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Rüdigerstraße 14,
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similar change was observed for log-transformed CRP ($+0.21$ mg/dL vs. -0.25 mg/dL, $p = 0.04$). The cytokines and complement activation markers remained similar in the two groups.

Conclusion   The found slight increases in FVIII:C and CRP levels might support the hypothesis that a vancomycin-induced gram-negative shift in the gut microbiome could induce increased systemic inflammation and thereby a procoagulant state.

Introduction

Venous thromboembolism (VTE) is a common cardiovascular disease with an annual incidence of 1 to 2 per 1,000 in the adult population with severe short- and long-term complications including death.[1] In contrast to arterial cardiovascular disease like myocardial infarction and stroke, the incidence is not declining.[2] Therefore, there is an urgent need to unravel molecular mechanisms that could be targets for preventive treatment.

Growing evidence supports an important role for immunothrombosis in the pathogenesis of VTE. Inflammation and coagulation are closely linked through interactions between the complement, the coagulation, and fibrinolytic systems,[3,4] and through interactions between these and the proinflammatory cytokine network.[4] Furthermore, several systemic infectious and inflammatory conditions, i.e., human immunodeficiency virus (HIV),[5] inflammatory bowel disease (IBD),[6] and acute infections,[7–9] are established risk factors for VTE. A novel and yet rather unexplored source of chronic systemic inflammation is the gut microbiome.

In healthy individuals, the gut microbiome is dominated by two bacterial phyla, the *Bacteroidetes*, consisting of gram-negative bacteria, and the *Firmicutes*, consisting of mainly gram-positive bacteria.[10] Moreover, a healthy gut microbiome has been associated with high bacterial diversity.[11] Conversely, a less diverse composition of the gut microbiome has been demonstrated in obesity,[12] IBD,[13] and *Clostridium difficile* infection,[14] and a reduction in health promoting bacteria is seen in cancer.[15] Besides these chronic diseases, oral antibiotic use also induces dysbiosis. Oral vancomycin use diminishes the richness and diversity of the gut microbiome.[16] Furthermore, it alters the gut microbiome composition in a gram-negative direction with more genera belonging to the Proteobacteria phylum (i.e., *Escherichia* and *Shigella)*, which is associated with infectious conditions.[17]

Animal studies have shown that dysbiosis can result in metabolic endotoxemia by the production of high levels of lipopolysaccharides (LPSs) and increased intestinal permeability by the loss of integrity of tight junctions between epithelial cells.[18,19] A possible mechanism by which gut microbiome dysbiosis may affect coagulation is through low-grade inflammation driven by translocation of LPS from gram-negative bacteria in the gut to the systemic circulation.[20] Gut-translocated LPS has been suggested to stimulate factor (F) VIII release from endothelial cells in liver

cirrhosis patients.[21] Furthermore, intravenously administered LPS has been demonstrated to increase FVIII levels and other procoagulant factors, in healthy volunteers.[22] Coagulation FVIII plays a central role in the coagulation cascade as a cofactor for activated FIX in the intrinsic pathway. Procoagulant FVIII activity (FVIII:C) is associated with both first[23] and recurrent VTE.[24]

In the exploration of the link between inflammation and coagulation, inflammatory markers have been linked to VTE risk. C-Reactive protein (CRP), a marker of systemic inflammation, has been associated with a short-term VTE risk.[25] As a mediator of the innate immune system, CRP activates the classical complement pathway[26] and plasma levels of complement C3 and the terminal complement complex (TCC) are associated with increased VTE risk.[27,28] Interleukin 6 (IL-6) is involved in vascular inflammation,[29] but its association with VTE risk is somewhat debated.[30–32]

Currently, little is known on the role of the gut microbiome in the pathophysiology of VTE. As a first translational step, we hypothesized that dysbiosis of the gut microbiome might elicit a systemic inflammatory response, which in turn can increase plasma FVIII:C levels. To investigate this hypothesis, we performed a randomized controlled trial in healthy volunteers, to explore whether a shift in the gut microbiome in a gram-negative direction influences systemic inflammation and plasma FVIII:C levels.

Methods

Participants and Trial Design

This randomized, controlled, investigator-blinded trial had a double-arm parallel design, and 21 participants were recruited to the intervention group and 22 to the control group. Previous studies have reported differences in the gut microbiome composition in lean and overweight/obese subjects. To allow for subgroup analysis, we recruited a minimum of 10 lean (body mass index [BMI] < 25 kg/m^2) and 10 overweight/obese (BMI $\geq$ 25 kg/m^2) subjects to both the intervention and the control group. No changes to methods were applied after trial commencement.

Exclusion criteria were the use of antibiotics for a minimum duration of 2 months or the use of any medication (except oral contraceptives) in the last month before entering the study, pregnancy or postpartum state, chronic inflammatory conditions (IBD, rheumatic disease, diabetes mellitus, HIV infection), gastrointestinal disease in the last month before entering the study, a history of irritable bowel disease,

allergy for vancomycin or teicoplanin, liver or kidney disease, a feverish episode the last 2 weeks, planned diet change, the start of systematic use of probiotics during the last 2 months, and travel outside of Europe in the last month before entering the study. Liver and kidney functions were evaluated by serum alanine aminotransferase, alkaline phosphatase, and creatinine, and pregnancy testing was performed by serum human chorionic gonadotropin in women before study start.

Subjects were recruited via advertisements at UiT—The Arctic University of Norway in Tromsø, Norway. The study took place at the Clinical Research Unit at the University Hospital of North Norway. Volunteers contacted the study physician by e-mail for written information about the study, and those still interested were invited for a screening/information visit with the study doctor. Eligible subjects who signed a written consent were randomized and enrolled for three study visits where blood and feces samples were collected. The randomization was performed by the central randomization unit at the University Hospital of North Norway through block randomization (block sizes of 2 and 4) with stratification for BMI. Detailed information on recruitment is provided in ►Fig. 1 (flow diagram).

