ARTICLE

# Extracellular matrix protein signature of recurrent spontaneous cervical artery dissection

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## Abstract

### Objective

To assess whether connective tissue disorder is evident in patients with spontaneous cervical artery dissection and therefore identify patients at risk of recurrence using a cutting-edge quantitative proteomics approach.

### Methods

In the ReSect study, all patients with spontaneous cervical artery dissection treated at the Innsbruck University Hospital since 1996 were invited to attend a standardized clinical follow-up examination. Protein abundance in skin punch biopsies (n = 50) was evaluated by a cutting-edge quantitative proteomics approach (liquid chromatography–mass spectrometry) that has hitherto not been applied to such patients.

### Results

Patients with 1-time single-vessel (n = 19) or multiple-vessel (n = 13) dissections did not differ between each other or compared to healthy controls (n = 12) in protein composition. Patients with recurrent spontaneous cervical artery dissection (n = 6), however, showed significantly different expression of 25 proteins compared to the other groups combined. Literature review and Gene Ontology term annotation check revealed that 13 of the differently expressed proteins play a major role in the structural integrity of connective tissue or are linked to connective tissue disorders. These proteins showed clustering to a collagen/elastin cluster and one consisting of desmosome related proteins.

### Conclusion

This study unravels an extracellular matrix protein signature of recurrent spontaneous cervical artery dissection. In the long run and after large-scale validation, our findings may well assist in identifying patients at risk of recurrent spontaneous cervical artery dissection and thus guide therapy.

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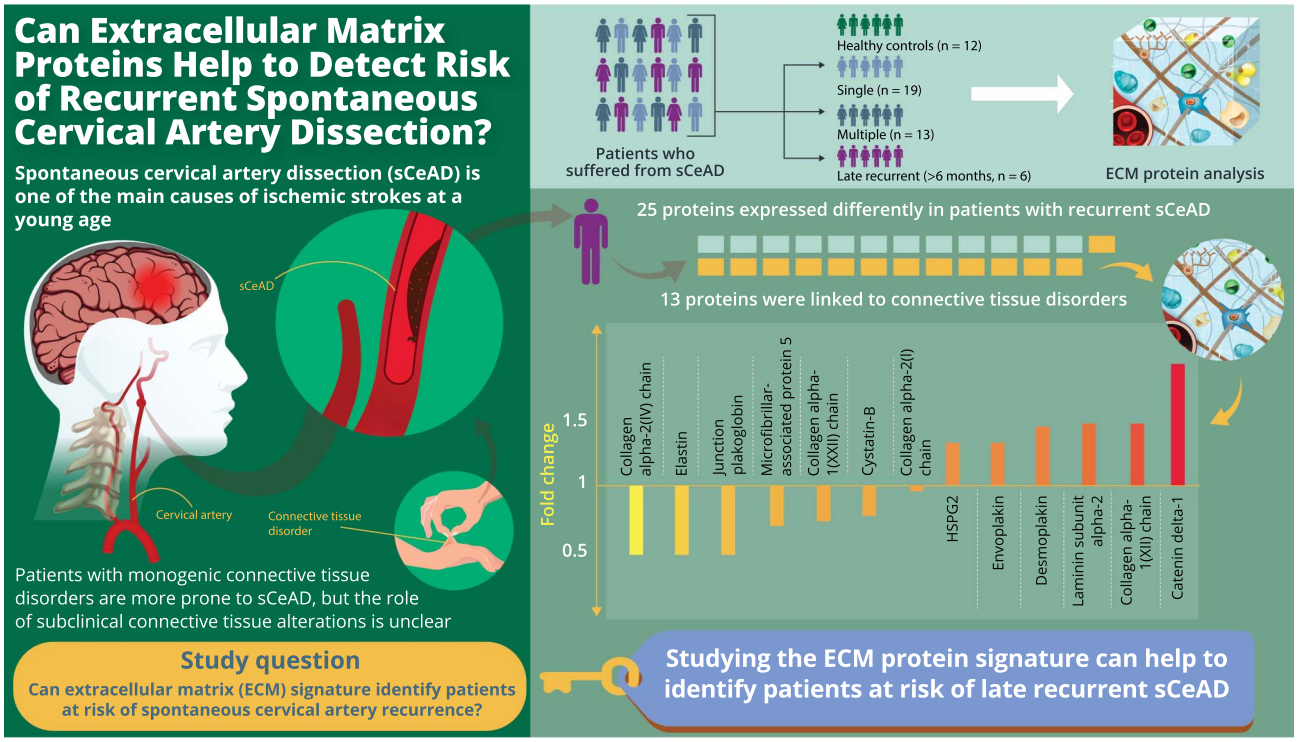
## Glossary

**CeAD** = cervical artery dissection; **ECM** = extracellular matrix; **GO** = Gene Ontology; **LC** = liquid chromatography; **MS/MS** = tandem mass spectrometry; **NCBI** = National Center for Biotechnology Information; **ReSect** = Recurrent disSection; **sCeAD** = spontaneous CeAD.

