A laboratory micro-CT technique is useful to visualize and characterize dermal skin components in a 3D manner

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Received August 24, 2025; Accepted February 18, 2026

DOI: 10.3892/etm.2026.13116

Abstract. Bowel and skin biopsies from patients with gastrointestinal disorders have revealed neuropathic changes and altered connective tissue. Patients with postural orthostatic tachycardia syndrome (POTS) often suffer from gastrointestinal symptoms and concomitant hypermobility spectrum disorders, such as hypermobile Ehlers-Danlos syndrome (hEDS). Since the skin is more accessible than the bowel, the aim of the present study was to evaluate skin biopsies in a 3D manner using micro-CT in patients with POTS and controls and relate the findings to symptoms presented. Healthy controls (n=13) and patients with POTS with (n=11) or without hEDS/EDS (n=26) were evaluated. Skin biopsies were taken proximally to the lateral malleolus using a 3 mm needle, fixed in formaldehyde and embedded in paraffin. The samples were harvested using a 1.5 mm punch (length, 2-5 mm) and scanned using a laboratory X-ray phase-contrast micro-CT. Scans were evaluated in a blinded manner and the regularity, thickness and tightness of collagen fiber bundles were assessed. All dermal structures were visible without staining. Intraepidermal nerves were not visible and a number of cell types could not be separated. The percentage of disorganized collagen bundles differed between groups, due to the majority being disorganized in hEDS/EDS (P=0.030). The proportion of any disorganized and parallel bundles throughout the biopsy differed within both patient groups (P<0.001) but not within the control group (P=0.175). There were no differences in symptoms between participants with disorganized bundles and participants without disorganized bundles. In conclusion, X-ray phase-contrast micro-CT was suitable to visualize and characterize dermal skin components in 3D. Patients with POTS and hEDS/EDS exhibited more disorganized collagen bundles; however, the technique cannot currently be used for diagnostic purposes until more patients are examined.

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Key words: collagen fiber bundles, hypermobile Ehler-Danlos syndrome, X-ray phase-contrast micro-CT, postural orthostatic tachycardia syndrome, 3D

Introduction

Postural orthostatic tachycardia syndrome (POTS) is a disease defined by symptoms of orthostatic intolerance and a heart rate increase of >30 bpm from a recumbent to a standing position or >120 bpm in a standing position in the absence of orthostatic hypotension (1,2). The etiology and pathophysiology of POTS are unknown; however, signs of autonomic neuropathy, such as orthostatic intolerance, pre-syncope and palpitations, and small fiber neuropathy with intraepidermal nerve fiber density less than the 5th percentile of normative values, have previously been described (3).

Ehlers-Danlos syndrome (EDS) comprises 13 heritable connective tissue disorders and is characterized by joint hypermobility, skin hyperextensibility and tissue fragility, among other clinical features (4). The classification and diagnosis of EDS is based upon clinical examination findings; however, a definite diagnosis is primarily made based upon molecular determination, except in cases of hypermobile EDS (hEDS), whereby the genetic variants remain unknown (5). Furthermore, in patients with symptomatic joint hypermobility who do not completely fulfill the criteria for EDS or hEDS, the term hypermobility spectrum disorder (HSD) is used (5,6). Genetically, EDS is heterogenous with varying mutations of protein-coding genes, including genes coding for collagen (5). Histopathologically, EDS is characterized by disorganized collagen fibers and fiber bundles (7,8). HSD and hEDS are more frequent in patients with POTS compared with the general population (9,10).

Gastrointestinal symptoms in POTS and HSD/hEDS occur frequently, both as functional gastrointestinal disorders and as more severe intestinal dysmotility disorders (10-12). Dysmotility may be due to pathology in the nervous system, muscles and/or connective tissue, (13) and diagnosing severe gastrointestinal dysmotility is challenging, as gastrointestinal motility varies widely among healthy individuals, with full-thickness bowel biopsies only occasionally being performed (11,14). Neuropathy is often generalized (15) and thus, biopsy sampling for diagnostic purposes should be prioritized in easily accessible organs, such as the skin. Our previous studies have reported that phase-contrast tomographic imaging with 3D illustration could reveal disorganized dermal collagen fiber bundles in a patient with POTS and hEDS and severe intestinal dysmotility, who also exhibited a reduced intraepidermal nerve fiber density (16,17). An additional patient with dysmotility exhibited an increased amount of inter-fibrillar ground substance (17). Notably, Histomography® GmbH has developed a laboratory-based micro-CT system, specifically optimized for paraffin-embedded biopsy cores. This technique enables detailed 3D structural analysis of soft tissue samples (18,19).

Therefore, the primary aim of the present study was to examine skin biopsies using virtual 3D histology to evaluate the suitability of the method to describe the dermal architecture and any presence of nerve fibers in a series of patients with POTS, with or without hEDS/EDS, and in healthy controls. The secondary aim was to relate the obtained dermal findings to clinical data and gastrointestinal symptoms.

Materials and methods

Recruitment of participants and skin biopsy procedure. A previously established cohort of 43 patients with POTS and 61 healthy controls without gastrointestinal symptoms was recruited from Skåne University Hospital (Lund, Sweden) between October 2020 and January 2022 (20). From this cohort, 38 patients and 13 healthy controls underwent skin biopsy sampling. POTS was diagnosed based on cardiovascular autonomic tests with tilt testing and continuous hemodynamic monitoring prior to study inclusion (21). The inclusion criteria were an age of 18-70 years with a diagnosis of POTS, ability to fully understand the study information and a home base within a reasonable proximity to Malmö, Sweden. Exclusion criteria were severe somatic comorbidity or mental illness and alcohol and drug abuse.

Among the patients with POTS, 12 patients also exhibited concomitant hEDS/EDS, which was diagnosed based on clinical examination by a physiotherapist or physician, without any genetic characterization. After study inclusion, their medical records were scrutinized and the types of diagnostic tests used, classification of subtypes and concomitant diagnoses were recorded.

A total of two skin biopsies were taken from non-lesional skin 10 cm proximally to the lateral malleolus as a 3 mm punch biopsy during local subcutaneous anesthesia (0.5 ml Carbocain®; 10 mg/ml). The skin defects were closed with one 4-0 suture each and covered by a small dressing allowing for free physical activities. Biopsy samples were immediately fixed in 4% buffered formaldehyde for ≥24 h at room temperature before dehydration in alcohol and paraffin-embedding according to clinical routines in the accredited hospital laboratory (16). Furthermore, one sample from each participant was then harvested for 3D imaging with a 1.5 mm punch using a dissection microscope and placed into a Kapton® tube (Paramount Tube), with a biopsy length of 2-5 mm. All other paraffin-embedded blocks were sectioned at 5 μm and immunohistochemically stained with a rabbit polyclonal protein gene product 9.5 (PGP9.5) antibody (cat. no. 318A-1; dilution, 1:3,000; Cell Marque™), according to the manufacturer's protocol. The biopsies were examined in a Sectra IDS7 workstation (Sectra AB), where the scanned tissue sections could be viewed and examined microscopically according to clinical routines (22).

