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Bridging the Diagnostic Gap for Hypermobile Ehlers-Danlos Syndrome and Hypermobility Spectrum Disorders: Evidence of a Common Extracellular Matrix Fragmentation Pattern in Patient Plasma as a Potential Biomarker

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ABSTRACT

Diagnosing hypermobile Ehlers-Danlos syndrome (hEDS) and hypermobility spectrum disorders (HSD), common overlapping multisystemic conditions featuring symptomatic joint hypermobility, is challenging due to lack of established causes and diagnostic tools. Currently, the 2017 diagnostic criteria for hEDS are used, with non-qualifying cases classified as HSD, although the distinction remains debated. We previously showed extracellular matrix (ECM) disorganization in both hEDS and HSD dermal fibroblasts involving fibronectin (FN), type I collagen (COLLI), and tenascin (TN), with matrix metalloproteinase-generated fragments in conditioned media. Here, we investigated these fragments in patient plasma using Western blotting across diverse cohorts, including patients with hEDS, HSD, classical EDS (cEDS), vascular EDS (vEDS), rheumatoid arthritis (RA), psoriatic arthritis (PsA), and osteoarthritis (OA), and healthy donors, uncovering distinctive patterns. Notably, hEDS/HSD displayed a shared FN and COLLI fragment signature, supporting their classification as a single disorder and prompting reconsideration of the hEDS criteria. Our results hold the promise for the first blood test for diagnosing hEDS/HSD, present insights into the pathomechanisms, and open the door for therapeutic trials focused on restoring ECM homeostasis using an objective marker. Additionally, our findings offer potential biomarkers also for OA, RA, and PsA, advancing diagnostic and therapeutic strategies in these prevalent joint diseases.

[Correction added on 11 September 2024, after first online publication: The spelling error in author name "Silvia Ebe Lucia Della Pinna" to "Silvia Ebe Lucia Della Pina" has been corrected.]

Nicoletta Zoppi and Marina Colombi equally contribute to the work.

1 | Introduction

Hypermobile Ehlers-Danlos syndrome (hEDS) and hypermobility spectrum disorders (HSD) pose significant challenge in medical science, with far-reaching implications for millions worldwide. Classified within heritable connective tissue disorders (HCTDs) and mainly characterized by joint hypermobility and musculoskeletal complaints (Carroll 2023; Castori et al. 2017; Malfait et al. 2020; Morlino and Castori 2023; Tinkle et al. 2017), hEDS and HSD are estimated to affect over 1 in 300–500 individuals. Recent data from the NIH "All of Us" database and studies of medical records in Wales (Demmler et al. 2019) suggest that more than one million people in the USA, and 15 million worldwide, may suffer from these conditions. However, unlike other well-defined EDS subtypes such as classical EDS (cEDS) (Bowen et al. 2017) or vascular EDS (vEDS) (Byers et al. 2017), which result from specific genetic mutations involving collagen production and extracellular matrix (ECM) homeostasis, the underlying etiologies of hEDS and HSD remain elusive despite decades of intensive research (Gensemer et al. 2021; Scicluna et al. 2021; Syx et al. 2015; Vandersteen et al. 2023). Consequently, differentiation between hEDS, HSD, and other related HCTDs only relies on clinical classification criteria established through expert consensus in 2017 (Malfait et al. 2017). Based on these criteria, hEDS is defined by the simultaneous presence of generalized joint hypermobility (gJHM) according to an age specific Beighton score (BS) along with a combination of at least 5 out of 12 signs of multisystemic involvement plus either a positive family history and/or at least one musculoskeletal manifestation. Additionally other HCTDs, as well as autoimmune rheumatologic conditions such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA), must be ruled out. Individuals with symptomatic JHM not fulfilling the 2017 hEDS criteria and without signs and symptoms of other JHM-associated conditions are currently characterized as having HSD (Carroll 2023; Castori et al. 2017; Malfait et al. 2020; Morlino and Castori 2023).

The updated hEDS diagnostic criteria have faced criticism for their limited ability to identify the most severely affected patients and their failure to account for numerous extra-musculoskeletal manifestations, leading to a contentious debate regarding the categorization of hEDS and HSD, as they exhibit substantial clinical overlap (Anderson and Lane 2021; Aubry-Rozier et al. 2021; Copetti et al. 2019; Hakim 2019; Martin 2019; Williams 2019). Our recent work contributed to this discussion by highlighting the excessive stringency of the 2017 criteria and their inability to capture the extensive phenotypic heterogeneity in hEDS and HSD (Ritelli et al. 2024). Indeed, while JHM and its complications represent the hallmark features, both conditions encompass a broad spectrum of multisystemic manifestations not covered by diagnostic criteria, often more debilitating than joint symptoms. These associated conditions, recognized with varying levels of evidence as JHM-associated comorbidities, include chronic pain and fatigue, functional gastrointestinal disorders, cardiovascular dysautonomia, gynecological and bladder concerns, neurological symptoms, psychological and psychiatric issues, temporomandibular joint disorders, increased susceptibility to osteoarthritis (OA), orthopedic concerns, and immune system alterations (Brock, Prendergast, and Maitland 2021; Fernandez et al. 2022; Gagnon et al. 2023; Hakim, Tinkle, and Francomano 2021; Lam et al. 2021; Pietri-Toro et al. 2023; Ritelli et al. 2024; Vermeulen et al. 2022; Wasim et al. 2019; Zloof et al. 2023).

The lack of consensus within this field, along with the ongoing discussion on whether hEDS and HSD are distinct disease entities or part of a phenotypic continuum, mainly stem from the absence of objective biomarkers and reliable diagnostic laboratory tests to assist in clinical decision-making. Consequently, progress in understanding these HCTDs heavily depends on laboratory-based research aimed at uncovering pathophysiological clues and candidate biomarkers. Our prior efforts aimed to address this unmet need through comprehensive omics analyses in a large cohort of hEDS and HSD dermal fibroblasts (Chiarelli et al. 2019; Chiarelli, Zoppi, Ritelli, et al. 2021; Chiarelli, Zoppi, Venturini, et al. 2021; Ritelli et al. 2022; Zoppi et al. 2018). These findings have provided compelling evidence that hEDS and HSD fibroblasts share a common cellular trait, suggesting that these conditions may not be separate disorders. Indeed, our studies demonstrated that patient cells share a proinflammatory matrix-degrading phenotype typical of myofibroblasts (Zoppi et al. 2018), which can be induced in control fibroblasts when treated with conditioned media (CM) from hEDS and HSD cells (Chiarelli, Zoppi, Ritelli, et al. 2021). Analysis of the CM revealed the presence of degradation fragments of fibronectin (FN-fs), type I collagen (COLLI-fs), and tenascin (TN-fs), along with elevated amounts of ECM-degrading matrix metalloproteinases (MMPs) (Chiarelli, Zoppi, Venturini, et al. 2021; Zoppi et al. 2018). The detrimental impact of MMPs was further proven by treating hEDS and HSD cells with the nonselective MMP inhibitor doxycycline, which restored proper ECM organization and attenuated their myofibroblast-like features (Chiarelli, Zoppi, Venturini, et al. 2021). RNA-seq revealed common gene expression perturbation linked to various ECM-associated processes with differentially expressed genes including structural ECM components and regulators (including MMPs) and several proinflammatory mediators (Ritelli et al. 2022). Overall, these in vitro findings suggest a detrimental relationship between a pathological ECM and an uncontrolled inflammatory response as a driving force behind the pathophysiology of hEDS and HSD. Specifically, an imbalanced ECM turnover involving proteases may trigger a vicious cycle where ECM degradation products and other proinflammatory mediators synergistically impair connective tissue functionality, ultimately leading to the patients' multisystemic presentations. However, the existence of such pathophysiological mechanisms in vivo remains to be determined.

