Received: 4 August 2020 | Revised: 18 October 2020 | Accepted: 21 October 2020

DOI: 10.1111/hae.14195

Haemophilia WILEY

ORIGINAL ARTICLE

Clinical haemophilia

Collagen remodelling and plasma ascorbic acid levels in patients suspected of inherited bleeding disorders harbouring germline variants in collagen-related genes

Marcus Fager Ferrari[1] | Eva Zetterberg[1] | Maria Rossing[2] | Tina Manon-Jensen[3] | Martin Pehrsson[3] | Morten A. Karsdal[3] | Jens Lykkesfeldt[4] | Eva Leinoe[5]

[1]Clinical Coagulation Research Unit, Department of Translational Medicine, Lund University, Malmö, Sweden

[2]Center for Genomic Medicine, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

[3]Nordic Bioscience A/S, Biomarkers & Research, Herlev, Denmark

[4]Department of Veterinary & Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark

[5]Department of Hematology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

Correspondence
Marcus Fager Ferrari, Clinical Coagulation Research Unit, Skåne University Hospital, Jan Waldenströms gata 14, 205 02, Malmö, Sweden.
Email: marcus.fager_ferrari@med.lu.se

Abstract

Introduction: Variants in collagen-related genes *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* are associated with Ehlers-Danlos syndrome (EDS), a heterogeneous group of connective tissue disorders strongly associated with increased bleeding. Of patients with incompletely explained bleeding diathesis, a relatively high proportion were shown to harbour at least one heterozygous variant of unknown significance (VUS) in one of these genes, the vast majority without meeting the clinical criteria for EDS.

Aim: To investigate the functional consequences of the identified variants by assessing the formation and degradation of types I, III and V collagen, in addition to plasma levels of ascorbic acid (AA).

Methods: A total of 31 patients harbouring at least one heterozygous VUS in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* and 20 healthy controls were assessed using monoclonal antibodies targeting neo-epitopes specific for collagen formation and degradation. Plasma AA levels were measured in patients using high-performance liquid chromatography.

Results: Serum levels of C5 M (degradation of type V collagen) were decreased in patients compared with healthy controls (p = .033). No significant differences were found in biomarkers for remodelling of types I and III collagen. A significant negative correlation between bleeding (ISTH-BAT score) and plasma AA levels was shown (r = −.42; r^2 = .17; p = .020). Suboptimal or marginally deficient AA status was found in 8/31 patients (26%).

Conclusion: Functional investigations of collagen remodelling were not able to identify any clear associations between the identified variants and increased bleeding. The negative correlation between plasma AA levels and ISTH-BAT score motivates further investigations.

KEYWORDS
ascorbic acid, collagen, Ehlers-Danlos syndrome, haemorrhage, high-throughput nucleotide sequencing

1 | INTRODUCTION

Inherited bleeding disorders constitute a highly heterogeneous group of disorders, giving rise to bleeding symptoms of variable character and severity. The pathophysiological causes are multiple, including deficiencies of plasma coagulation factors and platelets, as well as defects of the vascular wall and connective tissues.[1] Historically, the diagnostics of inherited bleeding disorders have been complicated by the complexity and limited availability of the highly specialized assays required. In recent years, next-generation sequencing (NGS) techniques have allowed for the use of genetic screening in clinical practice, establishing a molecular diagnosis in an increasing proportion of patients with inherited bleeding disorders.[2–5]

In 2013, the Minibridge project was initiated as a collaboration between Rigshospitalet, Copenhagen and Skåne University Hospital, Malmö, with the aim to improve the diagnostics of inherited bleeding disorders by implementing upfront genetic screening in routine clinical practice.[6,7] Of 280 included patients, a relatively high proportion, 36/280 (13%), were shown to harbour variants of unknown significance (VUS) in at least one of the collagen-related genes *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2*, associated with Ehlers-Danlos syndrome (EDS).

Ehlers-Danlos syndrome is an inheritable disorder of the connective tissues. According to the current classification, 13 different clinical subtypes are defined.[8] Common symptoms are joint hypermobility and skin hyperextensibility due to compromised properties of collagen. Variants in *COL5A1* and *COL5A2* are associated with classical EDS (cEDS).[9], while variants in *COL3A1* are associated with vascular EDS (vEDS).[10] Variants in *COL1A1* are primarily seen in arthrochalasia EDS (aEDS) but are also rarely causative of vEDS and cEDS.[11] A propensity to bleeding diathesis is a common symptom in most EDS subtypes.[8] The most prevalent subtypes associated with bleeding diathesis are cEDS and vEDS.[12] Other subtypes are very rare and most often associated with unusual phenotypes, often evident from birth. The prevalence of the rarer EDS subtypes is unknown, while the prevalence of cEDS and vEDS has been estimated to 1:20 000 and 1:90 000, respectively.[12] Collagen is indeed an important factor in hemostasis, being a central component for vascular stability and integrity. Further, collagens are known to interact with several central actors of hemostasis such as platelets, factor IX and von Willebrand factor (VWF).[13,14]

The Scientific and Standardization Subcommittee (SSC) on Genomics in Thrombosis and Hemostasis of the International Society on Thrombosis and Haemostasis (ISTH) recently generated a list of 91 genes associated with inherited bleeding, thrombotic and platelet disorders with conclusive evidence of being disease causing (TIER1 genes).[15]; the list has continuously been updated, now comprising 93 TIER1 genes (https://www.isth.org/page/GinTh_GeneLists). The association between bleeding symptoms and variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* in EDS patients was recognized by the authors. However, the genes were classified as non-TIER1 genes, since systematic investigations regarding this category of genes have yet to be performed in a patient population with unexplained bleeding symptoms.