Virtual 3D histology. For 3D imaging, the prepared tissue samples were sent to Histomography® GmbH. The analysis was conducted using X-rays in a laboratory micro-CT system specifically optimized for small formalin-fixed, paraffin-embedded core biopsies with a diameter of 1.5 mm. Imaging of soft tissue with X-rays presents a marked challenge due to its low X-ray absorption, especially compared with established applications in denser materials such as bone or metal (23). Exploiting the self-interference of partially coherent X-rays from laboratory X-ray sources offers a viable approach to address this issue. As the X-rays pass through the sample, minimal density variations induce phase shifts in the radiation. These phase shifts create interference patterns, which manifest on the detector image (as enhanced edge contrasts). Phase-contrast imaging was combined with advanced phase-retrieval algorithms and iterative tomographic reconstruction methods to markedly enhance image contrast, making it suitable for visualizing fine structures within soft tissues (18,19). The resulting 3D volumes had an isotropic voxel size of 840 nm. With a dynamic range of 16 bits, each dataset was ~10 GB in size, enabling detailed inspection of 3D tissue morphology. The datasets were subsequently provided through a browser-based volumetric viewer solution developed by Histomography® GmbH. The viewer enables interactive visualization of large 3D datasets and allows users to stream and interactively explore the data, including inspecting virtual slices of the 3D volume from numerous orientations and measurement of tissue structures in 3D, facilitating a comprehensive examination of tissue architecture (www.histomography.com).

Histopathological evaluation. Only one sample from a patient with POTS with hEDS was excluded as the final punching step of the tissue sample failed. All samples were pseudonymized and evaluated blinded to the examinator in a semi-quantitative manner by two independent examiners. After evaluation, biopsies that were differently evaluated by the two examiners (n=5) were discussed and a consensus was reached concerning the classification of the biopsies. The overall histology of the samples was studied to identify the different layers and structures. Each skin layer was clearly visible, including the horn layer, squamous epithelium, dermo-epidermal junction, adnexal structure in the dermal layer and subcutaneous fat (Fig. 1). Any presence of intraepidermal nerve fibers was examined.

The collagen fiber bundles in the dermal skin tissue were evaluated in 3D throughout the biopsy. The collagen arrangement was assessed qualitatively by visual inspection by trained pathologists and no quantitative metrics were applied. The degree of parallel or disorganized collagen fiber bundles was evaluated in the dermis and categorized into three groups:

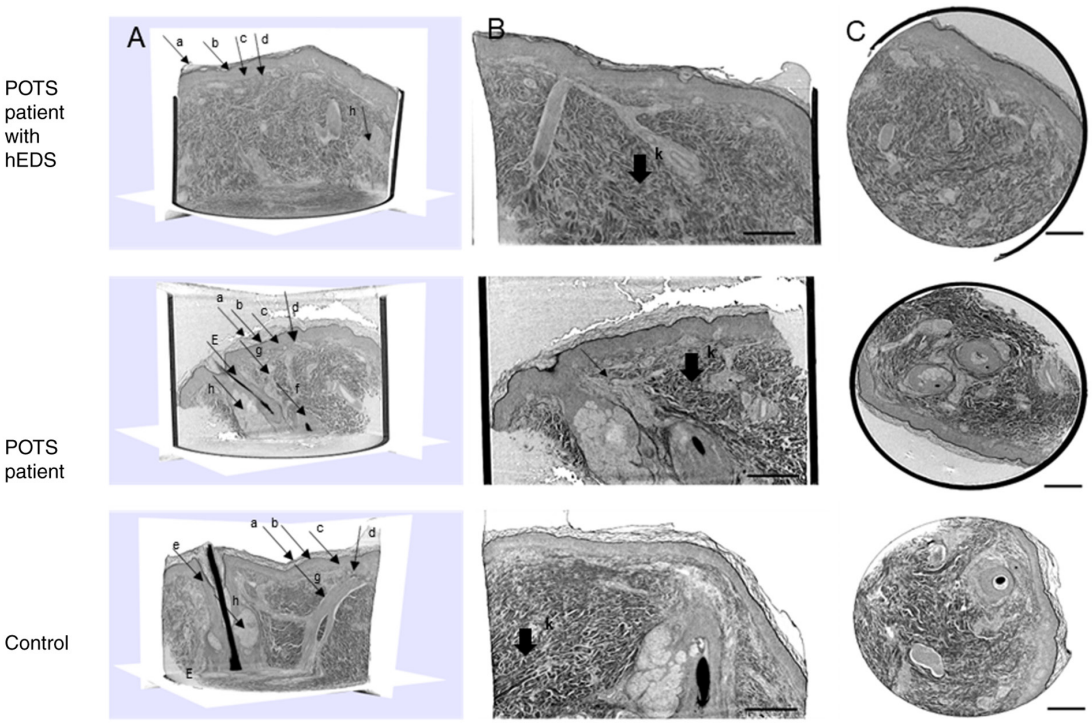

Figure 1. Skin tissue punch biopsy from patients with POTS, with and without hEDS, and 1 control, showing a scanned vertical overview. (A) Overview of skin biopsies. (B) The same scans with higher magnification illustrating the disorganized collagen fiber bundles throughout the biopsy in the patients with and without hEDS and more organized parallel fiber bundles throughout the biopsy in the control. (C) A transversal cross-section of the data set. Scale bars, 250 μm. a, stratum corneum; b, stratified granulosum; c, stratum spinosum; d, dermo epidermal junction; e, hair follicle with hair shaft; f, follicle bulb; g, arrector pili; h, sebaceous gland; k, collagen fiber bundles. hEDS, hypermobile Ehler-Danlos syndrome; POTS, postural orthostatic tachycardia syndrome.

i) Parallel bundles throughout the biopsy; ii) parallel bundles superficially and disorganized bundles in deeper layers; or iii) disorganized bundles throughout the biopsy. Furthermore, the thickness of collagen fiber bundles and presence of loosely organized fiber bundles with more ground substance between the bundles was estimated. Fiber bundles were subjectively divided into two categories: Thick or thin bundles; and loosely or tightly organized bundles. After the examination was finished, the code list of pseudo-anonymized samples was opened, and samples could be related to the participant, to identify the correct group identity.