The current study aimed to build on our prior cellular findings by evaluating the candidate FN-fs, COLLI-fs, and TN-fs within human plasma from individuals with hEDS and HSD, in order to identify potential biomarker signatures compared to healthy individuals. Our study included patients with cEDS and vEDS, whose fibroblast CM did not exhibit ECM-derived fragments despite presenting ECM disarray (Chiarelli et al. 2019; Zoppi et al. 2018), as well as individuals with RA (Smolen et al. 2018), PsA (FitzGerald et al. 2021), and OA (Martel-Pelletier et al. 2016), recognizing that in these inflammatory and degenerative joint diseases an excessive ECM proteolysis generates ECM degradation products acting as damage-associated molecular pattern molecules (DAMPs or alarmins). These DAMPs are well known

to perpetuate a degradative feedback loop driving chronic synovitis and progressive cartilage destruction through induction of proinflammatory mediators, cytokines, nitric oxide, and MMPs (Buckley et al. 2021; Gilbert, Bonnet, and Blain 2021; Grillet et al. 2023; Hasegawa, Yoshida, and Sudo 2020; Lambert et al. 2021; Nefla et al. 2016; Pérez-García et al. 2019; Roh and Sohn 2018; Wei et al. 2023).

2 | Materials and Methods

2.1 | Study Population

This multicenter study included a diverse range of adult participants (aged ≥18 years), encompassing patients diagnosed with hEDS, HSD, cEDS, vEDS, RA, PsA, and OA, as well as healthy donors for a total of 466 individuals. Most participants were recruited from 3 different outpatient clinics located in Brescia, Italy. Additionally, independent cohorts of hEDS, HSD, and healthy donors were enrolled by the Ehlers-Danlos Society in the USA. Specifically, between September 2022 and September 2023, 55 hEDS, 55 HSD, 12 vEDS, and 10 cEDS patients, along with 129 healthy individuals, were enrolled at the specialized outpatient clinic for HCTDs and EDS of the University Hospital ASST Spedali Civili of Brescia. Between May and September 2023, 40 RA and 40 PsA patients were consecutively enrolled at the Rheumatology and Clinical Immunology Unit of the same Institution. Furthermore, between April and September 2023, 40 consecutive patients with OA were recruited at the Orthopedics and Traumatology Unit of the Manerbio Hospital (ASST del Garda). The Ehlers-Danlos Society provided samples from 39 hEDS and 25 HSD patients, as well as 21 healthy donors.

Regarding the Italian cohorts of hEDS and HSD patients, the diagnosis of hEDS (or HSD) relied exclusively on direct clinical assessment according to the 2017 hEDS criteria (Malfait et al. 2017) with the now widely endorsed modification of considering gJHM (criterion 1) as positive for patients scoring one-point below the age-specific BS cut off (Malfait et al. 2020; Ritelli et al. 2024) in the presence of a positive 5-point questionnaire (5PQ) (Hakim and Grahame 2003). Symptomatic patients, with at least one musculoskeletal manifestation, not fulfilling these adjusted hEDS criteria were classified as HSD. In cases featuring a substantial overlap with other EDS types or HCTDs, the differential diagnosis and evaluation were broadened to include additional screenings, and, when necessary, application of other diagnostic criteria, as well as appropriate molecular studies ranging from targeted Sanger sequencing (e.g., *COL5A1*, *COL5A2*, *TNXB*) to a custom-made NGS panel for EDS and related disorders (Connective Tissue Panel, CTP) (Ritelli, Venturini, et al. 2020; Rymen et al. 2019).

As for the Italian hEDS patients, the inclusion criteria for the American hEDS group were the 5PQ-adjusted 2017 hEDS criteria, determined by (i) direct exam and interview or (ii) review of medical records and sometimes photographs of certain features amenable to photographic confirmation (e.g., high/narrow palate, skin hyperextensibility, striae distensae/rubrae, piezogenic papules, atrophic scarring, and arachnodactyly). As part of the HEDGE study, all hEDS patients underwent whole-genome sequencing, with data analysis currently underway, and registration in the "DICE EDS and HSD Global Registry," which is securely hosted on a REDCap system managed by The Ehlers-Danlos Society through Amazon Web Services to ensure GDPR and HIPAA compliance. The inclusion criteria for the HSD group were a 2018 or later HSD diagnosis, positive criterion 1, and less than 5 reported feature 2A findings in the REDCap survey from the EDS and HSD Registry, without additional medical record review or physical examination.

For both the Italian and American patient cohorts, a range of comorbid conditions were recorded through direct clinical evaluation and interview, patient-provided medical reports review, or self-reporting in the REDCap survey. These comorbidities were defined as follows: (i) functional gastrointestinal disorders: gastroesophageal reflux, gastroparesis, dysmotility, constipation, diarrhea, irritable bowel syndrome, abdominal pain; (ii) neurological issues: headaches/migraines, neuropathic pain, allodynia, paresthesia, peripheral neuropathy, dizziness, "brain fog", difficulty with memory and concentration; (iii) cardiovascular dysautonomia: abnormal heart rate responses, irregular heart rhythms, orthostatic intolerance, exercise intolerance, postural orthostatic tachycardia syndrome (POTS) confirmed by tilt table testing, (iv) psychological/psychiatric issues: depression, anxiety disorders, sleep and mood disorders, obsessive-compulsive disorder, attention-deficit/hyperactivity disorder; (v) bladder/urological issues: urinary incontinence, overactive bladder, neurogenic bladder, urinary retention, pelvic organ prolapse; (vi) gynecological concerns: meno/metrorrhagia, disabling dysmenorrhea, pelvic pain, dyspareunia, vulvodynia; (vi) chronic fatigue: persistent, unexplained, and severe fatigue lasting for at least 6 months and not relieved by rest or sleep; (vii) temporomandibular joint disorders: of the jaw muscles, temporomandibular joints, and the nerves associated with chronic facial pain; (vii) allergic/atopic issues: food/drug/insect allergies, asthma, atopic dermatitis, rhinitis/rhinoconjunctivitis, mast cell activation syndrome (MCAS) confirmed by an immunologist.

For the control group, which included both Italian and American participants (120 females and 30 males, mean age 41.7 years, SD 14.2, range 20–68), individuals had to be unrelated to anyone with hEDS or HSD, and they should not exhibit any signs or symptoms of these conditions upon physical examination and interview.

The inclusion criteria for cEDS e vEDS patients, most of whom have been previously reported by our group (Ritelli, Rovati, et al. 2020; Ritelli, Venturini, et al. 2020), were the presence of a clinical diagnosis according to the 2017 nosology (Malfait et al. 2017), along with a confirmed causative genetic variant. For new patients, pathogenic variants were identified through NGS with the CTP panel and confirmed by Sanger sequencing.

Patients with RA and PsA were clinically diagnosed based on the respective classification criteria: the "2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) rheumatoid arthritis classification criteria" (Aletaha et al. 2010) for RA and the "2006 classification criteria for psoriatic arthritis (CASPAR)" (Taylor et al. 2006) for PsA. Clinical disease activity was assessed with the 28-joints disease activity score based on C-reactive protein (CRP) (DAS28-CRP) for RA (Prevoo et al. 1995), and the psoriasis area severity index

(PASI) (Fredriksson and Pettersson 1978) for psoriasis, the primary mucocutaneous manifestation in PsA. During each visit, patients underwent clinical examination to rule out the presence of gJHM and other features that are typical of hEDS and HSD, except for pain, which is a shared symptom across these conditions.

Patients with OA were enrolled during the preoperative phase anticipating total hip or knee joint replacement surgery. During these visits, patients underwent examination and clinical interview to exclude gJHM and other characteristics commonly associated with hEDS and HSD.