To investigate the functional significance of the identified genetic variants in the collagen-related genes, we aimed to measure the formation and degradation of the corresponding collagen subtypes, types I, III and V collagen, using highly specific biomarkers. The use of biomarkers of collagen remodelling have previously been investigated in a variety of conditions with potential implications for diagnostics and disease monitoring.[16–18] To our knowledge, collagen remodelling related to genetic variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* has previously not been investigated in patients with unexplained bleeding symptoms.

Ascorbic acid (AA) is known to be crucial for the synthesis of mature collagen, being an important cofactor involved in the hydroxylation of lysine and proline residues in the procollagen chains forming the triple-helical collagen molecules.[19,20] Severe deficiency of AA is known to cause scurvy, with symptoms including impaired wound healing and bleeding diathesis, due to decreased collagen synthesis.[21] We hypothesized that a potentially altered collagen remodelling could be reflected in plasma levels of AA. Indeed, dietary supplements of AA have been proposed for patients with cEDS, in order to reduce easy bruising.[9,22] To our knowledge, levels of AA have not been systematically investigated in patients with EDS or isolated bleeding diathesis.

The aim of this study was to investigate collagen remodelling and plasma levels of AA in patients presenting with bleeding diathesis, harbouring genetic variants in collagen-related genes *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2*.

2 | MATERIALS AND METHODS

2.1 | Study population

Patients referred because of the suspicion of an inherited bleeding disorder of unknown cause were asked for participation if they were found to harbour at least one heterozygous variant in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*. In total, 31 patients were included. All patients were of at least 18 years of age. The patients were initially assessed for deficiencies of plasma coagulation factors using PK(INR), APTT, fibrinogen, factor VIII and VWF activity. Platelet counts, mean platelet volumes (MPV) and peripheral blood smears were also assessed. When available, platelet aggregation was assessed by Multiplate® or light transmission aggregometry (LTA).[23] Factor XIII levels were also analysed when possible. When motivated by the result of genetic screening, platelet flow cytometry (FC) and transmission electron microscopy (TEM) was used for functional/confirmatory testing.[7,23] Levels of C-reactive protein (CRP) were measured to exclude significant systemic inflammation, potentially affecting collagen remodelling. The patients had not been pregnant in the last 12 months prior to blood sampling. Bleeding tendencies were assessed using the International Society on Thrombosis and Haemostasis bleeding assessment tool (ISTH-BAT) scoring system.[24] A positive ISTH-BAT score is defined as ≥4 in adult males and ≥6 in adult females.[25] A positive family history

was defined as having at least one first-degree relative with a history of bleeding diathesis.

2.2 | Control population for assessment of collagen remodelling

For the assays of collagen remodelling, 20 healthy controls, age- and gender-matched on a group level, were recruited. All controls had normal ISTH-BAT scores.[24,25] They had not been pregnant for at least a period of 12 months prior to blood sampling, and CRP levels were normal.

2.3 | Ethics

Signed informed consent was retrieved from all patients and controls prior to inclusion.

The study was approved by the Ethics Committee, Lund University, Sweden (Dnr 2014/409) and the Regional Ethics Committee, Copenhagen, Denmark (H-15011677).

2.4 | Genetic screening

The patients were screened using an *in silico* 94-gene panel including 63 platelet-related and 31 non-platelet-related genes (Table S1). DNA sequencing and germline variant calling was performed as previously described.[6,7] Allele frequencies were derived from gnomAD v2.1.1 (https://gnomad.broadinstitute.org).[26] Variants with an allele frequency >1% in the background population at the time of analysis were excluded. The five-tier scheme recommended by the American College of Medical Genetics and Genomics was used to classify the identified variants regarding their potential significance.[27]

2.5 | Clinical assessment of EDS criteria

All patients were assessed by a trained physician regarding the presence of a clinical phenotype consistent with cEDS, vEDS or aEDS, according to the current international classification of EDS.[8]

2.6 | Remodelling of types I, III and V collagen

For each patient and control, collagen remodelling was assessed by competitive enzyme-linked immunosorbent assays (ELISAs), using monoclonal antibodies targeting specific neo-epitopes: Formation of types I, III and V collagen was assessed by the internal epitope in the N-terminal pro-peptide of type I collagen (PRO-C1).[28], by the N-terminal pro-peptide of type III collagen (PRO-C3).[29] and by the C-terminal pro-peptide of type V collagen (PRO-C5).[30,31] Degradation of types I, III and V collagen was assessed by the MMP-2, MMP-9 and MMP-13-derived fragment of type I collagen (C1 M).[32], by the MMP-9-derived fragment of type III collagen (C3 M).[33] and by the MMP-2 and MMP-9-derived fragment of type V collagen (C5 M).[34] As a measure of tissue balance, ratios were calculated as follows: PRO-C1/C1 M, PRO-C3/C3 M and PRO-C5/C5 M.[16,35] All assays were developed and produced by Nordic Bioscience A/S, Herlev, Denmark.