Questionnaires. All study participants were asked to complete a questionnaire regarding previous and current illnesses, family history and current pharmacological treatment. Furthermore, the validated visual analog scale for irritable bowel syndrome (VAS-IBS) was used to estimate the influence of abdominal pain, diarrhea, constipation, bloating and flatulence, vomiting and nausea, psychological well-being and intestinal symptoms on daily life using scales from 0-100 mm (whereby 0 mm represents no symptoms, and 100 mm represents severe symptoms). The scales were inverted from their original format (24). Reference values are available from healthy controls (25). The validated IBS-severity scoring system (IBS-SSS) was used to estimate abdominal pain, abdominal distension, satisfaction with bowel habits and the impact of bowel habits on daily life using visual analog scales (VASs) ranging from absent (0 mm) to very severe (100 mm) symptoms. The number of days with abdominal pain in the last 10 days was reported. The overall maximum achievable score was 500. Scores ranging between 75-174 indicated mild disease, scores between 175-299 indicated moderate disease and scores ≥300 indicated severe disease. Extraintestinal symptoms (nausea, difficulties eating a whole meal, headache, back pain, fatigue, belching/excess wind, reflux, urinary urgency, leg pain and muscle/joint pain) were also estimated on VASs with a maximal achievable score of 500 (26).

Statistical analysis. Statistical analyses were performed in SPSS (version 29; IBM Corp.). Differences between groups were compared using the non-parametric Mann-Whitney U test and Fisher's exact tests. P-values adjusted with Bonferroni correction due to multiple tests were given B values and were taken as the main result. A one-sample test was used to calculate differences in proportions of collagen bundles within each group of participants. Values are presented as the median (interquartile range) and number (percentage). P (or B when applicable) <0.05 was considered to indicate a statistically significant difference.

Results

Basal characteristics. All patients with POTS were divided into patients without hEDS/EDS (n=26) and patients with concomitant hEDS/EDS (n=11) groups for separate comparisons with the controls (n=13). Of the patients with concomitant hEDS/EDS, 6 patients were diagnosed with hEDS and 5 patients were diagnosed with unspecific EDS. Healthy controls had a higher education level, had less sick leave, drank more alcohol and were less physically active compared with POTS patients without hEDS/EDS (Table I).

Table I. Basal characteristics and symptoms in healthy controls compared with patients with POTS with or without concomitant hEDS/EDS.

Characteristic	Healthy controls (n=13)	POTS (n=26)	POTS with hEDS/ EDS (n=11)	P/B-value[a]	P/B-value[b]
Age, years	39 (36-41)	31 (27-44)	28 (28-40)	0.216/0.432	0.045/0.090
Sex, n (%)				1.000/1.000	0.482/0.964
Female	11 (84.6)	23 (88.5)	11 (100.0)		
Male	2 (15.4)	3 (11.5)	0 (0.0)		
BMI, kg/m^2	21.7 (20.6-24.0)	24.6 (21.3-26.6)	24.1 (21.6-29.6)	0.188/0.376	0.167/0.334
Education, n (%)				0.009/0.018	0.018/0.036
Primary school		2 (7.7)	1 (9.1)		
College		8 (30.8)	4 (36.4)		
≥1 more year of education	1 (7.7)	6 (23.1)	1 (9.1)		
University	12 (92.3)	10 (38.5)	5 (45.5)		
Occupation, n (%)				<0.001/0.002	0.002/0.004
Working 100%	8 (61.5)	4 (15.4)	1 (9.1)		
Working 99-51%	5 (38.5)	3 (11.5)	2 (18.2)		
Sick leave		13 (50.0)	5 (45.5)		
Unemployment		2 (7.7)	1 (9.1)		
Studying		4 (15.4)	2 (18.2)		
Marital status, n (%)				0.276/0.552	0.033/0.066
Living alone	2 (15.4)	9 (34.6)	7 (63.6)		
Married/living together	11 (84.6)	17 (65.4)	4 (36.4)		
Smoking, n (%)				0.557/1.000	0.845/1.000
Regularly	1 (7.7)	3 (11.5)	2 (18.2)		
Former smoker	3 (23.1)	2 (7.7)	2 (18.2)		
Never smoker	9 (69.2)	21 (80.8)	7 (63.6)		
Glasses of alcohol/week[c], n (%)				<0.001/0.002	<0.001/0.002
<1 glass	1 (7.7)	22 (84.6)	10 (90.9)		
1-4 glasses	10 (76.9)	4 (15.4)			
5-9 glasses	2 (15.4)		1 (9.1)		
Physical activity/week[d], n (%)				0.010/0.020	0.927/1.000
No time at all		6 (23.1)	1 (9.1)		
<30 min	2 (15.4)	2 (7.7)	3 (27.3)		
30-60 min	6 (46.2)	3 (11.5)	4 (36.4)		
60-90 min	2 (15.4)	10 (38.5)	1 (9.1)		
90-120 min	2 (15.4)		2 (18.2)		
>120 min	1 (7.7)	5 (19.2)			
Gastrointestinal symptom[e]					
Abdominal pain	0 (0-0)	30 (6-54)	63 (17-77)	<0.001/0.002	<0.001/0.002
Diarrhea	0 (0-0)	10 (0-61)	51 (0-72)	<0.001/0.002	0.003/0.006
Constipation	0 (0-16)	24 (2-72)	73 (34-91)	0.006/0.012	<0.001/0.002
Bloating and flatulence	3 (0-16)	73 (15-88)	62 (25-90)	<0.001/0.002	0.009/0.018
Vomiting and nausea	0 (0-6)	36 (12-69)	48 (30-70)	<0.001/0.002	<0.001/0.002
Intestinal symptoms' influence on daily life	0 (0-2)	49 (18-74)	74 (19-85)	<0.001/0.002	<0.001/0.002
Psychological well-being	0 (0-32)	48 (12-60)	57 (35-79)	0.008/0.016	0.001/0.002
Difficulties eating a meal	0 (0-0)	25 (0-52)	55 (5-79)	<0.001/0.002	<0.001/0.002
Headache	7 (2-24)	78 (44-94)	67 (48-80)	<0.001/0.002	<0.001/0.002
Back pain	7 (0-28)	50 (13-77)	50 (35-83)	0.003/0.006	<0.001/0.002

Table I. Continued.

Characteristic	Healthy controls (n=13)	POTS (n=26)	POTS with hEDS/ EDS (n=11)	P/B-value[a]	P/B-value[b]
Fatigue	10 (0-22)	94 (79-100)	92 (85-100)	<0.001/0.002	<0.001/0.002
Belching/excess wind	0 (0-2)	55 (22-89)	71 (36-81)	<0.001/0.002	<0.001/0.002
Reflux	0 (0-0)	17 (2-58)	59 (23-71)	<0.001/0.002	<0.001/0.002
Urinary urgency	0 (0-3)	54 (8-85)	63 (23-82)	<0.001/0.002	<0.001/0.002
Leg pain	0 (0-0)	8 (2-61)	44 (0-51)	<0.001/0.002	<0.001/0.002
Muscle/joint pain	0 (0-6)	68 (15-84)	90 (65-97)	<0.001/0.002	<0.001/0.002
Total IBS-SSS	8 (0-41)	204 (96-297)	288 (139-409)	<0.001/0.002	<0.001/0.002
Total extraintestinal IBS-SSS	38 (32-86)	485 (344-633)	675 (473-708)	<0.001/0.002	<0.001/0.002

Values are presented as n (%) or median (interquartile range) and analyzed using Mann-Whitney U tests or Fisher's exact tests. [a]Comparisons between controls and POTS. [b]Comparisons between controls and POTS with hEDS/EDS. [c]'Glasses' of alcohol represent standard glasses. [d]'Physical' denotes the degree of physical activity that led to shortness of breath. [e]Specific gastrointestinal symptoms were assessed by visual analog scale for IBS and specific extraintestinal symptoms, total gastrointestinal symptoms and total extraintestinal symptoms were assessed by IBS-SSS (24,26). P-values were adjusted for Bonferroni correction. P/B<0.05 were considered to indicate a statistically significant difference. EDS, Ehler-Danlos syndrome; hEDS, hypermobile EDS; POTS, postural orthostatic tachycardia syndrome; IBS, irritable bowel syndrome; IBS-SSS, IBS-severity scoring system.