2.2 | Storage and Handling of Plasma Samples

Processing and storage of samples from Italian participants followed a standardized protocol used across all contributing outpatient clinics. After study recruitment, 2 mL of venous peripheral blood was drawn into sterile S-Monovette K3 EDTA Tubes (Sarstedt). For patients with RA and PsA receiving infusion therapies, blood was collected before drug administration. Within 4 h of collection, samples were centrifuged at 2500 *g* for 15 min at room temperature and the plasma supernatant was removed, aliquoted, and stored at −80°C until analysis.

Blood samples from American participants were collected at their homes following standard operating procedures to minimize pre-analytical variation. Trained phlebotomists drew 4 mL of peripheral blood into BD (Becton Dickinson) Vacutainer plasma preparation tubes containing K3-EDTA anticoagulant during scheduled visits. Samples were stored at 4°C during transport to the processing laboratory in Baltimore within 24 h of collection. Upon arrival, samples were centrifuged at 2500 *g* for 15 min at room temperature to isolate plasma. Aliquots of the supernatant were transferred to cryovials and frozen at −80°C for long-term storage. Frozen aliquots were then shipped on dry ice to the laboratory in Italy for Western blotting analysis.

2.3 | Western Blotting (WB)

To analyze FN, COLLI, TN, and their fragments in plasma samples by WB, plasma protein concentrations were determined using the Bicinchoninic Acid Protein Determination Kit (#BCA1-1KT, Sigma Aldrich-Merck Life Science). A total of 30 μg proteins were separated under reducing conditions through 8% SDS-PAGE electrophoresis. Following the transfer to a nitrocellulose sheet, membranes were blocked O.N. at 37°C in 5% non-fat dry milk/TBS-0.1% Tween 20 (TBS-T) and then incubated with the following primary antibodies for 3 h at R.T.: rabbit anti-human FN Ab (#F3648, Sigma Aldrich-Merck Life Science) at 1:1000 dilution, undiluted f29 anti-human FN mAb recognizing the N-terminal gelatin/collagen binding domain of FN (Colombi et al. 2003), goat anti-human COLLI Ab (#AB758, Merck-Millipore) at 1:500 dilution, and anti-human TN mAb (clone BC-24, Sigma Aldrich-Merck Life Science) at 1 μg/mL recognizing an epitope located within the N-terminal EGF-like sequence present in all tenascin isoforms, all diluted in 5% milk/TBS-T. Following washing in TBS-T, membranes were incubated for 3 h at R.T. with HRP-conjugated anti-rabbit, anti-mouse (#A8275 and #A5906, respectively, Sigma Aldrich-Merck Life Science), and anti-goat IgGs (#401515, EMD Millipore-Merck Life Science), all diluted 1:1000 in 5% milk/TBS-T. Chemiluminescent signals were then developed using the ECL method (#34580, Thermo Fisher Scientific).

2.4 | ELISA Measurements

ELISA analysis was performed on all plasma samples obtained from patients with hEDS, HSD, RA, PsA, OA, and 40 randomly selected healthy donors using commercial ELISA kits (EUROIMMUN Medizinische Labordiagnostika AG), with anti-cyclic citrullinate peptide (CCP) IgG and rheumatoid factor (RF) IgM concentrations assessed, featuring sensitivity limits of 1 and 2 RU/mL, and intra-assay CV% below 5.9% and 8.2%, respectively. Results for CCP IgG were categorized as negative (<5 RU/mL), positive (5–200 RU/mL), and highly positive (>200 RU/mL), and RF IgM concentrations were classified as negative (<20 RU/mL), positive (20–200 RU/mL), and highly positive (>200 RU/mL), based on the standard curve readings from the kits.

2.5 | Statistical Analysis

Assessments between the presence/absence of the 2017 hEDS diagnostic criteria, investigated comorbidities in hEDS and HSD, and CCP and RF positivity across the different conditions were performed using the chi-square test with Yates's correction or Fisher's exact test when counts were insufficient. Analyses were carried out with the GraphPad Software, and statistical significance was determined at a threshold of $p < 0.05$.

3 | Results

3.1 | Characteristics of Study Population Cohorts

Detailed clinical features for individual patients are provided in the Additional Database S1, which is organized by clinical diagnoses into separate spreadsheets and also distinguishes cohorts by nationality for hEDS and HSD. Indeed, the hEDS cohort comprised 55 Italian and 39 American participants, while for HSD, 55 were Italians and 25 Americans. The 55 Italian hEDS patients involved in this study were distributed across 42 different families, with 10 families having 2 or more affected hEDS members. Additionally, there were 4 mixed families with both hEDS and HSD patients, where the probands were all hEDS cases, and 28 sporadic patients. Most Italian patients were female, comprising 51 females (92.7%) and 4 males (7.3%), resulting in a sex ratio of 12.7. Their age range at last examination was 18 to 68 years, with a mean of 38.4 years (standard deviation [SD] 12.6). In the American hEDS cohort, all patients were from different families, encompassing 35 females (89.7%) and 4 males (10.3%), yielding a sex ratio of 8.7; the age range at last examination was 22 to 71 years, with a mean of 37.9 years (SD 11.9). Turning to the Italian HSD cohort, the 55 enrolled patients were distributed across 38 different families (including the 4 mixed), with 11 families having 2 or more affected HSD members; 23 were sporadic patients. Compared to hEDS, the female-to-male ratio was lower (3.6), with 43 females (78.2%) and 12 males (21.8%). The patients'

age range at last examination was 18 to 72 years, with a mean of 36.7 years (SD 14.1). Among the 25 American HSD patients, all were females from different families, with an age range at last examination of 22 to 52 and a mean of 39.5 years (SD 7.7). A comparison of the clinical features among the Italian and American cohorts, along with graphical illustrations, is available in the Additional Results.

By merging the two distinct hEDS and HSD cohorts, this research involved a total of 174 patients, with 94 individuals meeting the 2017 hEDS criteria (86 females, 8 males) and 80 who did not (68 females and 12 males). Figure 1 graphically illustrates the occurrences of the three mandatory diagnostic criteria for an hEDS diagnosis observed in the entire cohort, with Additional Results providing the overall frequencies and statistically significant differences between hEDS and HSD. As depicted in Figure 1A, the primary reason for an HSD exclusion diagnosis in our study population, beyond the absence of gJHM according to the 5PQ-adjusted criterion 1, with a total of 133/174 (76.4%) positive patients, including 39/80 (48.8%) with HSD, was the negativity for criterion 2. Specifically, 110/174 (63.2%) patients resulted positive for criterion 2, with only 17/80 (20%) falling into the HSD category. This failure was primarily attributed to the absence of the required 5 items of feature A, with 103/174 (59.2%) positive patients. Specifically, while 89/94 (94.7%) hEDS patients tested positive, only 14/80 (17.5%) HSD patients reached the requested 5 items of feature A. More in detail, among items with frequencies >50%, bilateral piezogenic papules (89.4% vs. 70%), striae distensae/rubrae (82.9% vs. 57.5%), unusually soft or velvety skin (81.9% vs. 50%), mild skin hyperextensibility (78.7% vs. 38.8%), and dental crowding and high or narrow palate (69.2% vs. 37.5%), were all more prevalent in hEDS compared to HSD. Likewise, among features with rates <50%, mitral valve prolapse (46.8% vs. 12.5%), arachnodactyly (31.9% vs. 11.3%), pelvic floor, rectal and/or uterine prolapse (23.4% vs. 8.8%), and arm span-to-height ≥1.05 (10.6% vs. 1.3%), were significantly more frequent in hEDS, whereas the difference in atrophic scarring (41.5% vs. 27.5%) did not reach statistical significance. Finally, the rarely observed recurrent or multiple abdominal hernias (6.4% vs. 6.3%) and aortic root dilatation (1.1% vs. 1.3%) did not show any significant difference.