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Spontaneous (s) cervical artery dissection (CeAD) represents one of the main causes of ischemic stroke in the young.[1,2] Because patients with inherited connective tissue disorders such as vascular Ehlers-Danlos or Marfan syndrome are prone to sCeAD, connective tissue and vascular extracellular matrix (ECM) are of high interest, especially in individuals with recurrent sCeAD.[3]

To date, however, studies searching for associations between sCeAD and genes that are involved in known connective tissue disorders have been mostly negative. Only nonspecific, ultrastructural changes in connective tissue, especially collagen fibrils of the skin in patients with sporadic sCeAD compared to healthy controls, have been described.[4–9] Modern proteomics techniques to study the ECM have been applied successfully in the context of abdominal and thoracic aortic aneurysms from patients with Marfan syndrome but have not yet been used in sCeAD.[10,11]

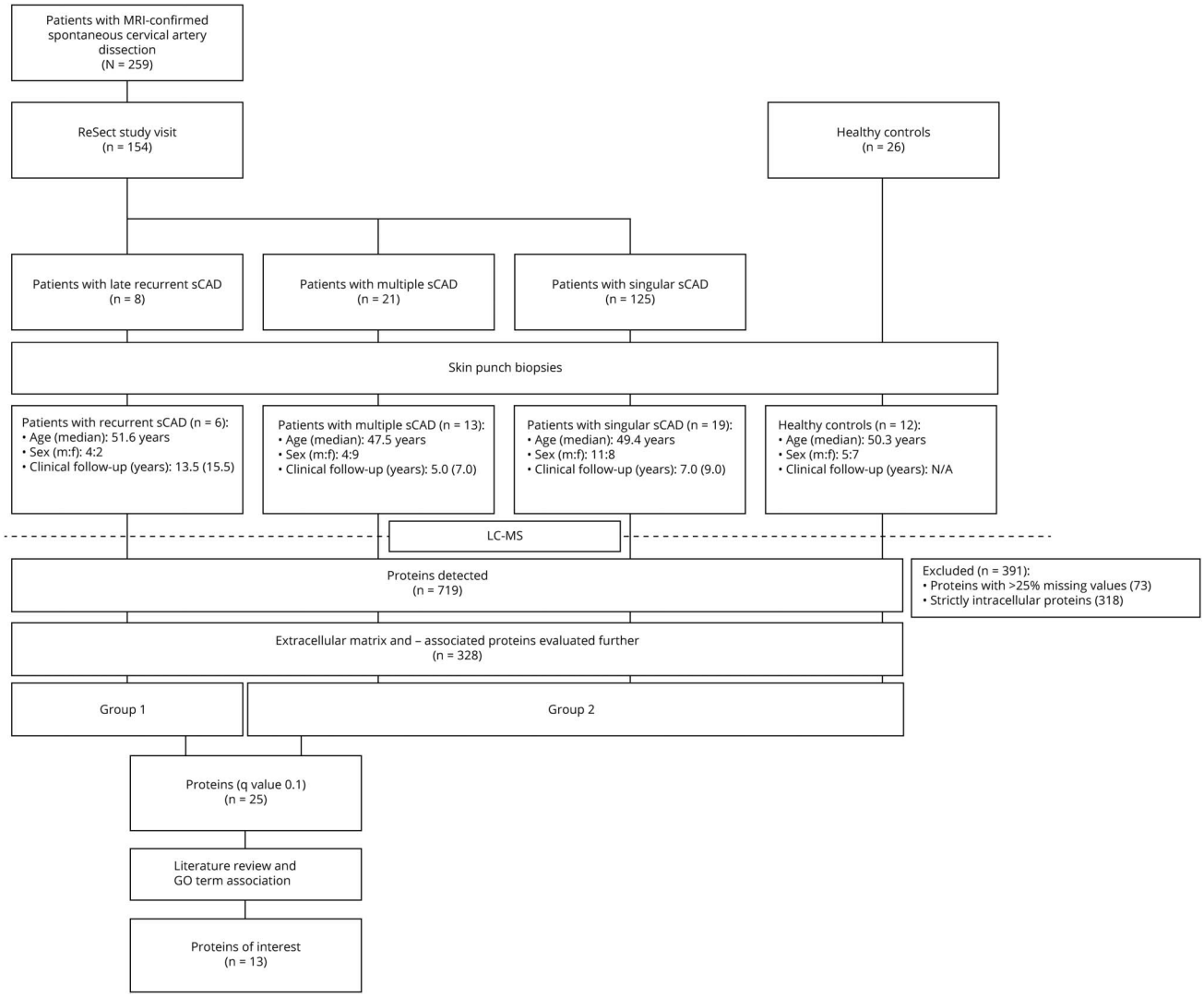
The aim of this study was to identify extracellular protein aberrations in patients with recurrent sCeAD by applying a state-of-the-art tissue proteomics approach.