Intervention

Participants were randomized to oral intake of vancomycin, 500 mg (4 capsules of 125 mg) three times per day for 7 consecutive days (the intervention group), similar to an earlier published study by Vrieze et al,[33] or to no intervention (the control group). The control group did not receive placebo, as objective laboratory measures were used as outcomes (see the Outcomes section). Vancomycin is effective against gram-positive bacteria,[34] and has shown in a trial on insulin sensitivity to provide the desired gram-negative shift of the microbiome using identical doses and duration of intervention.[33] The participants were asked to return capsules and drug packaging, and returned capsules were counted to assess compliance. All participants were asked to maintain their habitual physical activity pattern and dietary habits throughout the study. Hard physical activity and alcohol intake the day before blood sampling were discouraged. At baseline (T0), the day after the intervention stopped (T1), and 3 weeks after end of intervention (T2), blood was sampled after a normal breakfast and feces samples, produced within the last 24 (maximum 36) hours, were collected. In the control group, blood- and feces samplings were done at matching time points.

Outcomes

The predefined primary outcome was the difference in change (between T0 and T1) of coagulation FVIII:C between the intervention and the control group. Secondary outcomes were inflammatory parameters including hs-CRP,
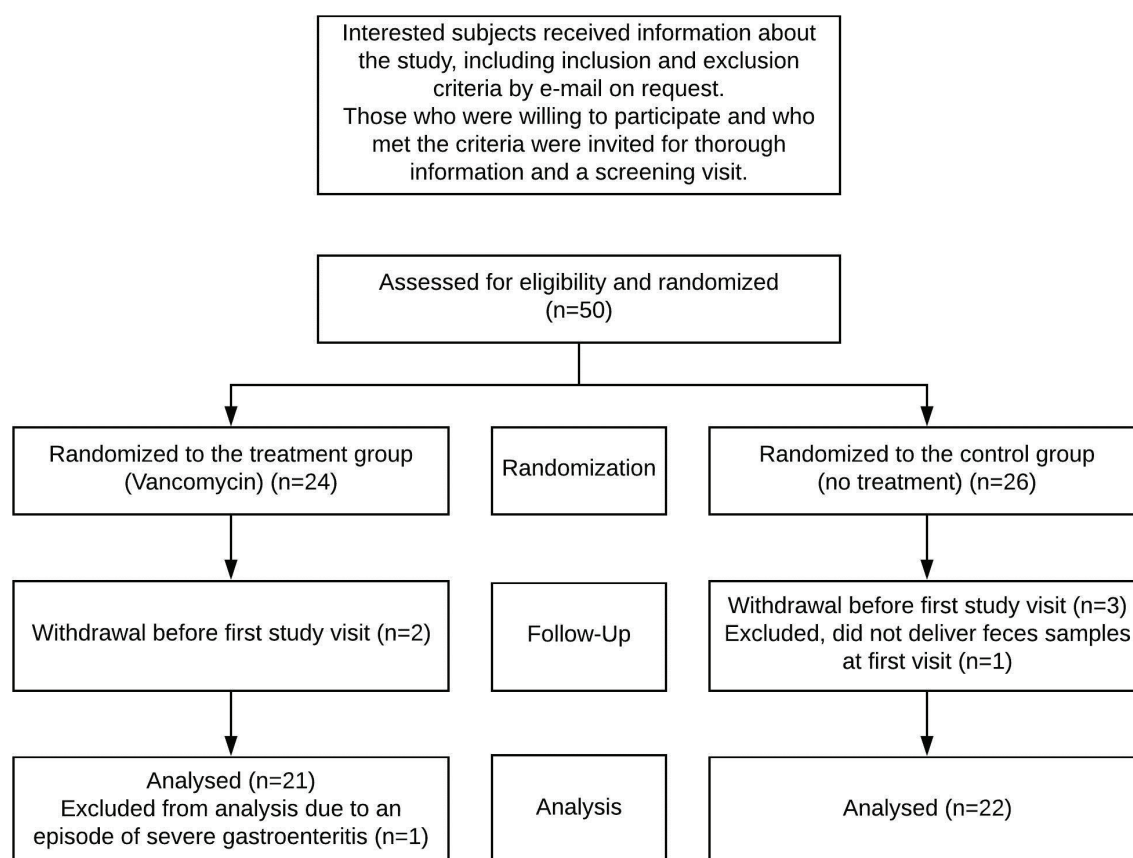
Fig. 1 Flow diagram showing recruitment of participants, randomization, and completion of the trial.

fibrinogen, IL-1β, IL-6, IL-8, IL-10, monocyte chemoattractant protein (MCP)-1, neutrophil cell count, and tumor necrosis factor (TNF). Zonulin, instead of LPS, was determined as a marker for gut permeability, as the reliability of plasma LPS assays is demonstrated to be inferior in comparison to plasma zonulin.[35,36] Complement activation products were assessed by C3bc, reflecting the proximal complement pathway and TCC. Gut microbiome composition was assessed to ensure that the intervention actually did change the balance between gram-positive and gram-negative bacteria. All outcomes were assessed at T0, T1, and T2.

Sample Size Calculation

An exploratory sample size calculation was performed to evaluate the feasibility of the study. The sample size was determined on the primary outcome, i.e., FVIII:C. As the concept of this study was quite novel in the field of VTE, estimated effect sizes could not be derived from currently available data. HIV infection is associated with a gram-negative shift in the microbiome and might resemble the effect of vancomycin in this study. Therefore, sample size calculations were based on the difference in FVIII:C plasma levels in treatment-naïve HIV patients compared with controls.[37] Treatment-naïve HIV patients had mean FVIII:C levels of 222 IU/dL (standard deviation [SD]: 11), whereas controls had mean FVIII:C levels of 100 IU/dL (SD: 11). With an α of 5% and 20 participants in both study arms, we would have >99% power to detect the same difference in FVIII:C as observed in the HIV study. We did not expect to reach this power, but this calculation demonstrated that this study could be feasible.