The predominant shared characteristic among patients with hEDS and HSD in our population was feature C positivity,

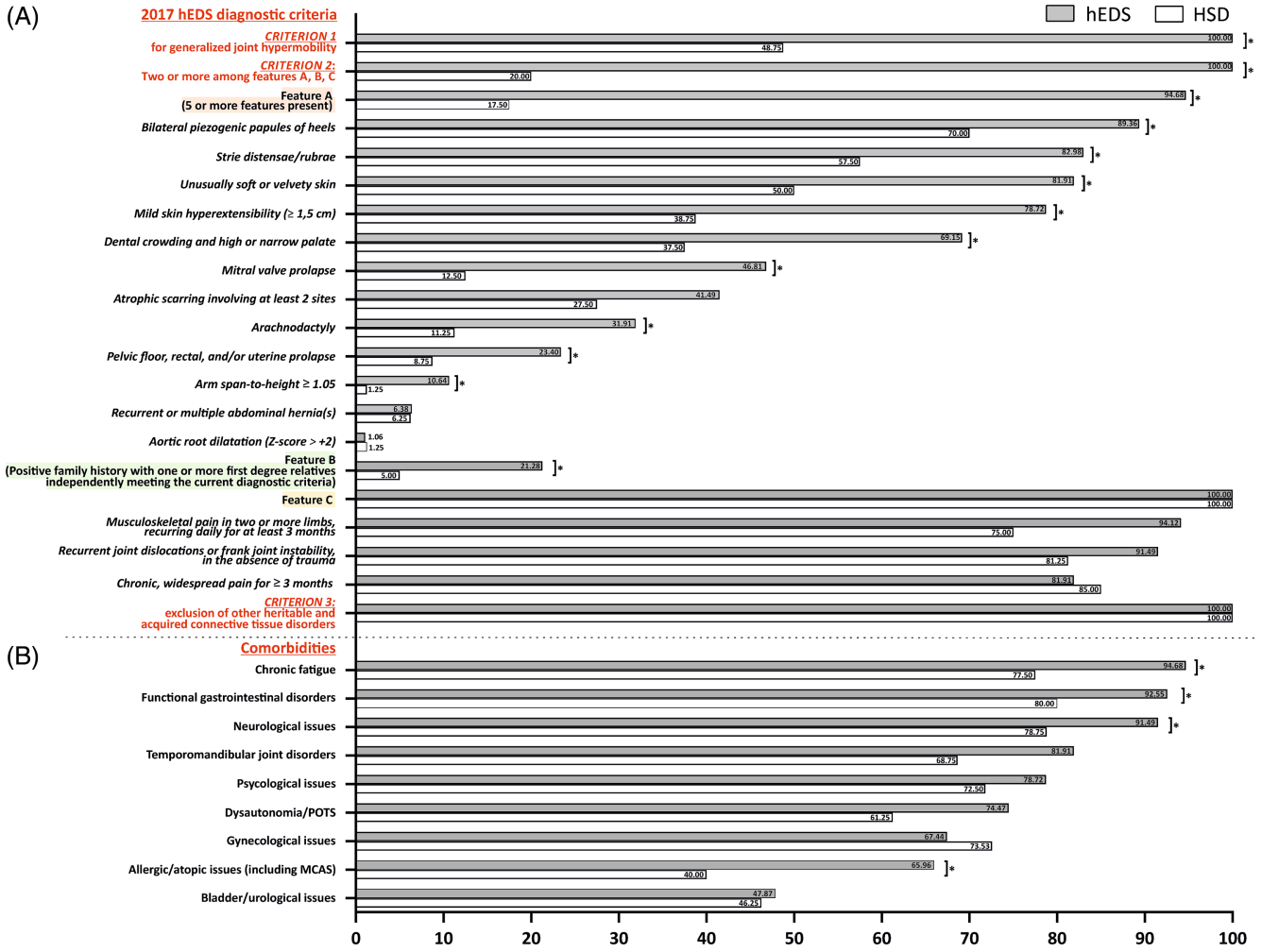

FIGURE 1 | (A) Prevalence of three mandatory diagnostic criteria for an hEDS diagnosis according to the 2017 EDS classification in the entire cohort of 174 patients, including 94 hEDS and 80 HSD individuals. Chronic pain was considered mutually exclusive with recurrent musculoskeletal pain. (B) Frequencies of comorbidities in the 2 different cohorts. *Presence of statistically significant differences between hEDS and HSD (for frequencies and *p*-values see Additional Results).

with all individuals demonstrating at least one musculoskeletal manifestation as defined by the 2017 hEDS criteria. Indeed, the differences observed between hEDS and HSD concerning the three items of feature C, that is, chronic, widespread pain (82% vs. 85%), musculoskeletal pain in 2 or more limbs recurring daily for at least 3 months (94.1% vs. 75%), which was considered mutually exclusive with chronic pain, and recurrent joint dislocations or frank joint instability in the absence of trauma (91.5% vs. 81.3%), were all not statistically significant.

Regarding assessed comorbidities, they were found to have a high prevalence across both hEDS and HSD patients, with only a few significant differences. Specifically, chronic fatigue (94.7% vs. 77.5%), functional gastrointestinal disorders (92.6% vs. 80%), neurological issues (91.6% vs. 78.6%), and allergic/atopic issues, including MCAS (66% vs. 40%) were more frequent in hEDS. On the other hand, the differences in temporomandibular joint disorders (81.9% vs. 68.8%), psychological issues (78.8% vs. 72.7%), dysautonomia/POTS (74.5% vs. 61.3%), gynecological concerns (67.4% vs. 73.5%), and bladder/urological issues (47.9% vs. 46.3%) did not reach statistical significance (Figure 1B and Additional Results).

In terms of anti-CCP IgG and RF IgM positivity, no statistically significant differences were observed between hEDS and HSD. Specifically, only 3/94 (3.2%) hEDS and 1/80 (1.3%) HSD patients tested positive for anti-CCP IgG (with none highly positive). RF IgM was present in 21/94 (22.3%) hEDS (with 2 highly positive) and 10/80 (12.5%) HSD patients (with none highly positive). These results were not statistically different from those observed in the 40 analyzed healthy individuals, all of whom were negative for anti-CCP IgG and with 25% of individuals testing positive for RF IgM (Additional Results).

Tables 1, 2, and 3 outline the main demographic, clinical, and laboratory findings of the RA, PsA, and OA patient cohorts, with a more detailed overview provided in the Additional Results, which also includes a summary of the cEDS and vEDS patient cohort characteristics.

3.2 | Biomarker Analysis

Based on our in vitro findings demonstrating the presence of degradation fragments of FN, COLLI, and TN in the CM of dermal fibroblasts derived from individuals with hEDS and HSD (Chiarelli, Zoppi, Venturini, et al. 2021), we employed the same antibodies to ascertain their potential as biomarkers in patients' plasma compared to samples from healthy donors. Notably, our investigations extended beyond hEDS and HSD to encompass inflammatory and degenerative joint diseases such as RA, PsA, and OA, alongside the two most frequent monogenic EDS types, cEDS and vEDS.

As illustrated in Figure 2, showing the different fragmentation patterns identified through WB in plasma samples from healthy donors and those affected with hEDS, HSD, OA, PsA, RA, cEDS, and vEDS, our analysis unveiled a shared fragment pattern for hEDS and HSD, while revealing distinct patterns for OA, PsA, and RA.

In particular, the polyclonal anti-FN Ab, besides recognizing the intact protein (≈250 kDa) across all tested subjects, highlighted the presence of a ≈52 kDa fragment in the plasma of all patients with hEDS and HSD. Notably, a distinct fragment of ≈38 kDa was consistently observed in all samples from patients with OA, whereas no FN-fs were detected in samples from healthy donors or patients with PsA, RA, cEDS or vEDS (Figure 2A). Moreover, the intact fibronectin (≈250 kDa) and its fragments of ≈52 and ≈38 kDa, observed respectively in patients with hEDS, HSD, and OA, were also recognized by the mAb f29 targeting the FN's N-terminal gelatin/collagen binding domain, thereby further delineating the protein region comprising the observed fragments (Figure 2D).