## Methods

### Patient recruitment and selection

Patients treated at the Department of Neurology at the Medical University of Innsbruck between July 1996 and May 2015 were screened by full-text search of electronic medical records of inpatients and outpatients for the terms dissection, dissected, dissecting, intramural, and flap and consequently assessed for eligibility. After that, we prospectively screened all inpatients or outpatients treated at the Department of Neurology until December 31, 2017. Patients were included if (1) the diagnosis of CeAD was confirmed by MRI documentation of the intramural hematoma in T1-weighted fat-saturated sequences, (2) the CeAD occurred spontaneously or after minimal trauma (e.g., hyperextension, rotation, or lateroversion of the neck), and (3) the CeAD had an extradural origin (extension of CeAD to the V4 segment was not an exclusion criterion). We excluded patients with high-impact trauma and signs of external or internal injury other than CeAD and those with sole intracranial artery dissection. We invited the remainder to an in-person follow-up visit. Patients were screened for clinically obvious signs of hereditary monogenetic connective tissue disease by an experienced

**Figure 1** Flowchart




Flowchart of patient recruitment and proteomics analysis. GO = Gene Ontology; LC-MS = liquid chromatography–mass spectrometry; sCAD = spontaneous cervical artery dissection.

dermatologist (general examination) and stroke neurologists (adapted structured examination[12]) separately and were excluded from this analysis if either physician expressed suspicion. We grouped all participants to (1) recurrent sCeAD occurring >6 months after the qualifying event, (2) 1-time multiple-vessel sCeAD at baseline (diagnosed at initial MRI or follow-up imaging within the first month) without recurrence during clinical follow-up, and (3) 1-time single-vessel sCeAD without recurrence during clinical follow-up. We additionally recruited healthy volunteers through public notice as a control group. Details of the so-called ReSect study were published previously.[13]

## Skin punch biopsy

Biopsies were taken from the lower trunk by the same experienced dermatologist using a 4-mm biopsy punch (Kai Medical, Solingen, Germany), immediately rinsed with NaCl 0,9% to get rid of obvious blood contaminants, and stored at −80°C.

## Proteomics

The protocol for ECM protein analysis was adapted from a previous publication.[14,15] ECM- and ECM-associated proteins were extracted with a published 3-step method.[14] The addition of 0.5 mol/L NaCl buffer to samples extracted newly synthesized and loosely bound proteins. We achieved tissue decellularization by using a low-concentration sodium dodecyl sulfate (0.1%) buffer to destabilize membranes and to remove intracellular components without disrupting more soluble, non-integral ECM components. The addition of a buffer containing 4 mol/L guanidine hydrochloride (GuHCl) extracted heavily crosslinked proteins and proteoglycans. Lastly, in adding PNGase-F, we enzymatically removed glycan portions from