Comorbidities were frequent among these patients with POTS, whereby irritable bowel syndrome was most frequently reported (n=7), followed by asthma (n=6), thyroid disorders (n=5), migraine (n=4), myalgic encephalomyelitis (n=3), dyspepsia (n=2), gastroparesis (n=2), fibromyalgia (n=2), post-coronavirus disease 19 (n=2), mast cell activation syndrome (n=2) and inappropriate sinus tachycardia (n=2) (data not shown). All specific gastrointestinal symptoms and extraintestinal symptoms, as well as total scores, were higher in patients with POTS without hEDS/EDS compared with controls, with B=0.002, except for constipation (B=0.012), psychological well-being (B=0.016) and back pain (B=0.006; Table I).

Upon comparison of healthy controls and patients with POTS with concomitant hEDS/EDS, control patients tended to be older and more often married or living together, with a higher education level and less sick leave taken. The controls also drank more alcohol compared with all other patients (Table I). Apart from hEDS/EDS, the patients also suffered from one or more autism spectrum disorders (n=4), myalgic encephalomyelitis (n=3), anxiety (n=2), depression (n=2), endometriosis (n=2), fibromyalgia (n=2), gastrointestinal dysmotility (n=2), sleeping disturbances (n=2), migraine (n=1) and mast cell activation syndrome (n=1) (data not shown). All specific gastrointestinal symptoms and extraintestinal symptoms, as well as total scores, were higher in patients with hEDS/EDS compared with controls, with B=0.002, except for diarrhea (B=0.006) and bloating and flatulence (B=0.018; Table I). There were no statistically significant differences in symptoms between patients with POTS with and without concomitant hEDS/EDS (data not shown).

Histopathology. Through the user-friendly and easy to navigate browser-based tool, provided by Histomography® GmbH, skin layers in 3D were scrutinized. The magnification gave a clear overview of the various tissue components in the skin biopsy (Fig. 1). The three layers epidermis, dermis and subcutis were studied in a vertical view. In the epidermis, the different layers stratum corneum, stratum granulosum, stratum spinosum and stratum basales could be separated. The dermo-epidermal junction appeared rather distinct. In the dermal layer, collagen bundles and hair follicles with hair shaft together with adnexal structures such as arrector pili and sebaceous glands were visible (Fig. 1A). There were no differences in adnexal structures between the patients and controls. Disorganized collagen fiber bundles were visualized in 2 patients with and without hEDS, in contrast to the organized parallel fiber bundles in a healthy participant (Fig. 1A-C). The same samples were also visualized at different transversal depths (Figs. 2-4). The magnification was not sufficient to separate varying cell types and for evaluation of intracellular changes in the skin. Neither was it possible to identify any intraepidermal nerve fibers.

Conventional immunohistochemistry using a PGP9.5 antibody was conducted and a representative image is shown for comparison with the micro-CT from 2 patients with POTS, 1 with hEDS and 1 without hEDs, and 1 healthy control, which demonstrated the different layers of the skin and adnexal structures (Fig. 5).

Collagen fiber bundles. Table II shows the distribution of the three categories of collagen fiber bundles: i) Parallel collagen fiber bundles throughout the biopsy; ii) superficial parallel fiber bundles with disorganized fiber bundles in deeper layers; and iii) disorganized fiber bundles throughout the biopsy. The number of participants with disorganized bundles tended to differ among the three groups of participants (controls, patients with POTS and patients with POTS and hEDS/EDS; P=0.056), due to primarily disorganized bundles exhibited

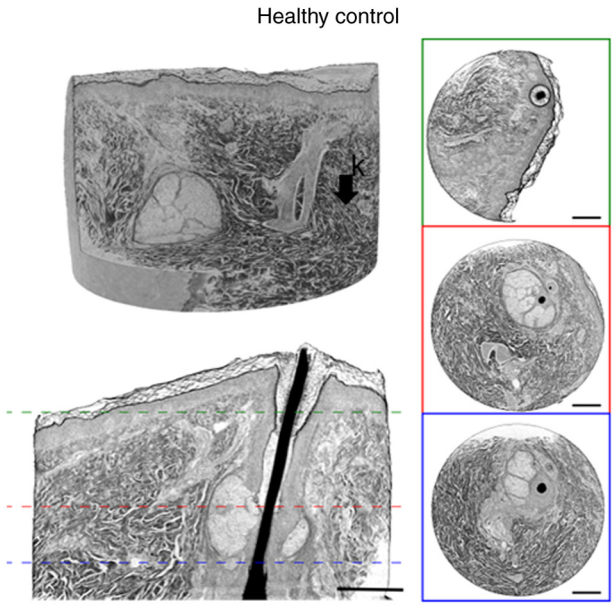

Figure 2. Skin biopsy from a healthy control participant. A control with organized collagen bundles throughout the skin biopsy. Vertical overview and transversal views at three different depths (marked in green, red and blue). k, organized collagen fiber bundles. Scale bars, 250 μm.

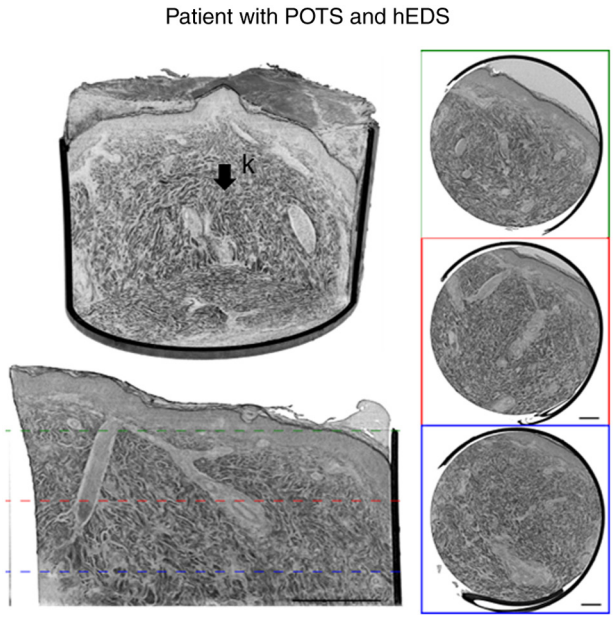

Figure 4. Skin biopsy from a patient with POTS and hEDS. Vertical overview and transversal views at three different depths (marked in green, red and blue). k, disorganized collagen fiber bundles. Scale bars, 250 μm. hEDS, hypermobile Ehler-Danlos syndrome; POTS, postural orthostatic tachycardia syndrome.