Regarding COLLI, WB using the polyclonal Ab did not reveal the intact protein in any analyzed plasma samples. However, a fragment of ≈45 kDa was consistently identified in all samples from patients with hEDS and HSD, while a fragment of ≈30 kDa was present in all OA patient samples. In the majority of PsA patient samples (87.5%), COLLI-fs were not detected, with only 12.5% of samples showing the same fragment identified in hEDS and HSD samples. This ≈45 kDa fragment was also present in the majority of RA patient samples (90%). Specifically, it was observed alone in 3/36 samples, in combination with a ≈60 kDa fragment in 10/36 samples, combined with a ≈80 kDa fragment in 7/36 samples, or concurrent with both the ≈60 and ≈80 kDa fragments in 16/36 samples. No COLLI-fs were evident in 10% of RA patient samples. The ≈60 kDa fragment was detected in all vEDS patient samples, while no COLLI-fs were evident in those of all healthy individuals and cEDS patients (Figure 2B,E).

Finally, the anti-TN mAb, besides recognizing the intact protein (≈250 kDa) across all tested healthy and patient subjects, revealed the presence of TN-fs exclusively in PsA and RA patient samples, but not in the plasma of healthy individuals or patients with the other conditions. Specifically, in the majority of PsA patient samples (95%), a fragment of ≈58 kDa was identified, while in the remaining samples, a fragment of ≈38 kDa was detected. This latter fragment was the most frequent fragment observed in RA patient samples (92.5%), either occurring alone in 31/37 samples or in combination with the ≈58 kDa fragment in 6 samples. In the remaining 7.5% of RA patient samples, the ≈58 kDa was instead evident (Figure 2C,F).

Overall, these findings not only underscore the diagnostic relevance of the FN fragment of ≈52 kDa as a possible biomarker for hEDS/HSD but also highlight the potential importance of the ≈38 kDa FN and ≈30 kDa COLLI fragments in the context of OA, as well as the TN-fs for two rheumatic diseases. Concerning the other COLLI-fs, while these individual fragments lack specificity as distinct biomarkers for a given condition, our findings suggest that further studies should be undertaken to assess whether they could contribute to enhancing diagnostic accuracy when considered in combination with the presence or absence of the other fragments identified in this study (Table 4).

4 | Discussion

The primary aim of our study was to explore the potential of ECM degradation fragments in plasma, with the goal of

TABLE 1 | Summary of demographic and clinical features of the RA patient cohort.

Females/Males, *n* (%)	32 (80)/8 (20)
Age, years	57.7 (12.9)
Disease duration, years	14.8 (7.2)
Ever smokers (current and past), *n* (%)	7 (17.5)
Erosive arthritis (radiography), *n* (%)	19 (47.5)
CRP-DAS28 score	2.78 (1.06)
Remission (≤2.6), *n* (%)	22 (55)
Low disease activity (2.6–3.2), *n* (%)	7 (17.5)
Moderate disease activity (>3.2–5.1), *n* (%)	10 (25)
High disease activity (>5.1), *n* (%)	1 (2.5)
Treatment	
Currently treated with corticosteroids, *n* (%)	25 (62.5)
Currently treated with csDMARDs, *n* (%)	29 (72.5)
MTX	22 (55)
LEF	4 (10)
SSZ	1 (2.5)
HCQ	8 (20)
2 or more concomitant csDMARDs	9 (22.5)
Currently treated with b/tsDMARDs, *n* (%)	37 (92.5)
TNFα-inhibitors	12 (30)
ABA	16 (40)
RTX	1 (2.5)
IL6-inhibitors	6 (15)
JAK-inhibitors	3 (7.5)
Number of previous csDMARDs	2.62 (1.6)
Number of previous b/tsDMARDs	2.87 (2.2)
Comorbidities, *n* (%)	
Diabetes mellitus	5 (12.5)
Dyslipidemia	19 (47.5)
Depression	5 (12.5)
Cancer	5 (12.5)
Arterial hypertension	16 (40)
Laboratory findings	
RF and CCP positive, *n* (%)	26 (65)
CCP only positive, *n* (%)	1 (2.5)
RF only positive, *n* (%)	7 (17.5)
RF and CCP negative, *n* (%)	6 (15)
CCP titer	88.9 (74.6)
RF titer	138.2 (85.2)
Elevated CRP, mg/L (n.v. <5), *n* (%)	3 (7.5)
CRP, mg/L	1.65 (2.9)

Note: Results are shown as mean (±SD), if not otherwise specified.
Abbreviations: ABA, abatacept; b/ts, biological/target synthetic; CCP, anti-cyclic citrullinate peptide IgG; CRP, C-reactive protein; cs, conventional synthetic; DAS28, disease activity score; DMARDs, disease modifying anti-rheumatic drugs; HCQ, hydroxychloroquine; IL, interleukin; LEF, leflunomide; MTX, methotrexate; n.v., normal value; RF, rheumatoid factor IgM; RTX, rituximab; SSZ, sulfasalazine.

identifying minimally invasive biomarkers that could significantly improve the diagnostic classification of hEDS and HSD. Despite considerable research efforts over the years, the daunting reality remains: the absence of any available molecular basis or laboratory test makes the diagnosis and classification of these patients extremely challenging. This diagnostic ambiguity not only places a substantial burden on healthcare practitioners but also significantly impacts patients, leading to prolonged diagnostic journeys, misdiagnosis, and potentially harmful delays in appropriate management (Anderson and Lane 2021; Carroll 2023; Halverson et al. 2021). Indeed, the average delay to diagnosis is 12 years, during which time these individuals endure misdiagnoses and inadequate treatments (Halverson et al. 2021; Schubart et al. 2021). Regrettably, due to a lack of awareness among healthcare professionals, these patients might be dismissed or stigmatized, with their symptoms attributed to malingering or psychiatric issues (Anderson and Lane 2021; Carroll 2023; Halverson et al. 2021). This lack of awareness perpetuates the suffering of patients, as they struggle to find healthcare providers who acknowledge and address their debilitating symptoms, which extend far beyond mere joint instability (Malfait et al. 2020).

Our study builds upon a growing body of evidence suggesting that hEDS and HSD are not distinct disorders but rather represent variants of the same entity (Chiarelli et al. 2019; Chiarelli, Zoppi, Ritelli, et al. 2021; Chiarelli, Zoppi, Venturini, et al. 2021; Ritelli et al. 2022, 2024; Zoppi et al. 2018). This perspective is reinforced by our current findings, which revealed a shared pathophysiological mechanism characterized by excessive ECM breakdown, observed through the identification of FN and COLLI degradation fragments in plasma. The presence of the ≈52 kDa FN fragment exhibited extraordinary sensitivity and specificity in distinguishing hEDS/HSD from healthy individuals and other disorders in differential diagnosis. While we recognize the need for validation studies, our findings represent a significant milestone in the field of hypermobility syndromes. First, they raise concerns about the current distinction between hEDS and HSD, highlighting the shortcomings of the current diagnostic criteria. In line with our recent clinical study (Ritelli et al. 2024) and work currently being undertaken by the hEDS/HSD working group of the International Consortium on EDS and HSD (https://www.ehlers-danlos.com/criteria-and-diagnostic-pathway-update/), the 2017 hEDS diagnostic criteria need to be revised to a more comprehensive framework that recognizes a broader phenotypic spectrum, including additional individuals

TABLE 2 | Summary of demographic and clinical features of the PsA patient cohort.