2.7 | Ascorbic acid

Plasma levels of AA were assessed in all patients using high-performance liquid chromatography with coulometric electrochemical detection, as previously described.[36] All patients were instructed to quit any dietary supplements containing AA at least one week prior to blood sampling and were in a fasting state at the time for blood sampling. The plasma levels of AA were interpreted as follows: <11 μM: severe deficiency; 11-23 μM: marginal deficiency; and 23-50 μM: suboptimal status.[37]

2.8 | Statistical analyses

Numerical data are presented as medians with lower and upper quartiles (Q_1 – Q_3), if not stated otherwise. Data were assessed for normality visually and by using the D'Agostino-Pearson omnibus K2 normality test. Outliers were identified using Tukey's fences. Outliers were identified for further assessment of the data distribution, but were never excluded in the analyses. For comparing variables between patients and controls, Mann-Whitney tests were consistently used. Correlations were investigated by creating scatter plots. Correlation coefficients and *p*-values were computed. Depending on the distribution of the data, either the Pearson or the nonparametric Spearman correlation coefficient was used. For all analyses, a *p*-value <.05 was considered significant. Data were analysed and graphed using GraphPad Prism v8.2.1 for Mac OS X (GraphPad Software).

3 | RESULTS

3.1 | Patient characteristics

In total, 31 patients were included, whereof three were males (9.7%) (Table 1). The median age was 41 years (21-67 years, min-max). The median ISTH-BAT score was 10 (6 – 13, Q_1 – Q_3), and 25/31 patients (81%) had significant ISTH-BAT scores. Patients without a significant ISTH-BAT score were initially included either because of an unexplained thrombocytopenia or a borderline significant ISTH-BAT score combined with a positive family history of increased bleeding. The vast majority of the patients, 94%, had normal levels of plasma coagulation factors and VWF. No abnormalities in plasma

TABLE 1 Patient characteristics including plasma AA levels.

Patient	Age	Sex	ISTH-BAT[e]	Family history[f]	Platelet count (x10^9/L)	Collagen-related variants[c]	Allele frequency[g]	Hypermobility[h]	Ascorbic acid (μM)
P1	21	F	5	Yes	282	*COL3A1*: c.2035G>A, p.Ala679 Thr *COL1A1*: c.436C>A, p.Pro146 Thr	1.016% 0.002886%	Yes	47.3[a]
P2	43	F	15[d]	Yes	266	*COL3A1*: c.2035G>A, p.Ala679 Thr (Ho) *COL1A1*: c.436C>A, p.Pro146 Thr	1.016% 0.002886%	No	13.2[b]
P3	39	F	8 [d]	Yes	298	*COL3A1*: c.3938A>G, p.Lys1313Arg *COL1A1*: c.4018G>A, p.Gly1340Ser	0.1533% 0.07447%	Yes	58.1
P4	64	F	9[d]	Yes	244	*COL3A1*: c.3938A>G, p.Lys1313Arg *COL1A1*: c.4018G>A, p.Gly1340Ser	0.1533% 0.07447%	Yes	79.7
P5	21	F	8 [d]	No	252	*COL5A2*: c.1301C>T, p.Thr434Met	0.1000%	No	72.7
P6	30	F	12 [d]	No	235	*COL5A1*: c.2852A>G, p.Asn951Ser	1.364%	Yes	63.7
P7	47	F	8 [d]	Yes	257	*COL3A1*: c.3938A>G, p.Lys1313Arg	0.1533%	Yes	62.8
P8	23	F	7 [d]	No	219	*COL1A1*: c.3665A>G, p.Asn1222Ser *COL5A1*: c.278C>T, p.Ala93Val	0.0007741% 0.3575%	Yes	88.6
P9	51	F	10 [d]	Yes	265	*COL1A1*: c.2467C>G, p.Pro823Ala	0.04746%	No	63.1
P10	61	F	13 [d]	Yes	239	*COL3A1*: c.2035G>A, p.Ala679 Thr	1.016%	Yes	57.6
P11	32	F	5	Yes	200	*COL3A1*: c.1804C>A, p.Pro602 Thr	0.6605%	No	84.8
P12	39	F	17 [d]	Yes	224	*COL5A1*: c.193C>T, p.Arg65Trp *COL5A1*: c.514G>T, p.Val172Ile	0.1606% 0.0007785%	Yes	60.0
P13	54	F	16 [d]	Yes	322	*COL3A1*: c.1804C>A, p.Pro602 Thr	0.6605%	Yes	18.8 [b]
P14	28	F	13 [d]	Yes	43 [d]	*COL3A1*: c.1804C>A, p.Pro602 Thr *COL5A1*: c.1976C>T, p.Pro659Leu *COL5A1*: c.5 T > C, p.Met2 Thr	0.6605% N/A N/A	Yes	37.4 [a]
P15	40	F	9 [d]	Yes	136 [d]	*COL3A1*: c.2035G>A, p.Ala679 Thr	1.016%	Yes	74.9
P16	51	F	13 [d]	Yes	188	*COL3A1*: c.2002C>A, p.Pro668 Thr	0.1000%	Yes	60.4
P17	42	F	10 [d]	Yes	122 [d]	*COL5A1*: c.1796C>T, p.Pro599Leu	0.001767%	Yes	71.1
P18	41	F	2	Yes	112 [d]	*COL5A1*: c.3491C>T, p.Pro1164Leu	0.01240%	Yes	75.4
P19	32	M	10 [d]	Yes	143 [d]	*COL5A1*: c.4135C>T, p.Pro1379Ser *COL5A2*: c.3689C>G, p.Thr1230Arg	0.9074% 0.8250%	No	81.5
P20	42	F	17 [d]	Yes	232	*COL3A1*: c.1337G>A, p.Arg446His	0.000%	Yes[i]	55.0
P21	51	M	6 [d]	Yes	215	*COL5A1*: c.278C>T, p.Ala93Val	0.3575%	No	48.2 [a]
P22	34	F	5	Yes	235	*COL5A1*: c.5350G>A, p.Ala1784 Thr	0.1013%	No	62.1
P23	61	F	10 [d]	Yes	219	*COL3A1*: c.1804C>A; p.Pro602 Thr	0.6605%	Yes	65.7
P24	41	F	5	Yes	244	*COL1A1*: c.613C>G, p.Pro205Ala	0.5057%	Yes	78.1
P25	35	M	13 [d]	No	162	*COL3A1*: c.505C>T, p.Leu169Phe	0.01707%	Yes	23.5 [a]