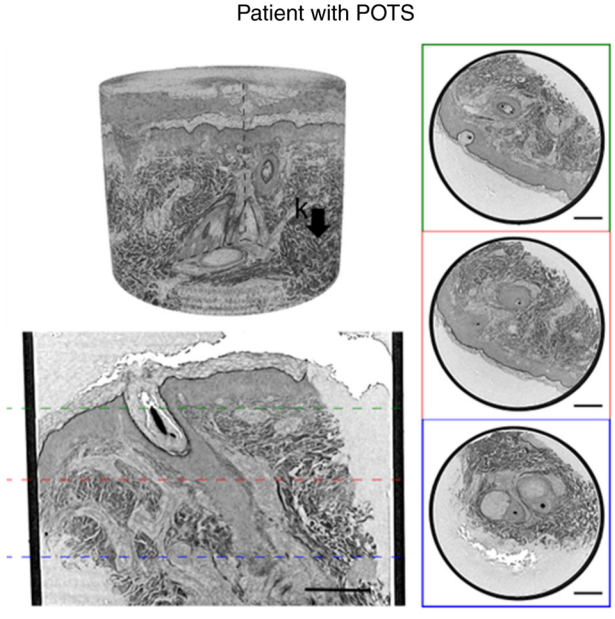

Figure 3. Skin biopsy from a patient with POTS. A patient with disorganized collagen bundles throughout the skin biopsy. Vertical overview and transversal views at three different depths (marked in green, red and blue). k, disorganized collagen fiber bundles. Scale bars, 250 μm. POTS, postural orthostatic tachycardia syndrome.

by the hEDS/EDS group. The number of patients in the three fiber organization categories tended to differ between patients with only POTS and patients with POTS and hEDS/EDS (B=0.057).

There was a significant difference among the three groups in the number of patients with parallel bundles throughout the biopsy or at least superficially (categories 1 and 2) compared with complete disorganized bundles (category 3) (P=0.030). Healthy controls and patients with POTS exhibited a more similar bundle distribution compared with patients with hEDS/EDS, with a significant difference between patients with only POTS and patients with POTS and hEDS/EDS (B=0.039; Table II).

One-sample test was used to calculate differences in proportions of different collagen bundles within each study group. The proportion of controls with complete disorganized bundles (category 3) did not differ compared with the proportion of controls with partly or complete parallel bundles (38.5 vs. 61.5%; P=0.427). Neither was there any difference between the proportion of any disorganized bundles (categories 2 and 3) and parallel bundles throughout the biopsy (69.2 vs. 30.8%; P=0.175). Within the POTS group without hEDS/EDS, the proportion of patients with complete disorganized bundles did not differ from the proportion of patients with parallel bundles at least superficially (34.6 vs. 65.4%; P=0.118), however proportions differed between patients with any disorganized bundles and patients with parallel bundles throughout the biopsy (84.6 vs. 15.4%; P<0.001). In patients with POTS and hEDS/EDS, the proportion of patients with complete disorganized bundles differed significantly from the proportion of patients with parallel bundles at least superficially (81.8 vs. 18.2%; P=0.026). The difference was more pronounced when comparing the proportions of patients with any disorganized bundles and patients with parallel bundles throughout the biopsy (90.9 vs. 9.1%; P<0.001). There were no differences in terms of the thickness of the bundles (P=1.000) or the structure of tight or loose bundles (P=0.872) between the groups (data not shown).

Clinical features stratified by fiber bundle structure. Participants with parallel fiber bundles at least in the superficial layers (n=27) did not differ in sociodemographic factors

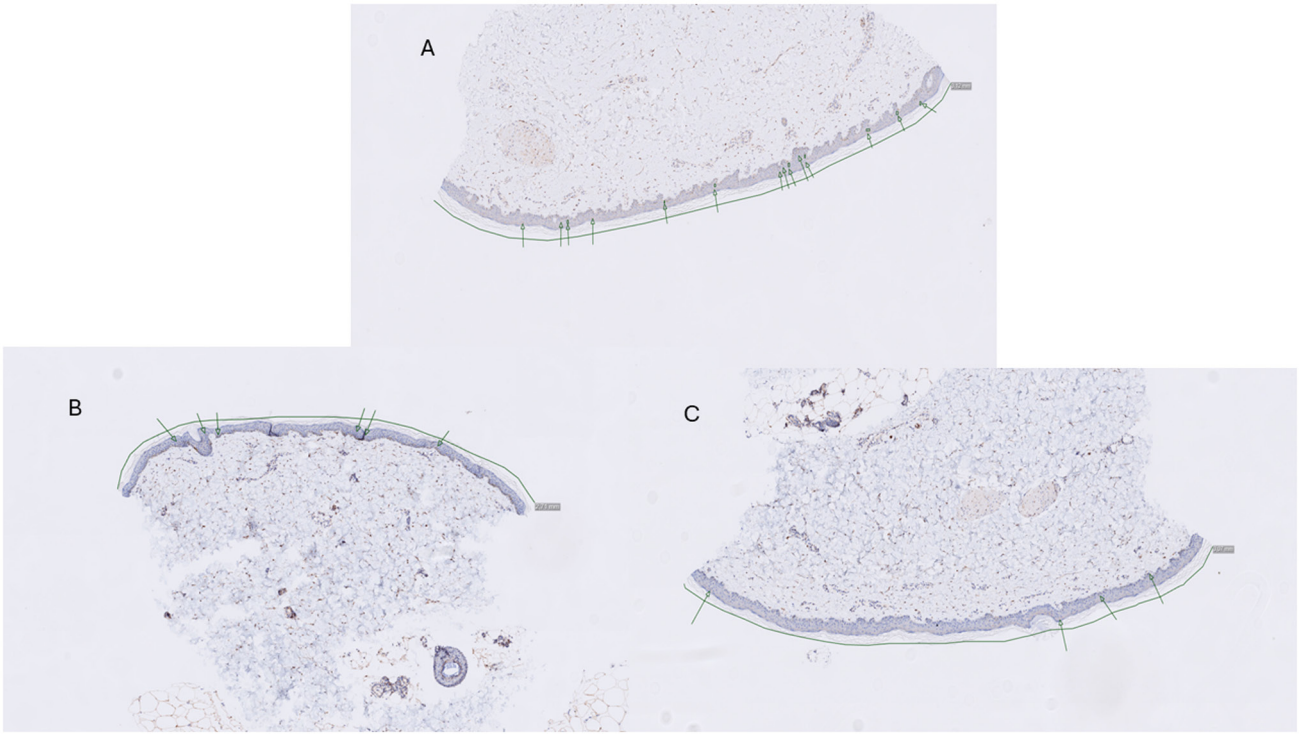

Figure 5. Immunohistochemistry of skin biopsy. Representative image from (A) healthy participant, (B) patient with POTS and (C) patient with POTS and hEDS. Skin with small intraepidermal nerves (arrows) in a 5 μm skin section shown through protein gene product 9.5 staining. Magnification, x50. hEDS, hypermobile Ehler-Danlos syndrome; POTS, postural orthostatic tachycardia syndrome.

or lifestyle habits compared with those with disorganized fiber bundles (n=23). Although participants with disorganized bundles exhibited more symptoms of, fpr example, abdominal pain, constipation, headache, muscle/joint pain, total IBS-SSS and total extraintestinal IBS-SSS, the difference between groups did not reach statistical significance (Table III).