Females/Males, *n* (%)	18 (45)/22 (55)
Age, years	54.6 (10.6)
Disease duration, years	9.7 (8.2)
Ever smokers (current and past), *n* (%)	7 (17.9)
Erosive arthritis (radiography), *n* (%)	10 (25)
Psoriasis, *n* (%)	31 (77.5)
CRP-DAS28 score	2.61 (1.1)
Remission (≤2.6), *n* (%)	24 (60)
Low disease activity (2.6–3.2), *n* (%)	4 (10)
Moderate disease activity (>3.2–5.1), *n* (%)	10 (25)
High disease activity (>5.1), *n* (%)	2 (5)
PASI score (range 0–72)	0.40 (1.3)
Treatment	
Currently treated with corticosteroids, *n* (%)	9 (22.5)
Currently treated with csDMARDs, *n* (%)	19 (47.5)
MTX	17 (42.5)
LEF	0 (0)
SSZ	2 (5)
HCQ	0 (0)
2 or more concomitant csDMARDs	0 (0)
Currently treated with b/tsDMARDs, *n* (%)	33 (82.5)
TNFα-inhibitors	8 (20)
JAK-inhibitors	3 (7.5)
IL17-inhibitors	9 (22.5)
Ustekinumab	6 (15)
Guselkumab	6 (15)
Risankizumab	1 (2.5)
Number of previous csDMARDs	1.72 (1.3)
Number of previous b/tsDMARDs	2.93 (3.06)
Comorbidities, *n* (%)	
Irritable bowel disease	2 (5)
Diabetes mellitus	5 (17.2)
Dyslipidemia	13 (44.8)
Depression	5 (17.2)
Cancer	3 (10.3)
Arterial hypertension	12 (41.4)
Laboratory findings	
RF and CCP positive, *n* (%)	1 (2.5)
CCP only positive, *n* (%)	0 (0)
RF only positive, *n* (%)	9 (22.5)
RF and CCP negative, *n* (%)	30 (75)
CCP titer	NE
RF titer	17.3 (38.9)
Elevated CRP, mg/L (n.v. <5), *n* (%)	3 (7.5)
CRP, mg/L	2.63 (2.4)

Note: Results are shown as mean (±SD), if not otherwise specified.
Abbreviations: b/ts, biological/target synthetic; CCP, anti-cyclic citrullinate peptide IgG; CRP, C-reactive protein; cs, conventional synthetic; DAS28, disease activity score; DMARDs, disease modifying anti-rheumatic drugs; HCQ, hydroxychloroquine; IL, interleukin; LEF, leflunomide; MTX, methotrexate; n.v., normal value; NE, not evaluable; PASI, psoriasis area severity index; RF, rheumatoid factor IgM; SSZ, sulfasalazine.

currently classified as HSD. This is particularly important as HSD is unrecognized in many nations, exacerbating care disparities, financial strains, and psychological distress (Morlino and Castori 2023; Ritelli et al. 2024). Since our discovery holds the promise for introducing the first and only blood test to definitively diagnose hEDS/HSD, the potential clinical implications of this breakthrough are extensive. Indeed, it could significantly improve diagnostic pathways, leading to the inclusion of more individuals in appropriate medical care, reduce diagnostic delays by increasing healthcare professionals' confidence in identifying the condition, and have substantial impacts on legal settings, insurance payment policies, and various other healthcare areas. Additionally, developing and validating a blood biomarker for hEDS/HSD could provide new insights into its underlying biology. Improved understanding could also expand patient cohorts available for future research studies. Larger, well-defined patient groups may also enhance genomic analyses by allowing comparisons within a more uniformly diagnosed population. Over time, aggregating data from multiple studies using a confirmed diagnostic biomarker may help deepen our scientific understanding of hEDS/HSD.

The work to assess revision of the hEDS diagnostic criteria is part of a wider International Consortium on EDS and HSD program called the "Road To 2026" with a primary goal of updating the classification framework for all types of EDS (https://www.ehlers-danlos.com/road-to-2026/). This revision process will involve reviewing evidence to potentially expand and restructure the criteria, thus advancing research and understanding of these conditions. Additionally, formal criteria for HSD will be defined, and an EDS diagnostic pathway will be established based on clinical and biological evidence. We are confident that our recently published proposals to improve the diagnostic criteria for hEDS (Ritelli et al. 2024) will be considered by this committee, especially now that we have potentially identified a common biomarker for hEDS and HSD. In our opinion, any revised framework should address the shortcomings of current criteria, notably the restrictive nature of criterion 1 and the insufficient objective multisystemic clinical signs and specific symptoms in criterion 2. We proposed broader use of the 5PQ in combination with the BS and/or alternative

TABLE 3 | Summary of demographic and clinical features of the OA patient cohort.

Females/Males, *n* (%)	18 (45)/22 (55)
Age, years	71.2 (8.1)
Surgical intervention, *n* (%)	
Total hip replacement (R)	15 (37.5)
Total hip replacement (L)	9 (22.5)
Total knee replacement (R)	7 (17.5)
Total knee replacement (L)	9 (22.5)
Comorbidities, *n* (%)	
Arterial hypertension	17 (42.5)
Heart disease	6 (15)
Type 2 diabetes	7 (17.5)
Gastroesophageal reflux	1 (2.5)
Fibromyalgia	1 (2.5)
Laboratory findings	
RF and CCP positive, *n* (%)	2/40 (6)
CCP only positive, *n* (%)	0/40 (0)
RF only positive, *n* (%)	11/40 (27.5)
RF and CCP negative *n* (%)	27/40 (67.5)
CCP titer	NE
RF titer	26.3 (55.5)

Note: Results are shown as mean (±SD), if not otherwise specified.
Abbreviations: CCP, anti-cyclic citrullinate peptide IgG; L, left; NE, not evaluable; R, right; RF, rheumatoid factor IgM.

assessment tools to evaluate JHM alongside significant restructuring of criterion 2 involving a weighted scoring system within an expanded feature A, while removing positive family history from this criterion (Ritelli et al. 2024). Current biomarker evidence supports such changes, as we identified the 52 kDa FN fragment in patients who fail to meet criterion 1 (even with a BS of 0 but with a positive 5PQ) and/or criterion 2 (due to the lack of the 5 required items of feature A), including several individuals who are family members of probands diagnosed with hEDS. Regarding feature A, although 9 out of 12 items were statistically more common in hEDS compared to HSD, the lack of any correlation with the FN fragment highlights that these signs should not serve as main indicators for differentiating HSD from hEDS. The same concern applies to the use of the BS in defining gJHM, highlighting the limitations of its application in delineating between HSD and hEDS. In addition to subjective interpretation by practitioners and intrinsic technical inaccuracies that vary depending on the physician's experience with the BS, the most significant shortcoming of the BS is its strong upper limb bias, limited number of joints assessed, and focus on motion in only the sagittal plane. The recently proposed Lower Limb Assessment Score and Upper Limb Hypermobility Assessment Tool, both 12-item tests covering the major joints of the upper and lower limbs in multiple planes of movement [Nicholson and Chan 2018; Meyer et al. 2017], could be effective alternatives, even if these multidimensional examinations require standard operating procedures, expert management, and further psychometric testing for validation. In our cohort, hEDS and HSD patients shared positivity for sign C, with all individuals exhibiting at least one musculoskeletal manifestation. Additionally, the majority of the investigated comorbidities were prevalent in both hEDS and HSD, with few statistically significant differences. These observations support a paradigm shift towards clinical features prompting confirmatory testing through FN fragment verification. These features should initially encompass a thorough assessment of musculoskeletal manifestations, including but not limited to JHM, joint instability, and any associated musculoskeletal pain or dysfunction. Moreover, the revised framework should include an expanded list of multisystemic signs and symptoms, also encompassing comorbidities commonly associated with hEDS and HSD that must be evaluated based on their established diagnostic definitions, as outlined in the recent framework for pediatric JHM (Tofts et al. 2023). On the other hand, we advocate for a less restrictive assessment of JHM that should encompass both present and historical manifestations, recognizing joint laxity's dynamic nature over time (Castori et al. 2017; Malfait et al. 2020; Ritelli et al. 2024; Tinkle et al. 2017). Before implementing any revised criteria incorporating the FN fragment biomarker, it is crucial to conduct large, independent confirmatory cohort studies and to expand the analyses to include pediatric patients. Additionally, further research should explore a broader range of populations, including asymptomatic individuals with gJHM, people with fibromyalgia who do not have JHM, people with MCAS who do not have JHM, and other relevant groups. Furthermore, to validate the FN fragment as a specific biomarker for hEDS and HSD, it is essential to establish its presence in other conditions with overlapping features, such as fibrosis, monoclonal gammopathies, other autoimmune diseases, and chronic infections. Ongoing and future studies encompassing these varied conditions will be critical in establishing the role of the identified biomarker and elucidating its diagnostic potential in hEDS and HSD.