**Figure 2** Overall differences in protein abundance between groups

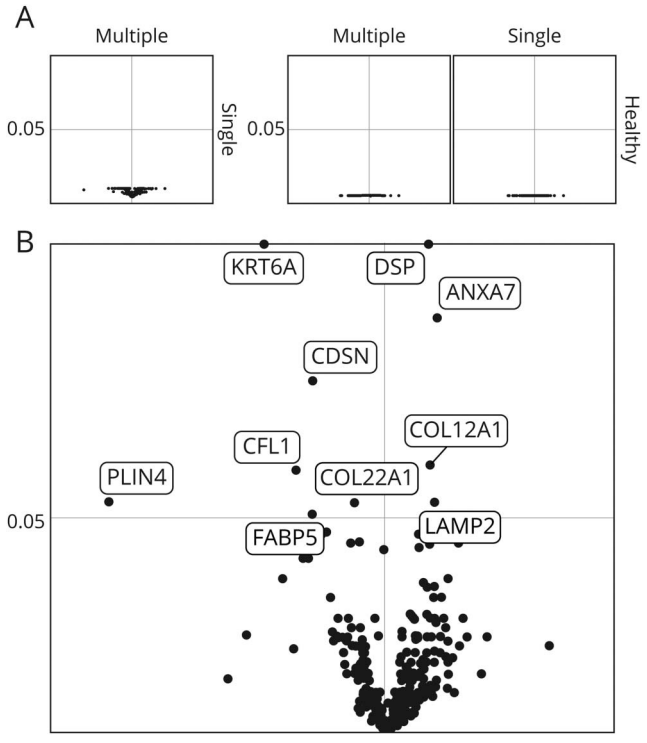



Illustration of (A) null findings in protein abundance between patients with 1-time single-vessel or multiple-vessel spontaneous cervical artery dissection (sCeAD) and healthy controls and (B) differences between those with recurrent sCeAD and the other groups combined.

core proteins to limit interference during liquid chromatography (LC)–tandem mass spectrometry (MS/MS).

### LC-MS/MS analysis

A nanoflow LC system separated the purified peptide samples before we injected them onto a trap column. A nano LC gradient separated the peptides. The eluate was sprayed into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Waltham, MA) operating in data-dependent top speed mode (cycle time 3 seconds). We acquired a survey full scan spectra over the mass-to-charge (m/z) range of 350 to 1,500 using Orbitrap detection (resolution 120,000 at 200 m/z). Dynamic exclusion duration was 60 seconds. The use of quadrupole isolation, collision-induced dissociation activation, and ion trap detection provided a data-dependent MS2 scan. Thermo Scientific Proteome Discoverer software (version 2.2.0.388) was used to search raw data files against the human database (UniProtKB/Swiss-Prot version January 2017) using Mascot (version 2.6.0, Matrix Science, Chicago, IL). The mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for fragment ions, keeping only high-confidence identifications. We used trypsin as a protein-digestion enzyme with up to 2 missed cleavages allowed. The chosen dynamic modifications were carbamidomethylation of cysteine; N-terminal acetylation; oxidation of methionine, lysine, and proline; and deamidation of asparagine in the presence of $^{18}O$ water. The last modification accounts for the detectable mass shift through deamidation of asparagine to aspartic acid during deglycosylation. We normalized the data to the total peptide amount.

### Western blot

Using the deglycosylated GuHCl samples, we validated the proteomics findings by immunoblotting. Antibodies for COL1A1 (sc-8783) were tested in 6 patients with recurrent sCeAD and 8 controls.

### Statistical methodology

We used the $\chi^2$ test and Kruskal-Wallis test for categorical variables and Wilcoxon test for continuous variables to examine group differences (i.e., age, sex, clinical characteristics) in baseline characteristics. Group differences in protein levels are expressed as ratio of means (fold change), and an unequal variance $t$ test tested significance. The Benjamini-Hochberg procedure controlled false discovery rate, with a value of $q < 0.1$ deeming significance.

### Gene set overrepresentation

We annotated proteins using the official National Center for Biotechnology Information (NCBI) Gene Symbol and added NCBI gene identifications for performing gene set overrepresentation analysis. The Database for Annotation, Visualization and Integrated Discovery version 6.8 tool was used for overrepresentation (Gene Ontology [GO] terms) analysis. We used NCBI gene identifications as identifiers and the STRING tool to construct a functional protein association network, which we imported into Cytoscape version 3.5.1 for additional data exploration.

### Standard protocol approvals, registration, and patient consents

The local ethics committee approved this analysis, and patients and healthy controls who took part in the ReSect study signed appropriate informed consent according to the Declaration of Helsinki.

### Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

## Results

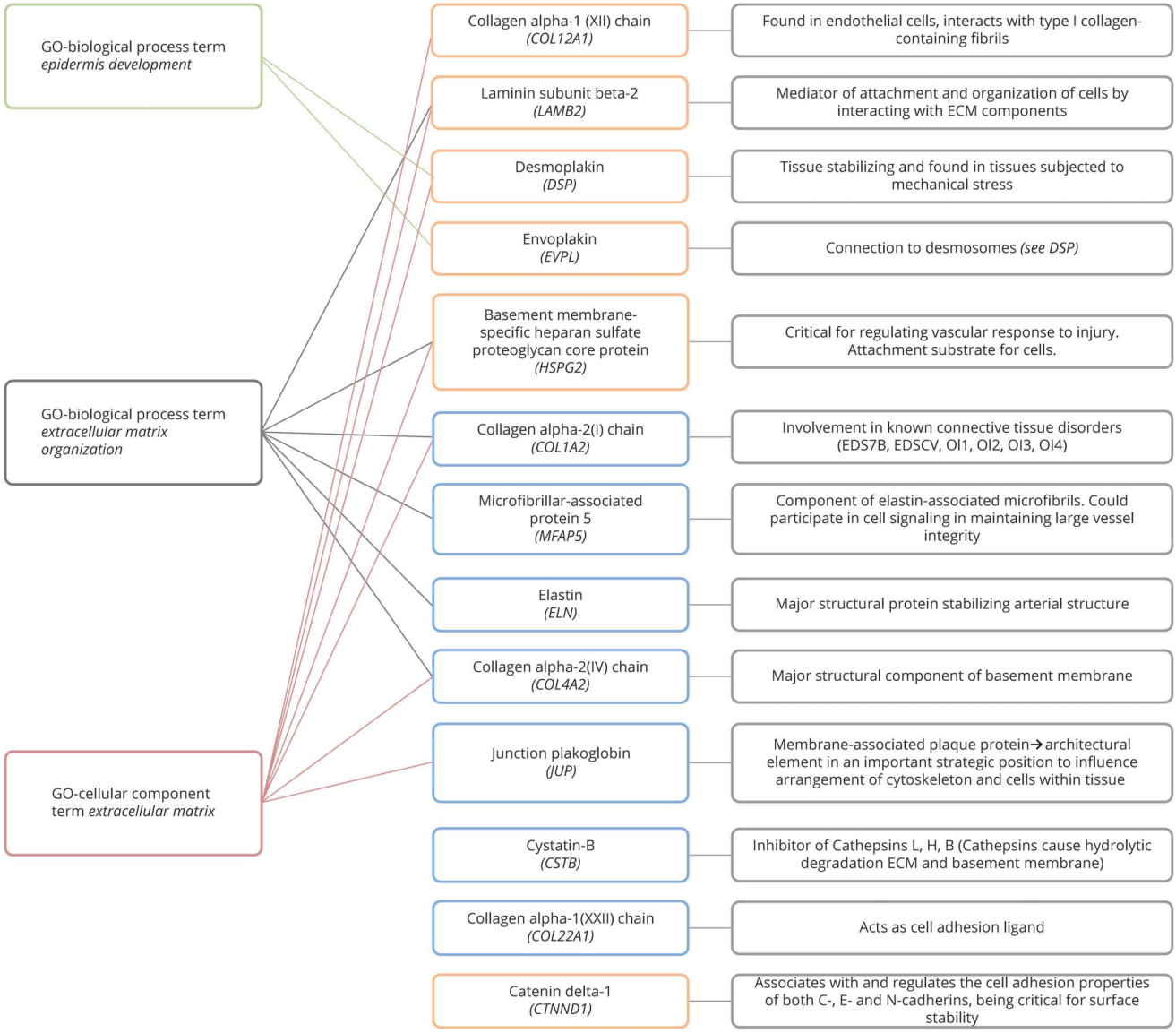
### Patient characteristics

The flowchart of patient recruitment and selected patient characteristics is shown in figure 1.

Patients who volunteered to have a skin punch biopsy taken did not significantly differ in relevant clinical characteristics from those who did not (data not shown). None of the patients had clinical stigmata suggestive of connective tissue disease.

There was no significant difference in clinical characteristics such as age, sex, presence of ischemia, prior minor trauma,

**Figure 3** Proteins of interest




List of proteins of interest after Gene Ontology (GO) term annotation check and literature research. Orange and blue indicate upregulation and downregulation, respectively. ECM = extracellular matrix.