Participants with parallel fiber bundles throughout the biopsy were significantly older compared with the remaining participants with disorganized fiber bundles at least in deeper layers (P=0.006). Furthermore, participants with disorganized bundles exhibited significantly more pronounced symptoms of muscle and joint pain compared with those with parallel bundles throughout the biopsy (P=0.031). No other parameters reached statistical significance (Table IV).

Discussion

Within the present study, the main findings indicated that the structures of the different layers in the skin could be visualized by 3D virtual histology using X-ray phase-contrast micro-CT. No intraepidermal nerve fibers could be visualized. The percentage of disorganized collagen fiber bundles was similar between healthy controls and patients with POTS but different in POTS with hEDS/EDS and the percentage of disorganized fibers in patients with concomitant hEDS/EDS differed from that in patients with only POTS. Furthermore, the proportion of participants with any disorganized collagen bundles and parallel bundles throughout the biopsy differed within both patient groups but not within the control group. However, a number of clinical differences were observed between participants with parallel and disorganized collagen fiber bundles.

The first EDS classification in 1998 assumed that all six EDS subtypes (or the majority of) were a consequence of alterations in fibrillar collagen genes or genes encoding collagen modifiers (27). Since then, numerous new EDS subtypes have been described (4,5). Using next-generation sequencing technologies, further mutations have been identified in genes not directly involved in collagen biosynthesis and/or structure, leading to the revised classification system with 13 EDS subtypes (5). Due to the vast genetic heterogeneity and phenotype variability of EDS subtypes, the definite diagnosis must rely on molecular determination of causative variants to optimize treatment and research purposes (5). The present patients were not examined by genotyping, as it cannot currently be used to diagnose hEDS, since genetic variants are unknown (5). Theoretically, the present patients with POTS with EDS and normal collagen bundles may have also had subtypes of EDS involving molecular components other than collagen or collagen-regulating factors.

Diagnoses based on subjective symptom reports and clinical examination may be difficult to evaluate and only those clinicians with specialist knowledge of in HSD, including EDS, may be able to correctly diagnose EDS according to the established guidelines (5). Therefore, histopathological findings may improve the ability to make a diagnosis and separate different entities, such as functional disorders and EDS. Full-thickness biopsies of the bowel to diagnose dysmotility are only chosen in selected cases, since they are performed under anesthesia which may be complicated by bowel rupture with severe complications (11). Histopathologically, EDS is characterized by insufficient contexture of the collagen elements in the cutis, subcutis and joint capsules (7). Despite disorderly

Table II. Prevalence of parallel or disorganized collagen fiber bundles in healthy controls compared with POTS patients with or without hEDS/EDS.

Collagen fibers	Healthy controls (n=13)	POTS (n=26)	hEDS/EDS (n=11)	P-value	P/B-value[a]	P/B-value[b]	P/B-value[c]
Bundles divided into three categories				0.056	0.461/ 1.000	0.144/ 0.432	0.019/ 0.057
Category 1, parallel bundles throughout the biopsy	4 (30.8)	4 (15.4)	1 (9.1)				
Category 2, parallel bundles superficially and disorganized deeply	4 (30.8)	13 (50.0)	1 (9.1)				
Category 3, disorganized bundles throughout the biopsy	5 (38.5)	9 (34.6)	9 (81.8)				
Bundles divided into two categories				0.030	1.000/ 1.000	0.047/ 0.141	0.013/ 0.039
Parallel bundles, at least superficially (categories 1 + 2)	8 (61.5)	17 (65.4)	2 (18.2)				
Disorganized bundles throughout the biopsy (category 3)	5 (38.5)	9 (34.6)	9 (81.8)				
Bundles divided into two categories				0.384			
Parallel bundles throughout the biopsy (category 1)	4 (30.8)	4 (15.4)	1 (9.1)				
Disorganized bundles, at least deeply (categories 2 and 3)	9 (69.2)	22 (84.6)	10 (90.9)				

Values presented as n (%) and analyzed using Fisher's exact test. P-values indicate comparisons of all three groups. [a]P/B indicates comparison of healthy controls with POTS. [b]P/B indicates comparison of healthy controls with POTS with hEDS/EDS. [c]P/B indicates comparison of POTS with or without hEDS/EDS. P-values were adjusted for Bonferroni correction. P/B<0.05 was considered to indicate a statistically significant difference. EDS, Ehlers-Danlos syndrome; hEDS, hypermobile EDS; POTS, postural orthostatic tachycardia syndrome.

arranged collagen bundles being a typical characteristic, there may be large interindividual differences in the magnitude of the ultrastructural changes (28). Electron microscopy (EM) is needed to study the structure of collagen fibrils, however the irregularity of collagen fibers and bundles is also observed using light microscopy (7,8). A number of stains may be used to visualize collagen and fibroblasts (8), yet in EM, there is no specific stain for this application. The validation of dermal structures using micro-CT compared with conventional staining was performed and described in our previous study, whereby a PGP9.5 image was used to determine the presence of nerve fibers (17). In the present study of POTS, an antibody to PGP9.5 was used to stain neural fibers, but other dermal structures were also visible. There were differences in the structure of collagen bundles between the different study groups, but nothing that may currently be used diagnostically due to the small group sizes.

The present results were in alignment with the previous observation in a patient with POTS and concomitant hEDS (17). Results indicated that 3D scanning without any staining is suitable to visualize and describe collagen fiber bundles. The image quality of the laboratory micro-CT was comparable to previously published data obtained at a synchrotron radiation facility in parallel beam geometry at a voxel size of 650 nm (17). This demonstrated that laboratory setups provided a viable alternative to synchrotron facilities for studying skin structure. Due to their accessibility and ease of use, laboratory-based systems may enable broader availability and facilitate long-term, multi-site studies in the future. Since the connective tissue is present in the whole body, 3D scanning of a greater area in unstained skin biopsies may give a relevant illustration of the tissue and could improve the diagnostic accuracy in patients with suspicious connective tissue disorders (17). Skin biopsy is relatively safe, with few mild complications, such as a small risk of local

Table III. Basal characteristics and symptoms depending on parallel, at least superficially or disorganized fiber bundles.