(Continues)

Table 1 (Continued)

Patient	Age	Sex	ISTH-BAT[e]	Family history[f]	Platelet count (x10^9/L)	Collagen-related variants[c]	Allele frequency[g]	Hypermobility[h]	Ascorbic acid (μM)
P26	40	F	17 [d]	Yes	307	*COL3A1*: c.2224G>T, p.Asp742 Tyr	0.006232%	No	62.1
P27	55	F	11 [d]	Yes	271	*COL5A2*: c.263C>A, p.Pro88His	0.02791%	Yes	35.7 [a]
P28	38	F	7 [d]	Yes	228	*COL5A1*: c.1831C>T, p.Arg611Trp	0.008795%	Yes	27.1 [a]
P29	57	F	6 [d]	Yes	185	*COL5A1*: c.367C>G, p.Gln123Glu	0.03953%	No	68.3
P30	67	F	10 [d]	No	223	*COL1A1*: c.613C>G, p.Pro205Ala	0.5057%	Yes	59.2
P31	54	F	5	Yes	66 [d]	*COL5A2*: c.3913G>A, p.Ala1305 Thr	0.002669%	Yes	50.4

Abbreviations: AA, ascorbic acid; Ho, homozygous; ISTH-BAT, International Society on Thrombosis and Haemostasis bleeding assessment tool; N/A, not applicable.

[a]Suboptimal AA status (23-50 μM);

[b]Marginal AA deficiency (11-23 μM);

[c]All genetic variants are heterozygous if not stated otherwise;

[d]Abnormal result;

[e]A positive ISTH-BAT score is defined as ≥4 in adult males and ≥6 in adult females;

[f]At least one first-degree relative with a history of bleeding diathesis;

[g]Allele frequencies were derived from the gnomAD browser;

[h]hypermobility was assessed using the Beighton score and the five-point questionnaire (5PQ).[8];

[i]P20 was previously diagnosed with hypermobile EDS (hEDS).

coagulation factors explaining the individual bleeding phenotypes were evident. Thrombocytopenia was present in 6/31 patients (19%) (Table 1). The prevalence of a positive family history of increased bleeding was high; 26/31 patients (84%). The following subjects were related: Patients P1 (daughter) and P2 (mother); Patients P3 (daughter) and P4 (mother); and Patients P13 (mother), P11 and P14 (daughters).

3.2 | Control population for assessment of collagen remodelling

The control group consisted of 20 subjects with a median age of 39 years (25-64 years, min-max). The proportion of males was 2/20 (10%). All controls had normal ISTH-BAT scores and negative medical histories regarding familial bleeding diathesis.

3.3 | Genetic variants

At least one heterozygous variant in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* was found in all 31 patients (Table 1). Patient P2 was found to be homozygous for a variant in *COL3A1*. In total, 28 unique variants in collagen-related genes were found (*COL1A1*, n = 5; *COL3A1*, n = 7; *COL5A1*, n = 12; *COL5A2*, n = 4). All variants were initially classified as VUS. Due to the continuously increasing information regarding population allele frequencies, two variants (*COL3A1*: c.2035G>A, p.Ala679 Thr and *COL5A1*: c.2852A>G, p.Asn951Ser) were found to have an allele frequency >1% following reassessments using updated databases (Table 1). In 25/31 patients (81%), additional variants were found, the majority assessed as benign (class 1) or likely benign (class 2) based on inheritance pattern, functional testing and/or clinical features (Table S2). In total, four likely pathogenic (class 4) or pathogenic (class 5) variants in non-collagen-related genes were found in four different patients (Table S2).