Laboratory Analyses of Blood

Blood was collected from an antecubital vein. Neutrophil cell count, fibrinogen, and hs-CRP were analyzed at the Department of Clinical Biochemistry at University Hospital of North Norway within a few hours after sampling. Neutrophil cell count was analyzed in EDTA-blood on an automated blood cell counter by a fluorescence flow-cytometric method (Sysmex XN, Sysmex Nordic ApS), with a coefficient of variation (CV) of <5%. Fibrinogen was analyzed in plasma prepared by centrifugation of sodium-citrated blood at $2,500 \times g$ for 15 minutes, and analyzed by a clotting method (STA-Liquid Fib, STA-R Evolution, Diagnostica Stago, France), with a CV of <5%. Hs-CRP was analyzed in serum prepared by centrifugation at $2,000 \times g$ for 15 minutes of blood sampled on a serum-separating tube, by an immunoturbidimetric assay on a Cobas 8000 autoanalyzer (Roche Hitachi, Mannheim, Germany). The CV for hs-CRP was 2.9% at CRP-level 1.65 mg/L, and 4.45% for all CRP-levels. The cytokines IL-1β, IL-6, IL-8, IL-10, MCP-1, TNF, and complement activation products (C3bc and TCC) were analyzed in EDTA-plasma prepared immediately after blood sampling. Whole blood was placed on crushed ice, centrifuged within 30 minutes at $2,500 \times g$ at 4°C for 15 minutes, and then stored at −70°C. Plasma concentrations of the cytokines were determined using a multiplex immunoassay system (Bio-Plex Multiplex System, Bio-Rad Laboratories, Inc. Hercules, California, United States). Values for IL-1β, IL-8, IL-10, and MCP-1 reported

below the lower detection limit were set at the lowest reported value. The complement activation products were analyzed using the enzyme-linked immunosorbent assay (ELISA). Analyses of FVIII:C was performed in plasma prepared from blood samples containing sodium citrate as the anticoagulant by centrifugation in two steps, first at $2,500 \times g$ for 15 minutes, and then at $10,000 \times g$ for 10 minutes. Plasma was stored in cryovials at −70°C until analysis. FVIII:C was measured using an ACL TOP 300 CTS (Instrumentation Laboratory, Massachusetts, United States) with SynthAsil APTT (Instrumentation Laboratory Werfen, New Delhi, India) reagents in FVIII-deficient citrated plasma, with a CV of <5% for FVIII:C levels in the normal range. Samples were tested on an automated coagulation analyzer. As CVs of these types of automated analyses are below 5%, also for samples that are higher than 100%, these assays were performed without duplicates or triplicates. When samples had strongly elevated FVIII levels (>400%), samples were further diluted and tested again. Zonulin was measured in duplicate using an enzyme immunoassay kit from CUSABIO (Wuhan, China) according to the manufacturer's protocol with intra- and inter-assay CVs <15%.

Laboratory Analysis of Gut Microbiome

DNA was extracted from fecal material using a repeated bead beating protocol at Microbiota Center of Amsterdam University Medical Centers.[38] DNA was purified using Maxwell RSC Whole Blood DNA Kit. 16S rRNA gene amplicons were generated using a single-step polymerase chain reaction (PCR) protocol targeting the V3–V4 region.[39] PCR products were purified using Ampure XP beads and the purified products were equimolar pooled. The libraries were sequenced using a MiSeq platform using V3 chemistry with 2×251 cycles. Data were submitted to European Nucleotide Archive repository under study accession number PRJEB25759. Forward and reverse reads were length-trimmed at 240 and 210 respectively and amplicon sequence variants (ASVs) were inferred and merged using DADA2 (V1.5.2).[40] Taxonomy was assigned using the RDP classifier and SILVA 16S ribosomal database V128.[40] Microbiota data were further analyzed and visualized using phyloseq,[41] vegan,[42] and picante[43] packages.

Gram stain classification of the ASVs was done based on phylum or, in the case of Firmicutes, class level taxonomy assignment.

Statistical Analyses

Statistical analyses of all nonfeces data were performed using STATA version 14.0 (Stata Corporation, College Station, Texas, United States) and R version 3.5.3 (https://www.R-project.org). All data were evaluated for normality and outliers by inspection of box plots. One outlier in the C3bc variable and one in the TNF variable were detected, and values at T0, T1, and T2 in these cases were set as missing. Both subjects with outlier values belonged to the control group. Normal distribution was present for all variables except for hs-CRP, and to achieve normal distribution, values for hs-CRP were logarithmically transformed for use in

statistical analyses. Data were presented by means ± SD. Differences between the intervention and the control group in changing from baseline to after intervention were tested with a two-sample, two-sided *t*-test. In case of significant results in the primary analyses, the analyses were further performed in the BMI subgroups.

Statistical analysis of the gut microbiome was performed using R version 3.4.4. Reads were rarefied to 35,000 reads and no samples were excluded. Differences in microbiome composition were tested using PERMANOVA on Bray–Curtis dissimilarity.[44] Effects of group/BMI and treatment on α diversity were tested using linear mixed models with subjects as a random effect. Alpha diversity indicates the within-sample microbiome variance. Beta-diversity associations were tested using PERMANOVA, which indicates variance of the microbiome composition between samples.[44]

Study Oversight

The study was approved by the Regional Committee for Medical and Health Research Ethics (2015/597/REK Nord) and the Norwegian Data Inspectorate in accordance with the Declaration of Helsinki (updated version 2013). All participants provided written informed consent. The trial was prospectively registered at Dutch Trial Register (Trial NL4971).

Role of the Funding Source

The trial was funded by an independent grant from Stiftelsen K. G. Jebsen. The investigators designed the trial, conducted the protocol, and wrote the manuscript.

Results

The study was conducted from September 15, 2016 until June 1, 2017. Fifty healthy volunteers aged 19 to 37 years were initially included in the study, and after withdrawals and exclusions, 43 (29 women and 14 men) completed the study and were included in our analyses (►**Fig. 1**). Baseline characteristics are shown in ►**Table 1**. There were no serious adverse events. Three participants experienced mild diarrhea of short duration during intervention with vancomycin.