Characteristic	Parallel bundles (n=27)	Disorganized bundles (n=23)	P-value
Age, years	39 (28-45)	32 (28-40)	0.259
Sex, n (%)			0.167
Female	26 (96.3)	19 (82.6)	
Male	1 (3.7)	4 (17.4)	
BMI, kg/m^2	21.7 (20.7-26.1)	23.5 (21.6-26.5)	0.396
Education, n (%)			0.785
Primary school	2 (7.4)	1 (4.3)	
College	5 (18.5)	7 (30.4)	
≥1 more year of education	5 (1.5)	3 (13.0)	
University	15 (55.6)	12 (52.2)	
Occupation, n (%)			0.244
Working 100%	7 (25.9)	6 (26.1)	
Working 99-51%	6 (22.2)	4 (17.4)	
Sick leave	9 (33.3)	9 (39.1)	
Unemployment		3 (13.0)	
Studying	5 (18.5)	1 (4.3)	
Marital status, n (%)			0.559
Living alone	11 (40.7)	7 (30.4)	
Married/living together	16 (59.3)	16 (69.6)	
Smoking, n (%)			0.788
Regularly	3 (11.1)	3 (13.0)	
Former smoker	3 (11.1)	4 (17.4)	
Never smoker	21 (77.8)	16 (69.6)	
Glasses of alcohol/week[a], n (%)			0.571
<1 glass	17 (63.0)	16 (69.6)	
1-4 glasses	9 (33.3)	5 (21.7)	
5-9 glasses	1 (3.7)	2 (8.7)	
Physical activity/week[b], n (%)			0.147
No time at all	3 (11.1)	4 (17.4)	
<30 min	2 (7.4)	5 (21.7)	
30-60 min	6 (22.2)	7 (30.4)	
60-90 min	8 (29.6)	5 (21.7)	
90-120 min	2 (7.4)	2 (8.7)	
>120 min	6 (22.2)		
Gastrointestinal symptom[c]			
Abdominal pain	11 (0-52)	30 (0-62)	0.450
Diarrhea	0 (0-54)	10 (0-64)	0.325
Constipation	15 (0-65)	51 (2-76)	0.160
Bloating and flatulence	50 (1-83)	62 (13-81)	0.435
Vomiting and nausea	31 (0-64)	29 (6-57)	0.797
Intestinal symptoms' influence on daily life	30 (0-74)	38 (13-77)	0.322
Psychological well-being	37 (2-61)	35 (10-57)	0.843
Difficulties eating a meal	5 (0-52)	12 (0-54)	0.722
Headache	38 (9-81)	69 (34-80)	0.285
Back pain	32 (0-71)	40 (9-70)	0.724
Fatigue	84 (20-95)	90 (49-100)	0.190
Belching/excess wind	40 (0-87)	36 (2-76)	0.796
Reflux	6 (0-38)	22 (0-70)	0.461
Urinary urgency	19 (0-82)	23 (6-76)	0.649

Table III. Continued.

Characteristic	Parallel bundles (n=27)	Disorganized bundles (n=23)	P-value
Leg pain	4 (0-51)	2 (0-50)	0.807
Muscle/joint pain	22 (0-84)	56 (19-90)	0.304
Total IBS-SSS	96 (4-286)	188 (42-315)	0.272
Total extraintestinal IBS-SSS	361 (40-629)	410 (141-636)	0.496

Values are presented as number and percentages or median (interquartile range) and analyzed using Mann-Whitney U tests or Fisher's exact tests. [a]'Glasses' of alcohol represent standard glasses. [b]'Physical' denotes the degree of physical activity that led to shortness of breath. [c]Specific gastrointestinal symptoms were assessed by visual analog scale for IBS and specific extraintestinal symptoms, total gastrointestinal symptoms and total extraintestinal symptoms were assessed by IBS-SSS (24,26). P<0.05 was considered to indicate a statistically significant difference. EDS, Ehler-Danlos syndrome; hEDS, hypermobile EDS; IBS, irritable bowel syndrome; IBS-SSS, IBS-severity scoring system.

Table IV. Basal characteristics and symptoms depending on parallel bundles throughout the biopsy or disorganized fiber bundles.

Characteristic	Parallel bundles (n=9)	Disorganized bundles (n=41)	P-value
Age, years	41 (39-44)	31 (27-40)	0.006
Sex, n (%)			0.570
Female	9 (100.0)	36 (87.8)	
Male	0 (0.0)	5 (12.2)	
BMI, kg/m^2	21.3 (20.5-27.6)	23.5 (21.4-26.0)	0.567
Education, n (%)			0.209
Primary school		3 (7.3)	
College	1 (11.1)	11 (26.8)	
≥1 more year of education		8 (19.5)	
University	8 (88.9)	19 (46.3)	
Occupation, n (%)			0.652
Working 100%	2 (22.2)	11 (26.8)	
Working 99-51%	3 (33.3)	7 (17.1)	
Sick leave	4 (44.4)	14 (34.1)	
Unemployment		3 (7.3)	
Studying		6 (14.6)	
Marital status, n (%)			1.000
Living alone	3 (33.3)	15 (36.6)	
Married/living together	6 (66.7)	26 (63.4)	
Smoking, n (%)			0.856
Regularly		6 (14.6)	
Former smoker	1 (11.1)	6 (14.6)	
Never smoker	8 (88.9)	29 (70.7)	
Glasses of alcohol/week[a], n (%)			0.167
<1 glass	4 (44.4)	29 (70.7)	
1-4 glasses	5 (55.6)	9 (22.0)	
5-9 glasses		3 (7.3)	
Physical activity/week[b], n (%)			0.708
No time at all		7 (17.1)	
<30 min	1 (11.1)	6 (14.6)	
30-60 min	4 (44.4)	9 (22.0)	
60-90 min	2 (22.2)	11 (26.8)	
90-120 min	1 (11.1)	3 (7.3)	
>120 min	1 (11.1)	5 (12.2)	

Table IV. Continued.