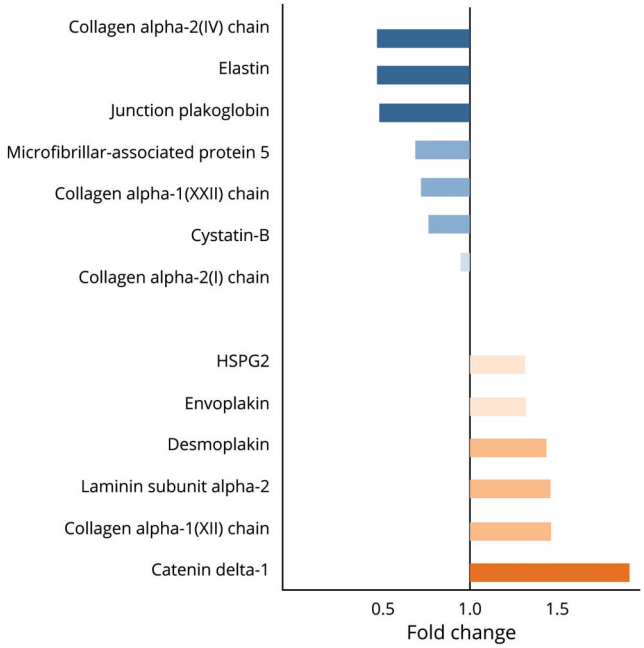
recent infection, vascular risk factors, or vessel status due to sCeAD between the various groups. Localization of initial sCeAD was significantly more likely to be in anterior circulation vessels in patients with recurrence compared to others (table 1, online repository, doi.org/10.5061/dryad.z34tmpg95). In addition, patients with recurrent sCeAD were less likely to have local symptoms, especially head/neck pain, compared to the other groups. These group differences did not remain significant after adjustment for multiple testing (data not shown). We found at least 1 subtle sign of connective tissue disorders in 18 of 38 (47.4%) patients with sCeAD and in 4 of 12 (33.3%) healthy controls. Prevalence of subtle signs of connective tissue disorders did not differ significantly in patients with late recurrent sCeAD (2 of 6, 33.3%), 1-time multiple-vessel sCeAD (5 of 13, 38.5%), and 1-time single-vessel sCeAD (11 of 19, 57.9%) and healthy controls (4 of 12, 33.3%). None of the included participants or healthy controls had a family history of sCeAD.

## Proteomics

The flowchart of patients includes LC-MS/MS results of proteins identified in the GuHCl fraction (figure 1). A total of 73 proteins that were not detectable in ≥25% (≥12 of 50) of samples were excluded. After the exclusion of 318 strictly intracellular proteins, 328 ECM and ECM-associated proteins were evaluated further.

Figure 2, A and B illustrates that there was no difference in protein abundance between patients with 1-time single-vessel

**Figure 4** Fold change in proteins of interest




Fold change in mean abundance of proteins of interest between patients with recurrent spontaneous cervical artery dissection and others. Orange and blue indicate upregulation and downregulation, respectively.

or multiple-vessel dissection or between these 2 groups and healthy controls, contrary to results comparing patients with recurrent sCeAD to all others. We uploaded 2 tables containing a summary of all identified proteins and the 25 differentially expressed ones with their relative difference in expression (fold-change) to the online data repository (tables 2 and 3, online repository, doi.org/10.5061/dryad.z34tmpg95).

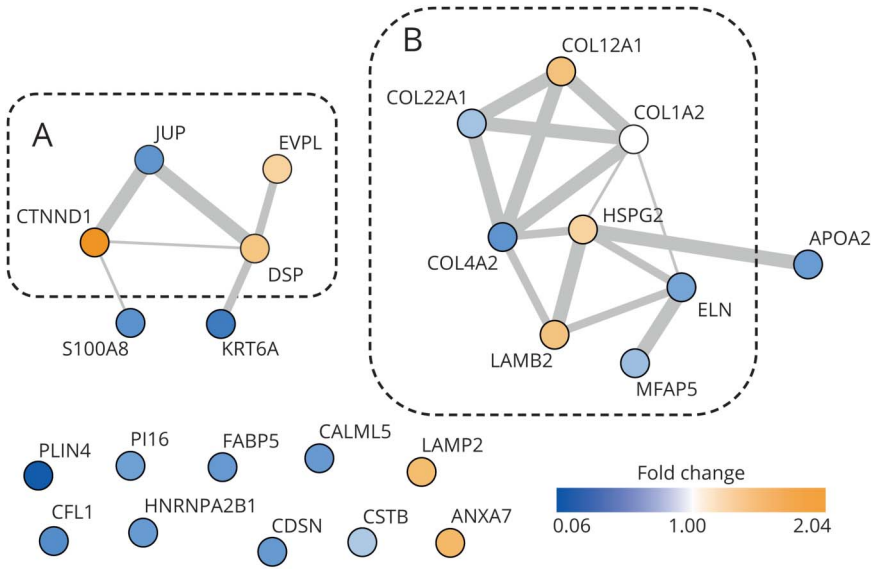
## Functional analysis

In a first step, we analyzed all 25 proteins by GO term annotation check, highlighting 3 GO terms relevant to the hypothesis of connective tissue disorder. (1) The biological process term epidermis development yielded 5 overrepresented proteins (DSP and EVPL upregulated; CALML5, FABP5 and CDSN downregulated; $p = 0.002$). (2) The biological process term ECM organization showed 6 proteins (LAMB2 and HSPG2 upregulated; MFAP5, ELN, COL4A2, and COL1A2 downregulated; $p = 0.0006$). (3) The cellular component term ECM provided 7 proteins (COL12A1, DSP, LAMB2, and HSPG2 upregulated; COL1A2, COL4A2, and JUP downregulated; $p < 0.0001$). In total, because 1 protein may be associated with multiple GO terms, the 3 selected GO terms highlighted 10 proteins.