Changes in the gut microbiome composition in the intervention subgroups, in contrast to the control subgroups (the control subgroup with BMI <25 kg/m² as the reference) at T0, T1, and T2 are shown in ►**Figs. 2** to **4**. Following intervention with vancomycin, the gut microbiome composition was
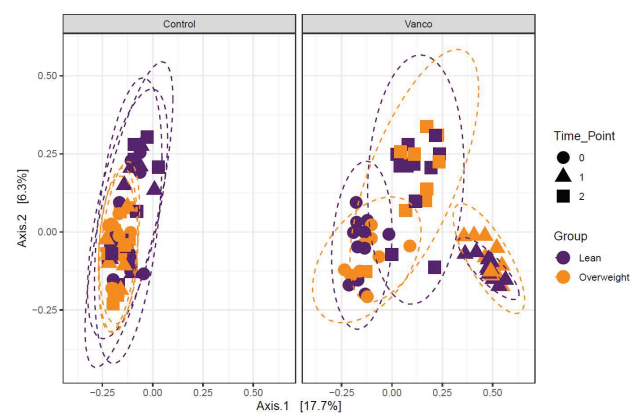
Fig. 2 Principal coordinate analysis plot with Bray-Curtis dissimilarity. Changes in the microbiome composition in the intervention subgroups, in contrast to the control subgroups at T0, T1, and T2, with the control subgroup with BMI <25 kg/m² (control lean) as reference. Each dot represents the gut microbiome composition from each sample in the study population. In the control group the gut microbiome composition remained stable at all time points. In the intervention group a clear change was observed from T0 to T1. No BMI specific effects were observed. Percentages on the axes represent the percentage of variance explained by principal component 1 (x-axis) and principal component 2 (y-axis).

markedly altered ($p = 0.001$; $R^2 = 0.26$) (►**Figs. 2** and **3**), and diversity metrics were significantly reduced ($p < 0.01$) (►**Fig. 4**). The ratio of gram-positive and gram-negative classes was significantly reduced, mainly due to a reduction in *Clostridia* and *Actinobacteria* and an increase of *Negativicutes*, *Proteobacteria*, and *Akkermansia* ($p < 10^{-11}$) (►**Fig. 3**). At T2, 3 weeks after the intervention was stopped, the gut microbiome became again more diverse, although still slightly different compared with baseline (Bray–Curtis, $p = 0.001$; $R^2 = 0.12$) (►**Fig. 4**). No BMI group-specific effects of the vancomycin treatment were observed in β diversity ($p = 0.37$, $R^2 = 0.02$). In controls, the gut microbiome remained stable throughout the entire study period regarding β diversity ($p = 0.91$, $R^2 = 0.005$).

Results of inflammation parameters and FVIII:C at baseline (T0) and after 7 days of oral vancomycin intake (T1) in the intervention (I) group and at corresponding time points in the control (C) group are shown in ►**Table 2**. There was a significant increase in FVIII:C in the intervention group when compared with the control group (ΔI: FVIII:C = 4 IU/dL vs. ΔC: FVIII:C = − 6 IU/dL, $p = 0.01$) (►**Figs. 5** and **6**). In the predefined subgroup analysis, this difference between the groups

Table 1 Baseline characteristics

	Intervention group			Control group		
	All (n = 21)	BMI < 25 (n = 11)	BMI ≥ 25 (n = 10)	All (n = 22)	BMI < 25 (n = 11)	BMI ≥ 25 (n = 11)
Age (mean, y)	26.7	24.6	28.9	25.0	24.5	25.5
Female/male (n)	14/7	7/4	7/3	15/7	8/3	7/4
Body mass index (mean, kg/m²)	25.2	22.8	27.9	26.3	22.3	30.4

Abbreviation: BMI, body mass index.
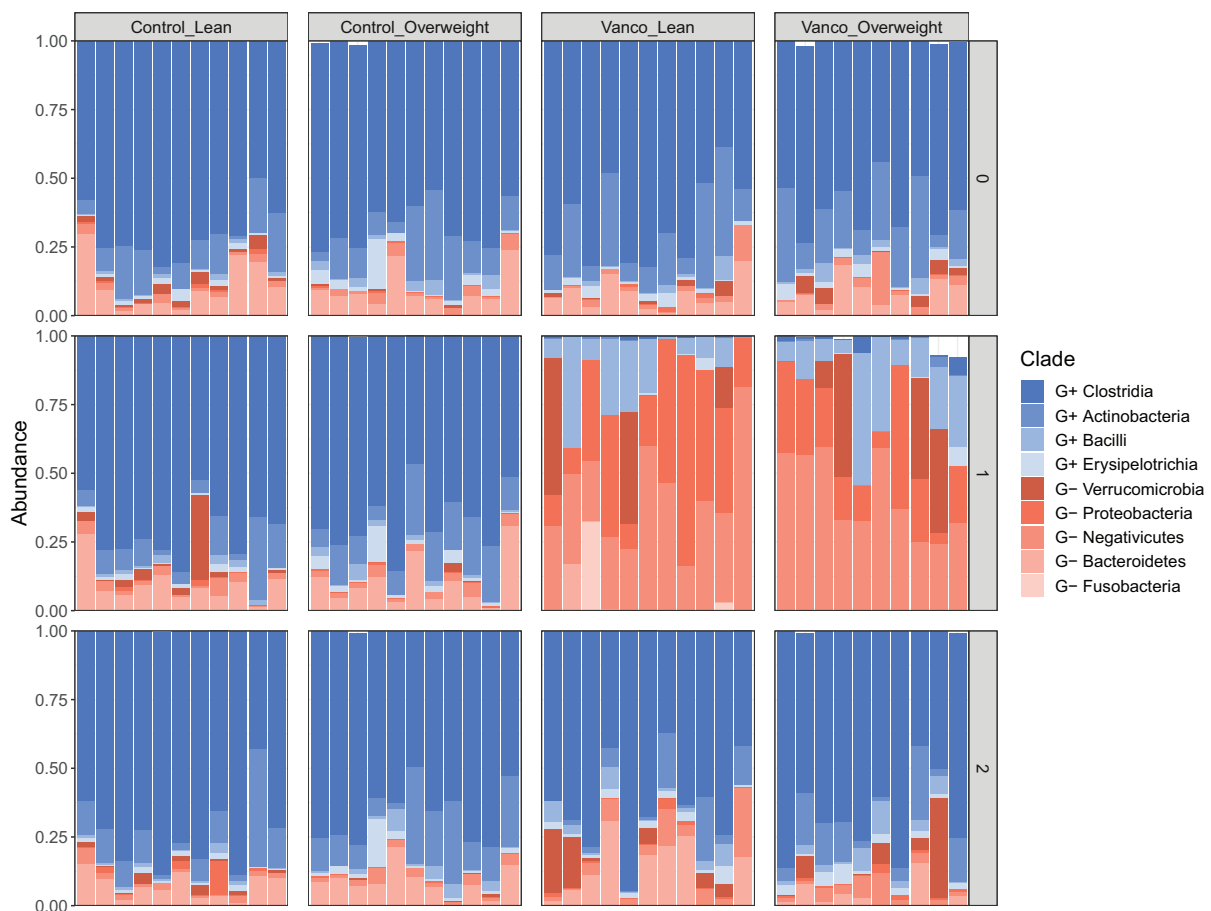
Fig. 3 Gut microbiome composition at T0 (top), T1 (middle), and T2 (bottom). A gram-negative shift occurred after treatment with vancomycin in the intervention group (Vanco_Lean and Vanco_Overweight) (T0 vs T1). In controls (Control_Lean and Control_Overweight), the gut microbiome remained stable throughout the entire study period. No BMI-specific group effects of the vancomycin treatment were observed.