Characteristic	Parallel bundles (n=9)	Disorganized bundles (n=41)	P-value
Gastrointestinal symptom[c]			
Abdominal pain	0 (0-37)	25 (0-62)	0.107
Diarrhea	0 (0-56)	5 (0-59)	0.369
Constipation	5 (0-42)	25 (2-74)	0.136
Bloating and flatulence	16 (0-82)	56 (5-82)	0.496
Vomiting and nausea	11 (0-72)	30 (5-57)	0.595
Intestinal symptoms' influence on daily life	0 (0-78)	38 (4-74)	0.311
Psychological well-being	50 (0-68)	35 (10-59)	0.926
Difficulties eating a meal	0 (0-41)	12 (0-54)	0.210
Headache	15 (5-74)	69 (24-82)	0.085
Back pain	27 (0-48)	43 (8-74)	0.226
Fatigue	79 (6-91)	89 (48-99)	0.149
Belching/excess wind	0 (0-86)	40 (2-78)	0.485
Reflux	0 (0-34)	15 (0-56)	0.231
Urinary urgency	0 (0-46)	38 (2-82)	0.168
Leg pain	0 (0-28)	4 (0-50)	0.159
Muscle/joint pain	0 (0-51)	65 (40-297)	0.031
Total IBS-SSS	54 (4-204)	177 (40-297)	0.170
Total extraintestinal IBS-SSS	137 (38-623)	469 (134-634)	0.238

Values are presented as number and percentages or median (interquartile range) and analyzed using Mann-Whitney U tests or Fisher's exact tests. [a]'Glasses' of alcohol represent standard glasses. [b]'Physical' denotes the degree of physical activity that led to shortness of breath. [c]Specific gastrointestinal symptoms were assessed by visual analog scale for IBS and specific extraintestinal symptoms, total gastrointestinal symptoms and total extraintestinal symptoms were assessed by IBS-SSS (24,26). P<0.05 was considered to indicate a statistically significant difference. EDS, Ehler-Danlos syndrome; hEDS, hypermobile EDS; IBS, irritable bowel syndrome; IBS-SSS, IBS-severity scoring system.

infection. The advantage of the present X-ray technique is the possibility to scan large and unstained preparations without physical sectioning, preserving sample integrity. However, this may also be a disadvantage, as it is not possible to differentiate a number of cell types from each other, such as fibroblasts and immune cells, due to absence of staining. Depending on the clinical question, alternative methods are needed. Through further development of 3D virtual histology, whole paraffin samples may be scanned, which may lead to even greater areas and more representative materials being examined, overcoming obstacles of histopathological dependency on 2D limitations and slice orientation. The primary contribution of the present study is as a feasibility analysis of laboratory-based phase-contrast micro-CT.

The alignment of structures in the data may be quantified using gray value-based algorithms which are primarily based upon a structure tensor analysis and describe the local orientation in the neighborhood (sigma) of each voxel. However, this algorithm works on the entire dataset and analyzes the surrounding paraffin, air and sample holder (tube). Furthermore, the results of structure tensor analysis highly depend on the sigma of the analysis. Thus, this cannot be used in clinical settings yet.

It has been established that severe gastrointestinal symptoms are frequent in POTS and HSD/hEDS (10,12,29). However, only a number of gastrointestinal or extraintestinal symptoms differed between participants with or without disorganized fibers in the present study. Furthermore, a number of healthy controls also exhibited disorganized collagen fiber bundles. Although the percentage of disorganized collagen fiber bundles differed between POTS patients with and without hEDS/EDS, gastrointestinal symptoms did not differ. Thus, the presence of disorganized collagen fiber bundles in the skin may support a diagnosis of hEDS/EDS and gastrointestinal dysmotility in a patient with clinical features of disease; however this cannot replace bowel biopsies.

Examination of the nervous system in varying organs has revealed similar findings represented by the same pathophysiology in the autonomic nervous system, the enteric nervous system and the peripheral nervous system in skin (16). Thus, utilization of skin biopsies may improve the diagnostic accuracy in a simpler way, exhibiting fewer risks and complications for patients. However, laboratory X-ray phase-contrast CT is not yet applicable for examining peripheral intraepidermal nerve fibers.

Micro-CT has recently been described as a complementary tool for histopathological diagnosis of oral soft tissue (30,31). To the best of our knowledge, since the present study was the first to describe skin biopsy analysis using this technique and a large dataset of 50 participants was evaluated, it is important to consider how the technique may be used in the future. Notably, a promising future application of this technique for skin examination could be to determine infiltration and the

radicality of surgery in basal cell carcinoma and to be able to separate different types of alopecia. The current 3D virtual histology using X-rays is an alternative option to evaluate the radicality of surgery in whole tissues, scanned from the bottom of the resected tissue. Scalp biopsy is currently the most reliable diagnostic technique to diagnose and classify different types of alopecia (32). Micro-CT is an alternative to multiple sectioning, offering a 3D view of the whole hair follicle and surrounding tissue.

The strength of the present study lies in the comparison of skin biopsies from patients with POTS with healthy controls, since a number of previous studies have not examined controls (33-35). However, one limitation was that the patients with hEDS/EDS were recruited from a POTS cohort, which may have led to different symptoms and basal characteristics than if patients with only EDS had been included. No patient had any molecular diagnostic tests of EDS, however all diagnoses were made after a clinical examination by a specialist in the field. For ~50% of patients, a diagnosis of EDS only was made, without any specification. This means that numerous patients may also have been suffering from hEDS or potentially the less specified diagnosis HSD, whereby not all criteria for hEDS were fulfilled (5,6). Upon study inclusion, the patients were not examined clinically regarding muscle/joint function and the Beighton score evaluating each joint with a goniometer was not calculated (5). Therefore, in future studies, patients with hEDS/EDS should be included based on their primary diagnosis, determined by clinical and molecular genetic testing for diagnosis. The markedly high prevalence of disorganized collagen fiber bundles in the POTS group without known hEDS/EDS may be explained by undiagnosed cases of hEDS/EDS in the present cohort. In addition, although there was a relatively large dataset of 50 participants, its division into three groups led to rather small groups, which made statistical calculations uncertain. If the groupings had been larger, a number of the present findings may have been statistically significant. Thus, future examination of larger groups is important to elucidate out the role of the skin architecture in this entity and investigate the role of skin sampling for diagnostic accuracy. Systematic benchmarking against conventional histological stains was also not performed and therefore the present findings require further validation in the diagnostic characterization of dermal components.

In conclusion, 3D virtual histology may be used for visualization of the architecture and structure in the skin but not for examination of intraepidermal nerve fibers. Furthermore, 3D virtual histology may contribute to the diagnostic workup in connective tissue disorders; however, further larger studies are needed to evaluate the use of 3D skin biopsies as a diagnostic tool in numerous skin disorders.

Acknowledgements

Thanks also go to Karolina Palmér, statistician, for assistance with statistical advice.

Funding

The present study was funded by The Ingrid and Sverker Persson's Foundation, The Development Foundation of Region Skåne (grant nos. 2022-Projekt0067 and 2021-01942), Skåne University Hospital (grant nos. 2022-882 and 2022-974) and The Swedish Research Foundation (grant no. 2021-01942).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KL, HT, LD, MR and BO designed and performed the present study. HT recruited the participants. LD performed the skin biopsy. MR performed the scanning. KL and BO evaluated the histopathology and analyzed the data. KL and BO confirm the authenticity of all the raw data. BO wrote the manuscript. All authors contributed to the intellectual process during the writing, and all authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki and approved by The Swedish Ethical Review Authority, with review occurring at The Uppsala University Board (approval nos. 2020-02432 and 2021-00049; Dates of approval, 26/08/2020 and 11/02/2021). All subjects gave their oral and written, informed consent to participate prior to inclusion and study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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