In light of our expanded exploration of ECM fragmentation patterns, the present results are significant not only within the realm of hEDS/HSD but are also relevant for rheumatologic diseases such as RA, PsA, and OA. Indeed, by comparing the ECM fragment signatures between hEDS/HSD and these conditions we have also identified potential biomarkers for these disorders.

Regarding OA, the presence of the specific 38 kDa FN and 30 kDa COLLI fragments, upon validation in larger cohorts, holds promise as potential plasma biomarkers for OA, particularly considering the current lack of specific blood tests for this disorder. Furthermore, it is noteworthy that all OA patients included in our study were at an advanced stage of the disease, requiring surgical intervention. As such, follow-up studies are warranted to investigate the persistence of these fragments post-clinical recovery and their potential prognostic value. Additionally, exploring the presence of these fragments in the initial stages of OA could provide valuable insights into their potential utility for intervention strategies at an early point in the disease progression.

In the autoimmune rheumatologic diseases, our analysis revealed distinct ECM fragmentation patterns between RA and PsA, as indicated by the presence or absence of TN and COLLI fragments. Specifically, the presence of TN-fs emerged as a

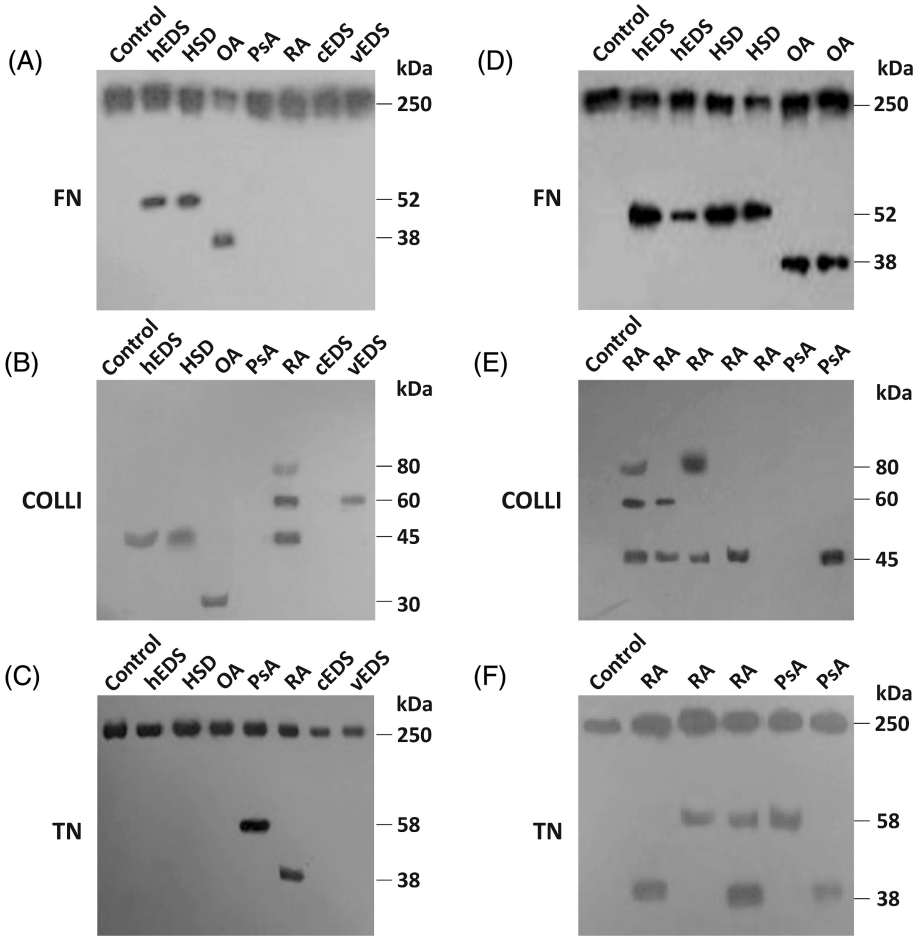

FIGURE 2 | Fragment analysis of fibronectin (FN), type I collagen (COLLI), and tenascin (TN) in plasma samples from control individuals and from patients with hypermobile Ehlers-Danlos syndrome (hEDS), hypermobility spectrum disorder (HSD), osteoarthritis (OA), psoriatic arthritis (PsA), rheumatoid arthritis (RA), classical Ehlers-Danlos syndrome (cEDS), and vascular Ehlers-Danlos syndrome (vEDS) revealed a shared fragment signature for hEDS and HSD, while distinct patterns emerged for OA, PsA, and RA. The images shown in panels A-C are representative of the most commonly observed patterns across all conditions obtained through Western blotting (WB) using the polyclonal anti-FN (#F3648) and anti-COLLI (#AB758) antibodies, as well as the monoclonal anti-TN (clone BC-24) antibody, while those shown in panels E and F represent all patterns of COLLI and TN fragments encountered in RA and PsA. Specifically, WB with the anti-FN antibody revealed no fragments in control (150), PsA (40), RA (40), vEDS (12), and cEDS (10) samples, whereas a ≈52 kDa fragment was detected in all hEDS (94) and HSD (80) samples, along with a ≈38 kDa fragment observed in all OA (40) samples (A). Both these fragments, specific to hEDS/HSD and OA, were also detected by the monoclonal f29 antibody that recognizes the N-terminal gelatin/collagen binding domain of FN (D). WB with the anti-COLLI antibody revealed absence of fragments in all control and cEDS samples, in contrast to the consistent detection of a ≈45 kDa fragment in all hEDS and HSD samples, as well as a ≈30 kDa fragment in all OA samples (B). The majority of PsA samples (35/40) did not display COLLI fragments (B), with only 5 showing the ≈45 kDa fragment (E). In RA samples, the most frequent pattern (16/40) was the simultaneous presence of the ≈45, ≈60, and ≈80 kDa fragments (B), followed by the combinations of ≈45/≈60 kDa (10/40) and ≈45/≈80 kDa (7/40). Absence of fragments was observed in 4/40 samples, and the ≈45 kDa fragment alone in 3/40 samples (E). Finally, in all vEDS samples, only the ≈60 kDa COLLI fragment was detected (B). WB with the anti-TN antibody consistently revealed fragments only in PsA and RA (C). In PsA, nearly all samples (38/40) showed a ≈58 kDa fragment, with only 2 samples exhibiting a ≈38 kDa fragment (F). In RA samples, the presence of the single ≈38 kDa fragment was the most common finding (31/40), with only three samples exhibiting the ≈58 kDa fragment and six showing the combination of both fragments (E).