was present only in those with BMI $<25 \, \text{kg/m}^2$ (ΔI: FVIII: C = 6 IU/dL vs. ΔC: FVIII:C = − 7 IU/dL, $p = 0.04$), and not in those with BMI $\geq 25 \, \text{kg/m}^2$. Similar results were obtained for log-transformed CRP (ΔI: logCRP = 0.21 mg/dL vs. ΔC: logCRP = − 0.25 mg/dL, $p = 0.04$) (►**Figs. 5** and **6**). For untransformed CRP this corresponded to $\Delta I = 0.49$ mg/dL vs. $\Delta C = − 0.38$ mg/dL in the control group. In the subgroup analysis, this difference was also solely present in the BMI $<25 \, \text{kg/m}^2$ group (ΔI: logCRP = 0.32 mg/dL vs. ΔC: logCRP= −0.55 mg/dL, $p = 0.02$). Untransformed this was a change of CRP of 1.05 mg/dL in the intervention group versus −0.67 mg/dL in the control group. Separate analysis of the BMI $<25 \, \text{kg/m}^2$ and the BMI $\geq 25 \, \text{kg/m}^2$ subgroups did not reveal any statistically significant association between changes in the gut microbiome composition on lower levels of taxonomy compared with phylum and the changes in CRP or FVIII:C levels. The change from T0 to T1 in IL-6 was slightly higher, although not statistically significant, in the intervention group than in the control group (ΔI: IL-6 = 0.1 pg/L vs. ΔC: IL-6 = − 0.3 pg/L, $p = 0.10$) (►**Fig. 5**). Changes in plasma levels of zonulin were not significantly different between the intervention and control groups (ΔI: zonulin = 0.02 ng/mL vs. ΔC: zonulin = 0.12 ng/mL, $p = 0.72$) (►**Figs. 5** and **6**). The plasma levels of FVIII:C, CRP, and IL-6 decreased from the day

after end of vancomycin treatment (T1) to the last study visit (T2) (►**Fig. 4**). For the other inflammatory variables, i.e., neutrophil count, fibrinogen, the cytokines IL-1β, IL-8, IL-10, MCP-1 and TNF, and the complement activation products (C3bc and TCC), changes in levels from T0 to T1 did not differ between the intervention and the control group (►**Table 2**).

Discussion

A short interval treatment with oral vancomycin resulted in a switch in the gut microbiome with a decrease of the total phylogenetic diversity, and an increase in the proportion of gram-negative bacteria at the expense of a decrease in the proportion of gram-positive bacteria. Our hypothesis was that this predicted change, towards a less diverse and more gram-negative gut microbiome, would lead to increased systemic inflammation and higher plasma levels of FVIII:C. We observed a small increase in hs-CRP and FVIII:C following vancomycin intervention, whereas a small decrease was observed in the control group. However, the gut permeability, assessed by plasma zonulin levels, did not change within or between the intervention and the control groups.