3.4 | Clinical assessment of EDS criteria

According to the current diagnostic criteria, 3/31 patients (9.7%) fulfilled the clinical criteria for EDS: P14 and P28 met the clinical criteria for cEDS, while P20 was previously diagnosed with hypermobile EDS (hEDS). The underlying molecular defect in hEDS is unknown.[8]

3.5 | Remodelling of types I, III and V collagen

On a group level, patients had significantly lower levels of C5 M compared with controls (p = 0.033) (Figure 1, H). There were no other significant differences between patients and controls regarding the remaining biomarkers of collagen remodelling (Figure 1). Patients with at least one specific collagen-related variant hypothesized to

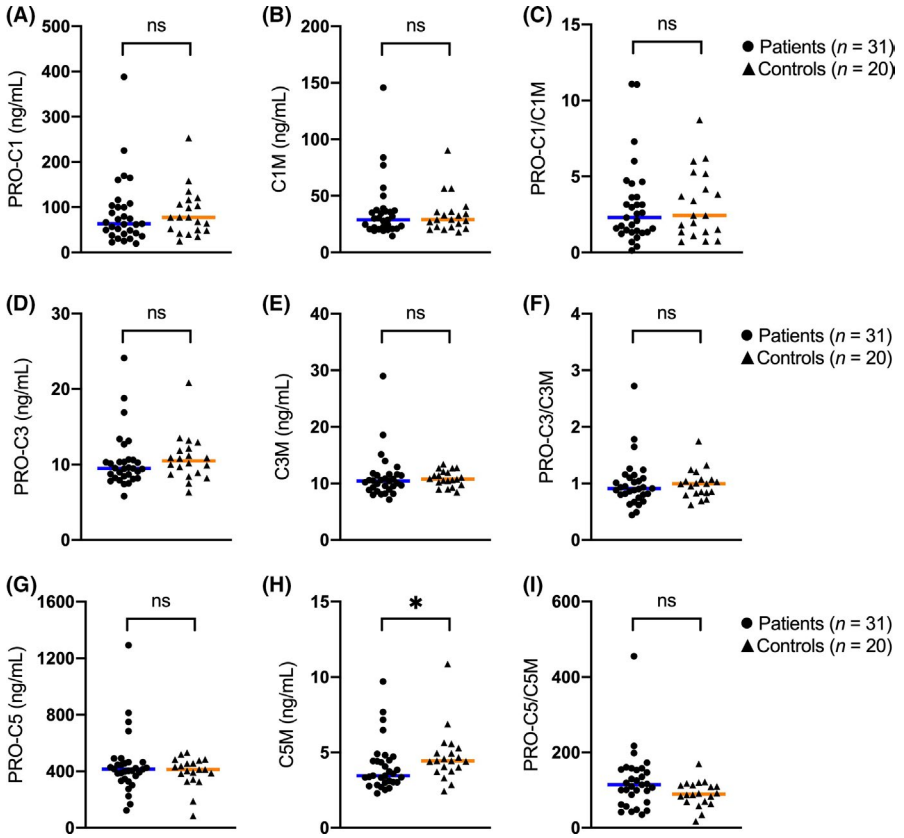

FIGURE 1 Remodelling of types I, III and V collagen. (A-I) Levels of PRO-C1, PRO-C3, PRO-C5, C1 M, C3 M and C5 M were analysed in patients and healthy controls. Collagen tissue balance was assessed using the following ratios: PRO-C1/C1 M, PRO-C3/C3 M and PRO-C5/C5 M. (H) The patients showed significantly lower levels of C5 M ($p = 0.033$). No additional significant differences were found. Median values are shown (blue line, patients; orange line, controls). ns, not significant; *, significant ($p < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

affect the associated collagen type (eg PRO-C1, C1 M and PRO-C1/C1 M in patients with variants in *COL1A1*) were grouped together and compared to the controls. No additional significant differences were found, except for significantly lower levels of C5 M in patients with at least one *COL5A1* variant compared to controls ($p = .017$) (data not shown). When looking at levels of C5 M, stratified according to all the different collagen-related variants, decreased levels were found compared to controls in patients harbouring at least one variant in *COL1A1* ($p = .029$) (data not shown). Further, no differences in levels of C5 M could be found compared to controls in patients stratified for exclusively harbouring collagen-related variants in either *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* (data not shown).

For each biomarker of collagen remodelling, the patient group was investigated regarding the presence of outlier values. In total, 13/31 patients (42%) presented as outliers regarding at least one biomarker of collagen remodelling. The proportion of patients being outliers for at least one biomarker related to a corresponding genetic variant was 7/31 (23%). The proportion of patients being outliers without harbouring a corresponding genetic variant was even higher, 10/31 (32%). None of the patients meeting the clinical criteria for EDS (P14, P20, P28) presented as outliers (data not shown).

PRO-C1, PRO-C3, PRO-C5, C1 M, C3 M, C5 M, PRO-C1/C1 M, PRO-C3/C3 M and PRO-C5/C5 M were separately analysed for correlations with AA levels and ISTH-BAT score. No significant correlations were found (data not shown).

3.6 | Ascorbic acid

The mean level of plasma AA in the patients was 58.3 ± 19.4 μM (mean ±SD, standard deviation). It was found that 6/31 patients (19%) had plasma levels consistent with suboptimal AA status, while 2/31 patients (6.5%) showed marginal AA deficiency. Consequently, 8/31 patients (26%) had at least suboptimal AA status (Figure 2, Table 1). Interestingly, both patients meeting the clinical criteria for cEDS (P14, P28) had suboptimal AA status.