signature pattern for both RA and PsA. The ≈58 kDa TN fragment was more prevalent in PsA, identified in 95% of samples, while the ≈38 kDa fragment appeared more RA-specific, present alone in 77.5% of RA patient samples. Interestingly, there was minimal overlap between RA and PsA based on these TN-fs, with only 2/40 PsA samples showing the ≈38 kDa fragment and 3/40 RA samples exhibiting the ≈58 kDa fragment. Furthermore, a small subset of 6/40 RA samples displayed both fragments, indicating potential heterogeneity within the RA population. In addition to TN-fs, the COLLI fragmentation patterns might help to distinguish between RA and PsA. Indeed, the absence of COLLI-fs was more PsA-specific, identified in 87.5% of samples, with only 5/40 samples showing the ≈45 kDa COLLI fragment. In contrast, various COLLI-fs were present in different combinations in 90% of RA patient samples, with only 4/40 samples lacking COLLI-fs. Given these promising findings, further evaluating the presence or absence of COLLI and TN fragments holds promise as novel biomarkers for RA and PsA. Larger validation studies are necessary to confirm their diagnostic utility, alone or in combination with established

TABLE 4 | Combination frequencies of FN, COLLI, and TN fragments identified in plasma samples of healthy donors and of hEDS, HSD, OA, PsA, RA, cEDS, and vEDS patients.

	FN (kDa)	COLLI (kDa)	TN (kDa)	*n* (%)
Healthy donors	—	—	—	150 (100%)
hEDS	52	45	—	94 (100%)
HSD	52	45		80 (100%)
OA	38	30	—	40 (100%)
PsA	—	—	58	33/40 (82.5%)
	—	45	58	5/40 (12.5%)
	—	—	38	2/40 (5%)
RA	—	45, 60, 80	38	12/40 (30%)
	—	45, 60	38	9/40 (22.5%)
	—	45, 80	38	6/40 (15%)
	—	45, 60, 80	38, 58	4/40 (10%)
	—	—	58	3/40 (7.5%)
	—	45	38	3/40 (7.5%)
	—	45, 60	38, 58	1/40 (2.5%)
	—	45, 80	38, 58	1/40 (2.5%)
	—	—	38	1/40 (2.5%)
cEDS	—	—	—	10 (100%)
vEDS	—	60	—	12 (100%)

markers like RF and anti-CCP antibodies, which have limitations in sensitivity and specificity (FitzGerald et al. 2021; Smolen et al. 2018). Integrating these novel markers into clinical practice could enhance diagnostic accuracy and guide more tailored clinical management decisions.

Regarding pathophysiology, our study has shed light on common pathogenetic mechanisms across hEDS/HSD, OA, RA, and PsA, providing valuable insights for future translational research. Our findings expand upon well-established knowledge about the role of ECM imbalance in rheumatic diseases (Buckley et al. 2021; Gilbert, Bonnet, and Blain 2021; Grillet et al. 2023; Nefla et al. 2016). In these conditions, ECM degradation leads to the release of various ECM-derived fragments that modulate the joint microenvironment by inducing changes in the expression of several proinflammatory genes, such as cytokines and ECM-degrading proteinases. This promotes a feedback loop of continued degradation and inflammation, recapitulating key features of the pathological phenotype (Hwang et al. 2015; Pérez-García et al. 2019; Reed et al. 2021). Several lines of evidence underscore the potential of these DAMPs as valuable biomarkers and therapeutic targets for OA, RA, and PsA (Hasegawa, Yoshida, and Sudo 2020; Lambert et al. 2021). Regulating DAMP signaling could reduce inflammation and provide effective treatment for these conditions (Roh and Sohn 2018; Wei et al. 2023). Here, we present the first in vivo evidence suggesting that ECM homeostasis disruption may play a central role in the disease progression of hEDS/HSD, corroborating our in vitro disease model (Chiarelli, Zoppi, Venturini, et al. 2021; Ritelli et al. 2022). Indeed, the presence of circulating FN and COLLI degradation products and other yet unidentified molecules may function as DAMPs, triggering detrimental responses by stimulating the overproduction of proinflammatory mediators, cytokines, and proteinases. This cascading process could result in additional ECM degradation, exacerbating the underlying pathological process. Such a self-perpetuating cycle of degradation and inflammation could help establish and maintain the disease state in hEDS/HSD.

While several questions remain regarding a more comprehensive mechanistic understanding of hEDS/HSD pathobiology, such as the specific tissue injuries generating ECM degradation fragments and the proteinase subclasses producing these DAMPs, exploring the therapeutic implications of targeting ECM fragments holds promise. This line of investigation could pave the way for innovative strategies aimed at modulating ECM remodeling and, ultimately, enhancing clinical outcomes for patients. Addressing the remaining unknown mechanisms involved in ECM breakdown and DAMP generation is essential and may elucidate further potential therapeutic targets for modulating disease progression in hEDS/HSD.

5 | Conclusions

In conclusion, our study represents a significant advance by highlighting the potential of plasma biomarkers to enhance

diagnostic classification not only for hEDS/HSD but also for the investigated inflammatory and degenerative joint diseases, while expanding our understanding of molecular mechanisms in these conditions. This lays the groundwork for developing of targeted diagnosis and treatment approaches, paving the way for improved patient care. These findings warrant consideration by the International Consortium on EDS and HSD in reference to the "Road to 2026" project considering revision of the 2017 diagnostic guidelines for hEDS and HSD.

Author Contributions

All authors have contributed to this article significantly. Conceptualization: Marco Ritelli, Nicola Chiarelli, Nicoletta Zoppi, Marina Colombi; formal analysis: Marco Ritelli, Nicola Chiarelli, Valeria Cinquina, Silvia Piantoni, Alessia Caproli, Nicoletta Zoppi, Marina Colombi; investigation: Valeria Cinquina, Valeria Bertini, Alessia Caproli, Silvia Ebe Lucia Della Pina, Nicoletta Zoppi; resources: Silvia Piantoni, Franco Franceschini, Guido Zarattini, Woodrow Gandy, Marina Venturini, Marina Colombi; data curation: Marco Ritelli, Nicola Chiarelli, Valeria Cinquina, Silvia Piantoni, Alessia Caproli, Nicoletta Zoppi, Marina Colombi; writing-original draft preparation, Marco Ritelli, Nicola Chiarelli, Marina Colombi; writing-review and editing, Woodrow Gandy, Silvia Piantoni, Franco Franceschini, Guido Zarattini, Marina Venturini, Nicoletta Zoppi; visualization: Marco Ritelli, Valeria Cinquina; supervision: Marco Ritelli, Nicoletta Zoppi, Marina Colombi; funding acquisition: Woodrow Gandy, Marina Colombi; project administration: Marina Colombi. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The study was approved by the local Ethical Committee "Comitato Etico di Brescia, ASST degli Spedali Civili, Brescia" in Italy (Protocol numbers NP5582, NP5832, NP5850) and by the "Genetic Alliance Institutional Review Board" in the USA (Federal Registration Number IORG0003358, Project number EDS002).

Consent

All subjects provided written informed consent for their inclusion in the study and for the use of their clinical data and samples for research purposes.

Conflicts of Interest

Marco Ritelli, Nicola Chiarelli, Nicoletta Zoppi, and Marina Colombi are co-authors of the patent application N. 102024000002376 (I0205230) entitled "Plasma biomarkers for hypermobile Ehlers-Danlos syndrome, hypermobility spectrum disorders, osteoarthritis, rheumatoid arthritis, and psoriatic arthritis." Woodrow Gandy is a board member of The Ehlers-Danlos Society. The remaining authors affirm that the research was conducted without any potential conflicts of interest arising from commercial or financial relationships.

Data Availability Statement

Most data generated or analyzed during this study are included in this published article and its Additional files. Additional data and materials are available from the corresponding author on reasonable request, subject to compliance with our obligations under human research ethics.

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Supporting Information

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