It should be noted that the statistical significance of the increase of FVIII:C and hs-CRP observed for the intervention
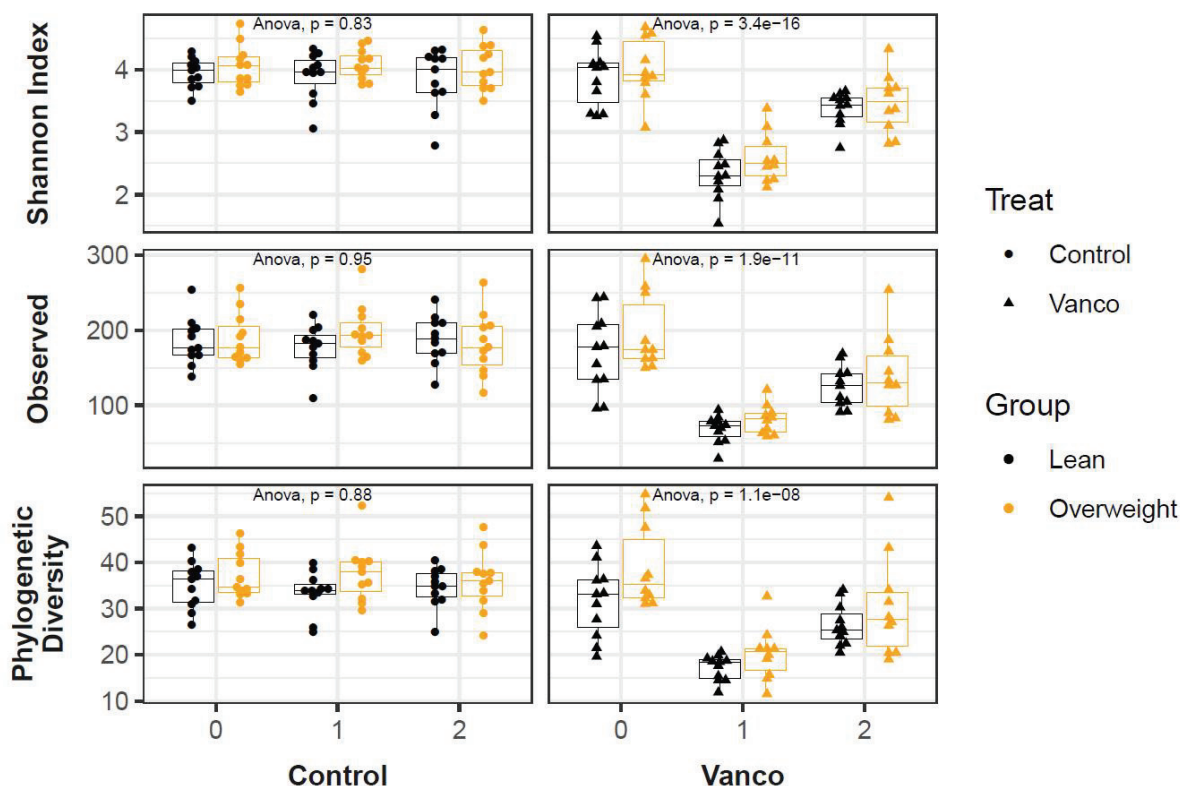
Fig. 4 Alpha diversity analysis at T0, T1, and T2. Shannon Index (left panel), observed species (middle panel) and Faith's phylogenetic distance (right panel) for intervention (Vanco) vs. control group (Control), stratified by BMI (lean <25 kg/m^2 vs. overweight ≥ 25 kg/m^2). The diversity was similar in lean and overweight subjects, and there was a clear effect of vancomycin on diversity ($p < 10^{-13}$) in both BMI groups.

versus control group was mainly driven by a significant decrease in FVIII:C and hs-CRP in the control group. As the gut microbiome remained unchanged in the control group, we did not expect any changes to occur in FVIII:C and hs-CRP. However, participants in the control group appeared to have slightly higher baseline levels of FVIII:C and hs-CRP with a larger variance compared with individuals in the intervention group. Because of the randomized nature of the study, these differences have probably occurred by chance. In addition, plasma FVIII:C and hs-CRP are known to be subject to short-time fluctuations determined by several environmental factors.[45] Either the decrease in these two parameters in the control group could therefore be considered as a "regression to the mean phenomenon" or an observation within natural variation. Although these reservations should be taken into consideration, the changes in FVIII:C and hs-CRP were in opposite directions for both parameters (i.e., an increase in the intervention group and a decrease in the control group). Therefore, our findings might provide support for the hypothesis that low-grade inflammation elicited by dysbiosis could result in elevation of plasma FVIII:C levels.

We could not substantiate the hypothesis that increases in FVIII:C levels would be the result of increased gut permeability, as we did not find a difference in plasma zonulin levels between the intervention and control groups. Findings similar to ours were reported in a randomized controlled trial of overweight and obese men, in which the effects of vancomycin and amoxicillin (a broad-spectrum antibiotic)

on the gut microbiome and inflammatory parameters in overweight and obese men were investigated.[46] As expected, a shift in the microbiome composition in a gram-negative direction was found in the vancomycin group, but not in the amoxicillin group. An increase in gut permeability, determined by urinary multisaccharide testing, and in the concentration of LPS-binding protein following vancomycin treatment was also not found.[46] In addition, the concentrations of IL-6, IL-8, or TNF did not change compared with the placebo group. Unfortunately, neither CRP nor FVIII was analyzed.

Ideally, systemic LPS levels should ideally have been measured in our study. However, sensitive and reliable methods for measuring low levels of LPS are unfortunately not available. Consequently, we measured plasma zonulin as a marker of gut permeability. Some controversy exists in the use of ELISA-based zonulin tests as a biomarker for gut permeability. Recently, it was shown that the ELISA-based test does not necessarily correlate with functional gut permeability tests (e.g., lactulose mannitol test) and that it does not only reflect actual zonulin levels, but also reflect levels of other related proteins.[47] Taken together, this implies that in our study increased gut permeability was either truly absent or was not detected by the used assay.