Correlations were analysed for AA levels and the following parameters: PRO-C1, PRO-C3, PRO-C5, C1 M, C3 M, C5 M, PRO-C1/C1 M, PRO-C3/C3 M, PRO-C5/C5 M and ISTH-BAT score. A significant negative correlation between plasma levels of AA and ISTH-BAT score was demonstrated ($r = -0.42$; $r^2 = .17$; $p = .020$) (Figure 2). Of importance, 6/31 (19%) of the patients were smokers. Smokers are known to have lower plasma concentrations of AA.[38] However,

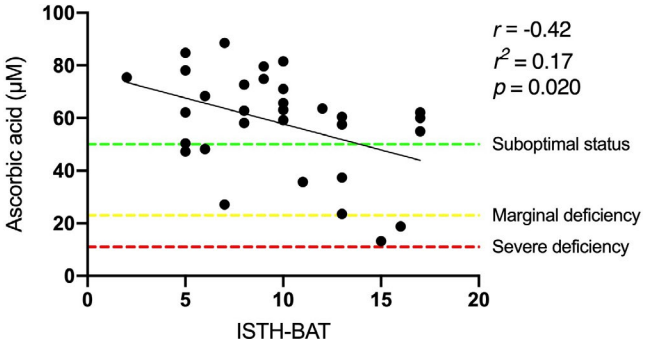

FIGURE 2 Correlation between plasma levels of AA and ISTH-BAT score in patients (n = 31). A significant negative correlation between plasma levels of AA and ISTH-BAT score was found (r = −0.42; r^2 = 0.17; p = 0.020). In addition, 8/31 patients (26%) showed at least suboptimal AA status. The levels of AA were interpreted as follows: <11 µM: severe deficiency; 11-23 µM: marginal deficiency; 23-50 µM: suboptimal status. AA, ascorbic acid; ISTH-BAT, International Society on Thrombosis and Haemostasis bleeding assessment tool. [Colour figure can be viewed at wileyonlinelibrary.com]

the negative correlation between plasma levels of AA and ISTH-BAT score was still significant when excluding the smokers (r_s = −.54; p = .0055). No significant correlations between AA levels and the other parameters were found (data not shown).

4 | DISCUSSION

In the present study, we investigated collagen remodelling in 31 patients with incompletely explained bleeding diathesis, harbouring variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2*, using biomarkers specific for collagen formation and degradation. No significant differences between patients and controls were found in the levels of biomarkers for remodelling of types I and III collagen. However, for type V collagen, the serum levels of C5 M were significantly decreased in the patient group compared with the control group. The lower levels of C5 M on a group level could be attributed to the patients with at least one variant in *COL5A1* (11/31, 36%) and patients with at least one variant in *COL1A1* (8/31, 26%). Only one patient was represented in both of the groups. When looking at patients exclusively harbouring collagen-related variants in either *COL5A1* (n = 8) or *COL1A1* (n = 3), there were no differences in the levels of C5 M compared to the controls. Hence, a clear correlation between the levels of C5 M and the different genes potentially affected was not possible to establish.

The integrity of vascular collagen is essential for mechanical stability and hemostasis. Types I, III, IV, VI, XV and XVIII collagen are present in the vascular wall and hence have the potential of affecting the vascular structure.[13] Of vascular collagens, type I collagen constitutes 60% while type III collagen constitutes 30%. Type V collagen is a fibrillar collagen important for the fibrillogenesis of collagen type I and III.[39] In theory, impaired remodelling of type V collagen could possibly affect the assembly of fibrillar types I and III collagen, thereby disrupting the overall stability of the vascular interstitial matrix, conferring increased bleeding. The significance of the decreased levels in C5 M observed on a group level, in combination with normal levels of PRO-C5 and a normal PRO-C5/C5 M ratio is, however, unknown.

When looking at the individual patients, 42% of the patients were shown to be outliers regarding at least one of the biomarkers of collagen remodelling. To investigate our hypothesis of collagen-related variants affecting collagen remodelling, an abnormal result in collagen biomarkers associated with a corresponding genetic variant were of particular interest (eg levels of PRO-C3, C3 M and PRO-C3/C3 M in patients with variants in *COL3A1*). However, when a patient presented as an outlier for a collagen biomarker, it was more common not to harbour a potentially corresponding genetic variant. The outliers identified could possibly be explained by a combination of a relatively low number of included subjects and a wide normal variation in the levels of the collagen biomarkers.

Reduced plasma levels of AA were found in 26% of the included patients. Furthermore, a significant negative correlation between AA levels and bleeding (ISTH-BAT score) was shown, even when excluding smokers, who are known to have lower levels of AA.[38] Ascorbic acid supplements are recommended in patients with cEDS, based on expert opinions.[9,22] Interestingly, suboptimal AA status was found in the two patients meeting the clinical criteria for cEDS. This result suggests a possible correlation, encouraging further investigations in a larger cohort of patients with isolated bleeding diathesis, as well as in patients with cEDS.

