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

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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; r² = .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
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. 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. 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 inherited disorder of the connective tissues. According to the current classification, 13 different clinical subtypes are defined. 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), while variants in COL3A1 are associated with vascular EDS (vEDS). Variants in COL1A1 are primarily seen in arthrochlasia EDS (aEDS) but are also rarely causative of vEDS and cEDS. A propensity to bleeding diathesis is a common symptom in most EDS subtypes. The most prevalent subtypes associated with bleeding diathesis are cEDS and vEDS. 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. 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).

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). 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. 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. Severe deficiency of AA is known to cause scurvy, with symptoms including impaired wound healing and bleeding diathesis, due to decreased collagen synthesis. 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. 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). 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. 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. A positive ISTH-BAT score is defined as ≥4 in adult males and ≥6 in adult females.
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. 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. Allele frequencies were derived from gnomAD v2.1.1 (https://gnomad.broadinstitute.org). 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.

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.

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 MMP-2, MMP-9 and MMP-13-derived fragment of type I collagen (C1 M), by the MMP-9-derived fragment of type III collagen (C3 M) and by the MMP-2 and MMP-9-derived fragment of type V collagen (C5 M). As a measure of tissue balance, ratios were calculated as follows: PRO-C1/C1 M, PRO-C3/C3 M and PRO-C5/C5 M. 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. 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.

2.8 | Statistical analyses

Numerical data are presented as medians with lower and upper quartiles (Q1 – Q3), 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, Q1 – Q3), 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>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>ISTH-BAT</th>
<th>Family history</th>
<th>Platelet count (x10^9/L)</th>
<th>Collagen-related variants</th>
<th>Allele frequency</th>
<th>Hypermobility</th>
<th>Ascorbic acid (µM)</th>
</tr>
</thead>
</table>
| P1      | 21  | F   | 5         | Yes            | 282                      | COL3A1: c.2035G>A, p.Ala679 Thr  
COL1A1: c.436C>A, p.Pro146 Thr | 1.016% 0.0002886% | 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% 0.0002886% | No | 13.2 b |
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 |
COL5A1: c.278C>T, p.Ala93Val | 0.1533% 0.07447% | Yes | 62.8 |
| P8      | 23  | F   | 7 d       | No             | 219                      | COL1A1: c.366SA>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 |
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.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 | 1.0000% | No | 60.4 |
| P17     | 42  | F   | 10 d      | Yes            | 122 d                    | COL5A1: c.1796C>T, p.Pro599Leu  
COL5A1: c.5 T > C, p.Met2 Thr | 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>T, p.Thr1230Arg | 0.9074% 0.8250% | No | 81.5 |
| P20     | 42  | F   | 17 d      | Yes            | 232                      | COL3A1: c.1337G>A, p.Arg446His | 0.0000% | Yes | 55.0 |
| P21     | 51  | F   | 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)
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 \( \text{COL1A1} \), \( \text{COL3A1} \), \( \text{COL5A1} \) or \( \text{COL5A2} \) was found in all 31 patients (Table 1). Patient P2 was found to be homozygous for a variant in \( \text{COL3A1} \). In total, 28 unique variants in collagen-related genes were found (\( \text{COL1A1} \), \( n = 5; \text{COL3A1} \), \( n = 7; \text{COL5A1} \), \( n = 12; \text{COL5A2} \), \( n = 4 \)). All variants were initially classified as VUS. Due to the continuously increasing information regarding population allele frequencies, two variants (\( \text{COL3A1} \): c.2035G>A, p.Ala679 Thr and \( \text{COL5A1} \): 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.

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
affect the associated collagen type (e.g., 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).

**3.6 | Ascorbic acid**

The mean level of plasma AA in the patients was $58.3 \pm 19.4$ 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 and 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,
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. 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. 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. Ascorbic acid supplements are recommended in patients with cEDS, based on expert opinions. 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. 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.

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.
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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.

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REFERENCES


**SUPPORTING INFORMATION**

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

Table S1

Table S2

Supplementary Material

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