The absence of an increased gut permeability does not exclude a possible effect of the microbiome on the FVIII:C levels. Constant bacterial and endotoxin translocation in the gut (metabolic endotoxemia) occurs in healthy individuals at
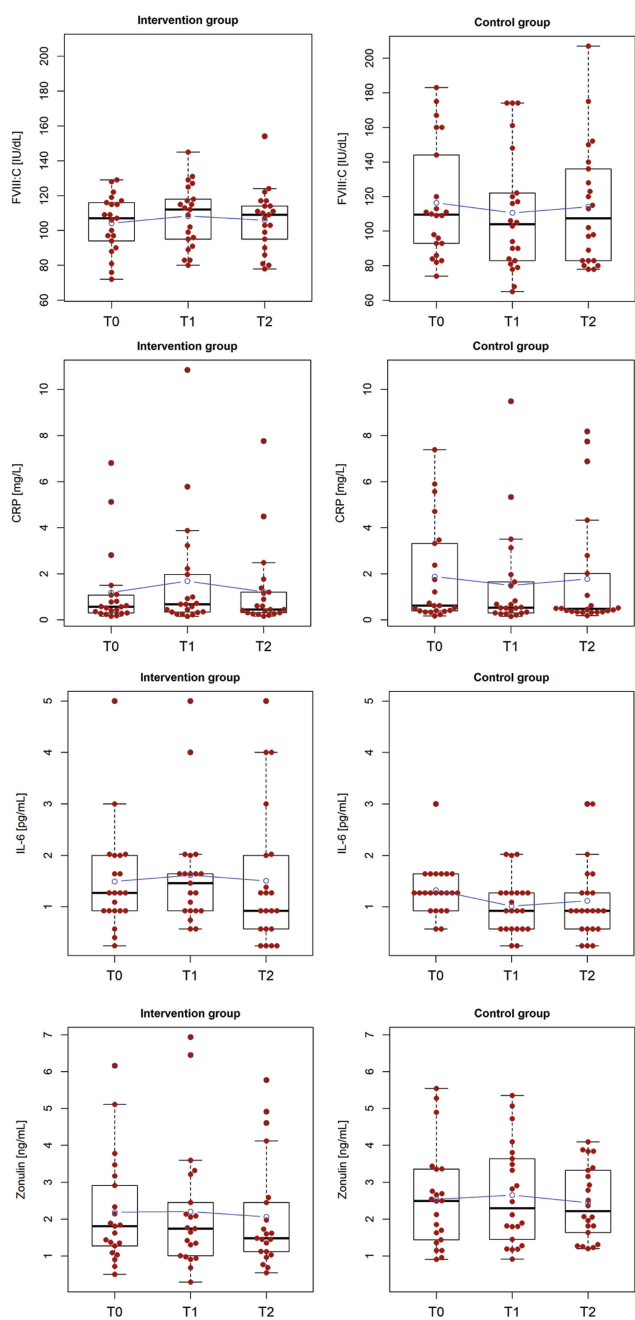
Table 2 Effects of 7 days of oral vancomycin intake versus no intervention on coagulation factor VIII:C and inflammatory parameters

Variable	Intervention group (I) (n = 21)			Control group (C) (n = 22)			t-Test	
	T0, mean (SD)	T1, mean (SD)	ΔI, T1–T0 (95% CI)	T0, mean (SD)	T1, mean (SD)	ΔC, T1–T0 (95% CI)	ΔI − ΔC	p^a
Factor VIII:C (IU/dL)	104 (16)	108 (18)	4 (−2 to 10)	116 (33)	110 (35)	−6 (−11 to −1)	10	0.013
log CRP[b]	−0.43 (1.0)	−0.22 (1.1)	0.21 (−0.17 to 0.58)	−0.03 (1.1)	−0.29 (1.1)	−0.25 (−0.5 to 0.01)	0.46	0.041
Fibrinogen (g/L)	2.6 (0.4)	2.7 (0.4)	0.1 (−0.0 to 0.2)	2.8 (0.5)	2.8 (0.5)	0.0 (−0.1 to 0.0)	0.1	0.061
IL-6 (pg/mL)	1.5 (1.0)	1.6 (1.1)	0.1 (−0.3 to 0.6)	1.3 (0.5)	1.0 (0.5)	−0.3 (−0.6 to 0)	0.4	0.100
IL-8 (pg/mL)	1.6 (1.7)	1.4 (1.7)	−0.2 (−0.7 to 0.3)	1.4 (0.9)	0.6 (0.6)	−0.8 (−1.2 to −0.3)	0.6	0.095
IL-10 (pg/mL)	2.9 (2.2)	2.6 (3.2)	−0.3 (−1.4 to 0.7)	1.6 (0.9)	0.9 (0.8)	−0.7 (−1.1 to −0.3)	0.4	0.50
IL-1β (pg/mL)	1.3 (0.7)	1.2 (0.7)	−0.1 (−0.3 to 0.1)	1.3 (0.6)	1.0 (0.5)	−0.3 (−0.6 to 0)	0.2	0.202
MCP-1 (pg)mL)	5.3 (6.0)	7.7 (8.9)	2.4 (−1.2 to 6.1)	3.0 (2.3)	5.7 (5.8)	2.7 (0.2 to 5.2)	−0.3	0.92
TNF (pg/mL)	18 (14)	14 (11)	−4 (−10 to 2)	16 (8)	10 (9)	−6 (−12 to −1)	2	0.50
Neutrophils (×10⁹/L)	2.9 (1.1)	3.1 (1.2)	0.2 (−0.4 to 0.7)	3.2 (1.2)	3.3 (1.1)	0.1 (−0.4 to 0.5)	0.1	0.74
C3bc (CAU/mL)	3.5 (2.4)	3.6 (2.3)	0.1 (−1.3 to 1.5)	4.7 (2.3)	3.6 (2.5)	−1.1 (−2.1 to −0.1)	1.2	0.145
TCC (CAU/mL)	0.4 (0.2)	0.4 (0.1)	0 (0 to 0.1)	0.8 (1.6)	0.8 (1.6)	0 (0 to 0)	0	0.36
Zonulin (ng/mL)	2.19 (1.46)	2.20 (1.73)	0.02 (−0.38 to 0.41)	2.53 (1.35)	2.65 (1.36)	0.12 (−0.31 to 0.54)	0.10	0.72

Abbreviations: CRP, C-reactive protein; IL, interleukin; MCP, monocyte chemoattractant protein; TCC, terminal complement complex; TNF, tumor necrosis factor.
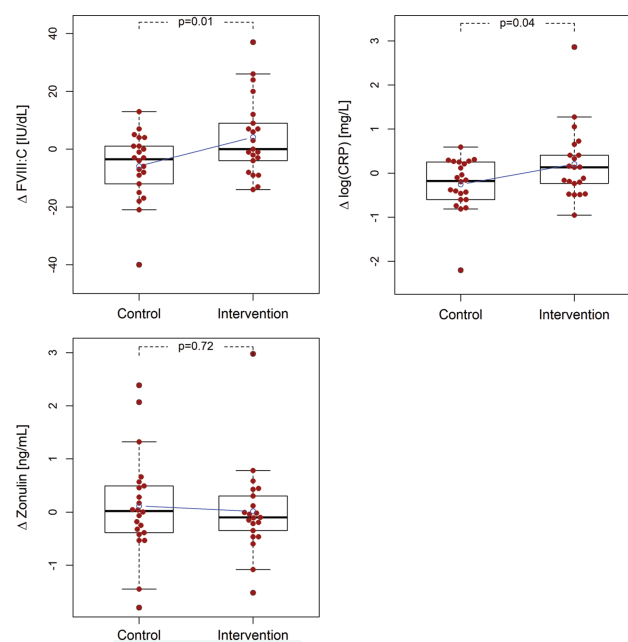[a]t-Test for difference in change from T0 to T1 between the intervention and the control group.
[b]High-sensitivity C-reactive protein (hs-CRP) was log-transformed to obtain normal distribution.