Limitations of the present study include the lack of a true functional test of collagen function and vascular stability. No strong correlations were identified between the biomarkers of collagen remodelling and the variants in collagen-related genes. Consequently, it remains to be elucidated to what extent collagen remodelling correlates with vascular instability. In addition, the low number of patients included increases the risk of type II errors, and thus, we might very likely have missed associations, becoming evident in a larger cohort. The average AA status of the present cohort was relatively good, and the washout period of one week may have been insufficient to reestablish a true baseline AA status. Finally, the plasma AA levels were interpreted using previously established cut-off values.[37] Since AA levels were not measured in the control population, a possible difference in AA status between the patients and controls is not possible to rule out. The effects of AA levels on the biomarkers of collagen remodelling are, however, unknown.

5 | CONCLUSION

In conclusion, the functional investigations of collagen remodelling were not able to verify any of the identified variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* as causative of increased bleeding. However, an interesting negative correlation between plasma AA levels and bleeding was found, motivating further investigations.

Taken together, further studies of the functional implications of collagen-related variants in hemostasis are warranted.

ACKNOWLEDGEMENTS

The authors would like to thank all the patients and controls who participated in this study. The nurses and the laboratory personnel at the Department of Hematology, Rigshospitalet and research nurse E. Lindén at the Clinical Coagulation Research Unit, Malmö are greatly acknowledged for their practical support during the project.

AUTHOR CONTRIBUTIONS

E. Leinoe, E. Zetterberg, T. Manon-Jensen, M. A. Karsdal, M. Rossing, M. Fager Ferrari and J. Lykkesfeldt participated in the design of the study. M. Fager Ferrari made the clinical assessments and collected and prepared the blood samples. M. Rossing and E. Leinoe provided the NGS data. T. Manon-Jensen, M. Pehrsson and M. A. Karsdal were responsible for the analyses of collagen remodelling. J. Lykkesfeldt provided the analyses of ascorbic acid. M. Fager Ferrari and M. Pehrsson performed the statistics. All authors were part of the interpretation of the data. M. Fager Ferrari wrote the manuscript, which was critically reviewed and approved by all authors.

DISCLOSURES

T. Manon-Jensen, M. Pehrsson and M. A. Karsdal are employed by Nordic Bioscience A/S. E. Zetterberg has received research grants from Shire and consulting fees from Sobi and CSL Behring. E. Leinoe has received a consulting fee from Amgen. The authors have no other potential conflicts of interest to declare.

ORCID

Marcus Fager Ferrari https://orcid.org/0000-0001-8651-101X
Eva Zetterberg https://orcid.org/0000-0003-1775-1727

REFERENCES

1. Boender J, Kruip MJ, Leebeek FW. A diagnostic approach to mild bleeding disorders. *J Thromb Haemost*. 2016;14(8):1507–1516.
2. Maclachlan A, Watson SP, Morgan NV. Inherited platelet disorders: Insight from platelet genomics using next-generation sequencing. *Platelets*. 2017;28(1):14–19.
3. Freson K, Turro E. High-throughput sequencing approaches for diagnosing hereditary bleeding and platelet disorders. *J Thromb Haemost*. 2017;15(7):1262–1272.
4. Sivapalaratnam S, Collins J, Gomez K. Diagnosis of inherited bleeding disorders in the genomic era. *Br J Haematol*. 2017;179(3):363–376.
5. Downes K, Megy K, Duarte D, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. *Blood*. 2019;134(23):2082–2091.
6. Leinoe E, Zetterberg E, Kinalis S, et al. Application of whole-exome sequencing to direct the specific functional testing and diagnosis of rare inherited bleeding disorders in patients from the Oresund Region, Scandinavia. *Br J Haematol*. 2017;179(2):308–322.
7. Leinoe E, Gabrielaite M, Ostrup O, et al. Outcome of an enhanced diagnostic pipeline for patients suspected of inherited thrombocytopenia. *Br J Haematol*. 2019;186(2):373–376.
8. Malfait F, Francomano C, Byers P, et al. The 2017 international classification of the Ehlers-Danlos syndromes. *Am J Med Genet C Semin Med Genet*. 2017;175(1):8–26.
9. Bowen JM, Sobey GJ, Burrows NP, et al. Ehlers-Danlos syndrome, classical type. *Am J Med Genet C Semin Med Genet*. 2017;175(1):27–39.
10. Byers PH, Belmont J, Black J, et al. Diagnosis, natural history, and management in vascular Ehlers-Danlos syndrome. *Am J Med Genet C Semin Med Genet*. 2017;175(1):40–47.
11. Brady AF, Demirdas S, Fournel-Gigleux S, et al. The Ehlers-Danlos syndromes, rare types. *Am J Med Genet C Semin Med Genet*. 2017;175(1):70–115.
12. Ghali N, Sobey G, Burrows N. Ehlers-Danlos syndromes. *BMJ*. 2019;366:l4966.
13. Manon-Jensen T, Kjeld NG, Karsdal MA. Collagen-mediated hemostasis. *J Thromb Haemost*. 2016;14(3):438–448.
14. Stafford DW. Extravascular FIX and coagulation. *Thromb J*. 2016;14(Suppl 1):35.
15. Megy K, Downes K, Simeoni I, et al. Curated disease-causing genes for bleeding, thrombotic, and platelet disorders: Communication from the SSC of the ISTH. *J Thromb Haemost*. 2019;17(8):1253–1260.
16. Henriksen NA, Mortensen JH, Lorentzen L, et al. Abdominal wall hernias-A local manifestation of systemically impaired quality of the extracellular matrix. *Surgery*. 2016;160(1):220–227.
17. Husakova M, Bay-Jensen AC, Forejtova S, et al. Metabolites of type I, II, III, and IV collagen may serve as markers of disease activity in axial spondyloarthritis. *Sci Rep*. 2019;9(1):11218.
18. Juhl P, Bay-Jensen AC, Karsdal M, Siebuhr AS, Franchimont N, Chavez J. Serum biomarkers of collagen turnover as potential diagnostic tools in diffuse systemic sclerosis: A cross-sectional study. *PLoS One*. 2018;13(12):e0207324.
19. Lykkesfeldt J, Michels AJ, Frei B. Vitamin C. *Adv Nutr*. 2014;5(1):16–18.
20. Granger M, Eck P. Dietary Vitamin C in Human Health. *Adv Food Nutr Res*. 2018;83:281–310.
21. Olmedo JM, Yiannias JA, Windgassen EB, Gornet MK. Scurvy: a disease almost forgotten. *Int J Dermatol*. 2006;45(8):909–913.
22. Malfait F, Wenstrup R, De Paepe A. Classic Ehlers-Danlos Syndrome. In: Adam MP, Ardinger HH, Pagon RA, et al., *GeneReviews® [Internet]*. Seattle, WA; 2007. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1244/
23. Fager Ferrari M, Leinoe E, Rossing M, et al. Germline heterozygous variants in genes associated with familial hemophagocytic lymphohistiocytosis as a cause of increased bleeding. *Platelets*. 2018;29(1):56–64.
24. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065.
25. Elbatarny M, Mollah S, Grabell J, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. *Haemophilia*. 2014;20(6):831–835.
26. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581(7809):434–443.
27. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405–424.
28. Leeming DJ, Larsen DV, Zhang C, et al. Enzyme-linked immunosorbent serum assays (ELISAs) for rat and human N-terminal pro-peptide of collagen type I (PINP)–assessment of corresponding epitopes. *Clin Biochem*. 2010;43(15):1249–1256.
29. Nielsen MJ, Nedergaard AF, Sun S, et al. The neo-epitope specific PRO-C3 ELISA measures true formation of type III collagen associated with liver and muscle parameters. *Am J Transl Res*. 2013;5(3):303–315.