In a second step, all 25 proteins with values of $q < 0.1$ underwent literature review for potential associations with connective tissue disorders or role in structural tissue integrity. The 10 proteins highlighted by GO term annotation check and 13 proteins with potential relationships to connective tissue disease according to literature review are depicted in figure 3. Figure 4 illustrates the fold-change differences of these 13 proteins between patients with recurrent sCeAD and others.

Two protein clusters of special interest were identified in protein-protein interaction analysis with STRING (figure 5). (1) The desmosome-associated protein cluster contains proteins with tissue stabilizing function in tissues subjected to mechanical stress (DSP), DSP-associated proteins (EVPL), strategically important elements for arrangement of cytoskeleton and cells within tissue (JUP), and critical proteins for surface stability (CTNND). (2) The collagen and elastin cluster consists of collagens (COL12A1, COL1A2, COL22A1, COL4A2), elastin and elastin components (ELN,

**Figure 5** Clustering of proteins




After the 13 proteins of interest were highlighted in the preconstructed Cytoscape network, 2 main clusters were identified: (A) desmosome-associated proteins cluster and (B) collagen and elastin cluster.

MFAP5), mediators of attachment and organization of cells interacting with ECM components (LAMB2, HSPG2), and proteins that are critical for regulating vascular response to injury, i.e., perlecan (HSPG2). Western blotting achieved the validation of one of the proteins of these clusters (COL1A1).

## Discussion

We present a proteomics-based analysis of patients with sCeAD revealing a specific ECM- and ECM-associated protein signature in individuals with sCeAD recurrence. Previous studies have suggested that sCeAD emerges in part on the basis of predisposing aberrations of connective tissue. (1) Clinical stigmata of connective tissue disease are more frequent in patients with sCeAD compared to those with ischemic stroke unrelated to sCeAD.[16] (2) On a molecular level, transmission electron microscopy previously demonstrated ultrastructural dermal connective tissue abnormalities in collagen fibril and elastic fiber formation in up to half of patients with sCeAD.[9,17,18] (3) Genetic studies highlighted variations of genes affecting the cardiovascular system in patients with sCeAD[19,20] and copy number variant enrichment in genes involved in ECM and collagen fibril organization[21] and, more recently, individuals with a family history of sCeAD.[20] However, both familial occurrence[22,23] and monogenetic inherited connective tissue disorders are rare in large cohorts of patients with sCeAD,[24–27] supporting the hypothesis of a polygenetic and multifactorial origin of disease.[12,28,29]

Our extracellular proteomics approach did not reveal differences in tissue protein-patterns between healthy individuals and patients with 1-time single-vessel or multiple-vessel sCeAD. However, there were substantial differences in skin biopsy protein expression profiles between patients who have had recurrent sCeAD and all others, indicating that connective tissue abnormalities may be relevant primarily to this subgroup of patients. In our analysis of protein abundance, we could identify 13 proteins that play a role in connective tissue integrity and functionality (figure 3). Functional protein association networking identified a clustering of these proteins (figure 5), including a cluster of structural collagen and elastin proteins and a desmosome-associated protein cluster.

To date, a connection between desmosome-associated proteins and recurrent sCeAD was not reported. There is compelling evidence that these proteins play an important role in the integrity of tissue subjected to mechanical stress through their function as adhesive intercellular junctions.[30] However, little is known about the role of desmosomes in endothelial cell and vessel wall development, formation, and healing. Electron microscopic evaluations of hypertension-induced arterial lesions suggested that desmosomes might be involved in late steps of endothelial healing.[31] Four proteins connected to desmosome function showed a significantly different expression pattern in patients with recurrent sCeAD, suggesting a pathophysiologic role of desmosome-related proteins in sCeAD recurrence.

An additional 8 proteins formed a collagen and elastin cluster. Several of these proteins are known to be related to or even to cause connective tissue diseases such as Marfan or Ehlers Danlos syndrome.[32] Recently, genetic variants with a causal link to sCeAD being evident primarily in those with a family history of sCeAD, not those with recurrence, has been reportet.[20] Still, patients with recurrent sCeAD had genetic variants suggestive of connective tissue aberration, especially in genes coding for different structurally integral collagens, which suggests the possibility of aberrations being elusive on a genetic level but evident on a proteome level.

Furthermore, cystatin B is of interest, even if it is not included in the previously discussed clusters, because it acts as an inhibitor of cathepsins L, H, and B. Lower expression in patients with recurrent sCeAD might cause overactivation of the cathepsins, resulting in hydrolytic degradation of ECM components.