Fig. 6 The effect of 7 days intervention with vancomycin compared to no intervention on change in plasma FVIII activity (FVIII:C), hs-CRP, and zonulin. The boxes represent the interquartile range (IQR), with the upper and lower edges of the boxes representing the 75th (Q3) and the 25th (Q1) percentiles respectively. The central horizontal lines within the boxes represent the median level for each group. The upper and lower whiskers follow the default definitions of the boxplot function in R and are defined as min (max(x), $Q_3 +1.5*IQR$) and max (min(x), $Q_1 +1.5*IQR$), respectively, where x is the data value. The blue dots represent the mean values.

Fig. 5 The effect of 7 days intervention with vancomycin or no intervention on plasma FVIII activity (FVIII:C), C-reactive protein (CRP), interleukin (IL)-6 and zonulin in all participants. The boxes represent the interquartile range (IQR), with the upper and lower edges of the boxes representing the 75th (Q3) and the 25th (Q1) percentiles respectively. The central horizontal lines within the boxes represent the median level for each group. The upper and lower whiskers follow the default definitions of the boxplot function in R and are defined as min (max(x), $Q_3 +1.5*IQR$) and max (min(x), $Q_1 +1.5*IQR$), respectively, where x is the data value. The blue dots represent the mean values.

a low baseline rate even with an intact gut barrier.[19] A potential explanation for the observed modest increase in FVIII:C and hs-CRP could be a relative increase in translocation of LPS (from degraded bacterial strains) into the systemic circulation induced by the relative increase of gram-negative bacteria even without an increased gut per-

meability. This is endorsed by Jäckel et al[48] who showed that the gut microbiome modulates hepatic von Willebrand factor (vWF) expression, and thereby plasma vWF and FVIII levels, via toll-like receptors. vWF and FVIII levels were decreased in germ-free mouse models, suggesting that qualitative changes in microbial products' leakage into the systemic circulation influences coagulation activity. This introduces the hypothesis that coagulation factor levels might be directly regulated by gut microbiome composition, also in the absence of increased intestinal permeability. Unfortunately, vWF levels were not measured in our study, and we could therefore not assess changes in vWF.

We found a slight increase in CRP after vancomycin treatment compared with controls, but no increase in the other cytokine and complement markers. Plasma levels of CRP may be considered the resultant marker of systemic inflammation by several cytokines operating both as a cascade and as a network. Stimulation of CRP production from several cytokines could potentially explain why CRP and not IL-6 reached statistically significant differences between groups. Furthermore, activation of complement system and release of cytokines may well occur locally without being reflected in the systemic circulation, due to the large dilution and short half-life of these mediators.

Our study has some strengths. The randomized controlled trial design is a clear strength. The inclusion of young and healthy participants as well as the rigorous exclusion criteria

reduced the risk of outcomes being influenced by factors other than the intervention. In subjects with an already leaky gut, the rate of LPS translocation might be greater than in healthy individuals, and could induce a higher degree of systemic inflammation and increased FVIII:C levels. By lack of available data, we based our sample size calculation on observations in HIV patients.[37] In hindsight, the effect in healthy individuals could not have been expected to be similar as in HIV patients. HIV infection is associated with a greater rate of bacterial translocation due to lymphoid tissue destruction in the intestinal wall.[49] Coherently, we found a smaller effect on FVIII:C levels in healthy individuals than in patients with HIV.[37] Posthoc power analysis based on the observed mean values with SDs for change in FVIII:C from T0 to T1 revealed that our study had a power of 70% with a significance level of 5%. This limited power may hinder drawing strong conclusions.

Whether the findings in this study should be considered clinically relevant can be questioned. The increases in FVIII:C and hs-CRP we observed in our population of young and healthy volunteers are probably too small to exert an effect on VTE. This small effect could also be explained by the absence of increased gut permeability in our population, even after vancomycin treatment in the intervention group. A gram-negative dominant gut microbiome could potentially yield a more pronounced effect on VTE risk in aging patients with comorbidities associated with an increased gut permeability (e.g., metabolic syndrome, chronic inflammatory disease, liver cirrhosis).[21,50] Thus, the found increase in FVIII:C in the intervention group should be considered as a translational concept of which the clinical relevance is still to be determined.

In conclusion, this randomized controlled trial in healthy volunteers might provide support for the hypothesis that a vancomycin-induced decreased gut microbiome diversity and a relatively increased gram-negative abundance might result in increased systemic inflammation, measured by hs-CRP, and increased FVIII:C. Future studies are warranted to investigate the relationship between the gut microbiome, inflammation, and coagulation in subjects with a (known condition that comes with a) higher rate of bacterial translocation and the impact on VTE risk.

What is known about this topic?

- Growing evidence supports a role for immunothrombosis in the pathogenesis of venous thromboembolism (VTE).
- Inflammation and an altered gut microbiome (dysbiosis) are a common feature in several conditions associated with an increased risk for VTE.
- Dysbiosis might be a source of systemic inflammation and increased coagulation activity.

What does this paper add?

- In this randomized controlled trial we induced a gram-negative shift in the gut microbiome by oral vancomycin in healthy participants.

- A slight increase in plasma factor VIII and hs-CRP was found after treatment with vancomycin compared with controls.
- These findings might support the hypothesis that a gram-negative shift in the microbiome might contribute to an increase in systemic inflammation and FVIII activity.

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