30. Vassiliadis E, Veidal SS, Simonsen H, et al. Immunological detection of the type V collagen propeptide fragment, PVCP-1230, in connective tissue remodeling associated with liver fibrosis. *Biomarkers*. 2011;16(5):426–433.
31. Leeming DJ, Veidal SS, Karsdal MA, et al. Pro-C5, a marker of true type V collagen formation and fibrillation, correlates with portal hypertension in patients with alcoholic cirrhosis. *Scand J Gastroenterol*. 2015;50(5):584–592.
32. Leeming D, He Y, Veidal S, et al. A novel marker for assessment of liver matrix remodeling: an enzyme-linked immunosorbent assay (ELISA) detecting a MMP generated type I collagen neo-epitope (C1M). *Biomarkers*. 2011;16(7):616–628.
33. Barascuk N, Veidal SS, Larsen L, et al. A novel assay for extracellular matrix remodeling associated with liver fibrosis: An enzyme-linked immunosorbent assay (ELISA) for a MMP-9 proteolytically revealed neo-epitope of type III collagen. *Clin Biochem*. 2010;43(10–11):899–904.
34. Veidal SS, Larsen DV, Chen X, et al. MMP mediated type V collagen degradation (C5M) is elevated in ankylosing spondylitis. *Clin Biochem*. 2012;45(7–8):541–546.
35. Karsdal MA, Henriksen K, Leeming DJ, et al. Biochemical markers and the FDA Critical Path: how biomarkers may contribute to the understanding of pathophysiology and provide unique and necessary tools for drug development. *Biomarkers*. 2009;14(3):181–202.
36. Lykkesfeldt J.^{M}easurement of ascorbic acid and dehydroascorbic acid in biological samples. Curr Protoc Toxicol. 2002;Chapter 7: Unit 7 6 1–15.
37. Lykkesfeldt J, Poulsen HE. Is vitamin C supplementation beneficial? Lessons learned from randomised controlled trials. *Br J Nutr*. 2010;103(9):1251–1259.
38. Langlois K, Cooper M, Colapinto CK. Vitamin C status of Canadian adults: Findings from the 2012/2013 Canadian Health Measures Survey. *Health Rep*. 2016;27(5):3–10.
39. Leeming DJ, Karsdal MA. Type V Collagen. In: Karsdal MA, ed. *Biochemistry of Collagens, Laminins and Elastin*, 1st edn. Academic Press; 2016:43–48.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1

Table S2

Supplementary Material

How to cite this article: Fager Ferrari M, Zetterberg E, Rossing M, et al. Collagen remodelling and plasma ascorbic acid levels in patients suspected of inherited bleeding disorders harbouring germline variants in collagen-related genes. *Haemophilia*. 2021;27:e69–e77. https://doi.org/10.1111/hae.14195