An unmet challenge in the clinical management of sCeAD patients is the proper identification of patients at risk of recurrence. Valid biomarkers in this context would support counseling of individual patients and would help define the duration of antithrombotic treatment, possibly even enabling the development of specific therapies once pathophysiologic pathways are fully elucidated. Our study lays the foundation for such developments.

So far, prior studies hypothesized that a substantial number of patients with sCeAD have subclinical connective tissue disorder. Concerning further research, our results indicate that it might be more rewarding to focus on differences in gene and protein expression between patients with and without recurrent sCeAD instead of comparing them with healthy controls or patients with stroke of other etiologies.

Strengths of this study are the stringent inclusion criteria of this well-characterized single-center cohort that includes only patients with a definite sCeAD diagnosis. Furthermore, the ReSect study relies on a long-term in-person follow-up of patients with sCeAD and is the first to use cutting-edge proteomics techniques. Limitations are that not all patients consented to have skin punch biopsies performed, yet all patients who had recurrent dissection did. One further limitation pertains to limited sample size. Because this study breaks novel ground, an explorative design has been used, and the findings await large-scale validation. To reduce the risk of false-positive findings, we used the Benjamini-Hochberg approach to account for multiple testing. Protein expression characterization in skin rather than vessel samples also is a limitation, but most of the identified extracellular proteins in the skin are present in vessels as well. Finally, patients attributed to the 1-time dissection groups may experience recurrence later on, although this is unlikely considering the low overall risk of recurrence and mean follow-up time beyond 5 years.

This study unravels an extracellular protein signature of recurrent sCeAD suggestive of connective tissue disease in these patients, with the prospect of future clinical translation.

## Study funding

The data were acquired through the ReSect study performed at the Medical University Innsbruck called. The ReSect study is funded by the OeNB Anniversary Fund (No. 15644). Stefan Kiechl, Johann Willeit, Manuel Mayr, and Michael Knoflach are also supported by VASCage, Research Centre on Vascular Ageing and Stroke (No. 868624). As a COMET center, VASCage is funded within the COMET program: Competence Centers for Excellent Technologies by the Austrian Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology, the Austrian Ministry for Digital and Economic Affairs, and the federal states Tyrol, Salzburg, and Vienna. Manuel Mayr is a British Heart Foundation (BHF) chair holder (CH/16/3/32406) with BHF program grant support (RG/16/14/32397). Javier Barallobre-Barreiro is supported by a BHF Intermediate Basic Science Research Fellowship (FS/19/33/34328).

## Disclosure

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## Publication history

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| **Raimund Pechlaner, MD, PhD** | Department of Neurology, Medical University Innsbruck, Austria | Data analysis, provided figures |
| **Javier Barallobre-Barreiro, PhD** | Cardiovascular Division, King's College London, UK | Experiment organization, sample extraction and quantification, Western blot, extraction method paragraph |
| **Christian Boehme, MD** | Department of Neurology, Medical University Innsbruck, Austria | Data acquisition and critical revision of the manuscript |
| **Thomas Toell, MD** | Department of Neurology, Medical University Innsbruck, Austria | Data acquisition and critical revision of the manuscript |
| **Marc Lynch, PhD** | Cardiovascular Division, King's College London, UK | Sample deglycosylation, digestion, and C18 clean-up |
| **Xiaoke Yin, PhD** | Cardiovascular Division, King's College London, UK | MS method design and MS operation, provided Methods section for the manuscript |
| **Johann Willeit, MD** | Department of Neurology, Medical University Innsbruck, Austria | Data acquisition and critical revision of the manuscript |
| **Elke R. Gizewski, MD** | Department of Neuroradiology, Medical University Innsbruck, Austria | Data acquisition and critical revision of the manuscript |
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| **Gudrun Ratzinger, MD** | Department of Dermatology, Medical University Innsbruck, Austria | Data acquisition and critical revision of the manuscript |
| **Stefan Kiechl, MD** | Department of Neurology, Medical University Innsbruck, Austria | Clinical examinations, data interpretation, and critical revision of the manuscript |
| **Manuel Mayr, MD, PhD** | Cardiovascular Division, King's College London, UK | M.M.'s group (M.L., X.Y., J.B.-B.) performed protein extractions and MS analyses and Western blots, critical revision of the manuscript |
| **Michael Knoflach, MD** | Department of Neurology, Medical University Innsbruck, Austria | Conceptualized the study, literature review for individual proteins, assisted in drafting of the manuscript |

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