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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1038/JID.2015.367>.

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Sub6 (Tri r 2), an Onychomycosis Marker Revealed by Proteomics Analysis of *Trichophyton rubrum* Secreted Proteins in Patient Nail Samples



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TO THE EDITOR

Onychomycosis, the most prevalent nail disease, is mainly caused by two dermatophyte species, *Trichophyton rubrum* and *Trichophyton interdigitale*, with a frequency in the range of 80% and 20%, respectively (Monod et al., 2002). To determine if the proteases secreted by *T. rubrum* in vitro during keratin digestion were involved in nail degradation, we investigated the fungus secretome in onychomycosis by proteomics analysis.

In a first experiment, mass spectrometry analyses were performed using a pool of extracts from 12 donors infected by *T. rubrum* as described in the [Supplementary Materials and Methods](#) online. Patient consent for experiments was not required because French laws consider human tissue leftover from surgery as discarded material. Proteins were extracted from

each sample using a nonionic acid labile surfactant (ALS-400). The secretion of the following four secreted proteases of *T. rubrum* proteins extracted from nail beds was identified after subsequent SDS-PAGE separation and *in-gel* digestion coupled to mass spectrometry analysis: subtilisin-like protease 6 (Sub6, Q9UW97), subtilisin-like protease 7 (Sub7, Q8NID9), dipeptidyl-peptidase 5 (DppV, Q9UW98), and leucine aminopeptidase 2 (Lap2, Q5QHG6) (Table 1 and [Supplementary Table S1](#) online). In particular, 12 unique peptides were found for Sub6, suggesting that this protease was abundantly secreted during nail infection. No *T. rubrum* proteins were detected in the collected samples from abnormal nails with trauma but without fungal infection (data not shown). No additional proteins were identified from

a sequential second extract, suggesting that all soluble secreted proteins were already extracted in the first extraction ([Supplementary Materials](#) online). The high amount of Sub6 secreted by *T. rubrum* in onychomycosis and the presence of DppV were confirmed by Western blot analysis and by a shotgun protein identification experiment in SDS-PAGE gels using the same pooled extract for mass spectrometry analysis and specific antisera ([Supplementary Table S2 and Figure S1](#) online). Surprisingly, most proteases secreted by the fungus during its in vitro growth in a keratin medium including subtilisin-like protease 3 (Sub3, B8XGQ6), subtilisin-like protease 4 (Sub4, A7UKV6), leucine aminopeptidase 1 (Lap1, Q5QHG5), dipeptidyl peptidase 4 (DppIV, Q5J6J3), and metalloproteinase (M14A, A6XGK3) (Giddey et al., 2007; Zaugg et al., 2008) were not detected either by mass spectrometry or by Western blot analysis ([Supplementary Table S3 and Figure S1](#) online).

Abbreviations: MRM, multiple reaction monitoring

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Table 1. *Trichophyton rubrum* proteins identified by LC-MS/MS in infected nail beds samples

Accession	Description	Score	Coverage	No. of proteins	No. of unique peptides	Band
Q9UW97	Subtilisin-like protease 6	205.96	46.12	1	12	1, 2, 3, 4, 5, 6, 7
F2SX42	Polyubiquitin	119.92	70.74	1	0	
F2SEJ4	Ubiquitin	62.22	26.8	1	0	
Q6QUM1	Actin	29.27	18.4	1	0	
Q8NID9	Subtilisin-like protease 7	18.22	22	1	4	5
F2SH28	Enolase	8.34	12.56	1	2	
Q9UW98	Dipeptidyl-peptidase 5	6.48	7.16	1	3	1
F2SUU6	Glucan 1,3-beta-glucosidase	6.24	18.16	1	3	
F2SLY0	Putative uncharacterized protein	6.13	34.25	1	3	
F2SZI9	Beta-glucosidase	4.59	7.79	1	2	
F2SN23	Superoxide dismutase [Cu-Zn]	3.95	25.32	1	2	
F2SU38	Hsp70-like protein	2.26	6.88	1	1	
Q5QH66	Leucine aminopeptidase 2			1	3	3

Proteins were extracted from infected nail beds samples using nonionic surfactant ALS-400. Proteins were separated by SDS-PAGE, and then digested using trypsin before peptide identification by mass spectrometry (Supplementary Table S1). Proteins of *T. rubrum* were identified in the protein grouping mode. Similarly proteases of *T. rubrum* proteins were also identified by mass spectrometry analysis after SDS-PAGE from pieces of gel corresponding to positive immunostaining observed in Western blot analysis as indicated in Supplementary Figure S1. In the right column, the numbers of bands in which the proteases were identified are indicated. Lap2 was identified by SDS-PAGE/LC/MS/MS in the second analysis coupled to Western blot. Twelve peptides of Sub6 could be identified by LC/MS/MS after separation of proteins using SDS-PAGE with an Orbitrap velos (Thermo Scientific, Bremen, Germany) as a mass spectrometer. In crude extracts from infected nail samples, only four Sub6 peptides could be identified with high confidence without extensive sample preparation and SDS-PAGE using TripleTOF 5600 System (ABSciex, Redwood Shores, Canada), Orbitrap EliteTM (Thermo Scientific, Bremen, Germany), and Maxis impact (Bruker, Bremen, Germany) mass spectrometers (data not shown). No additional proteins of *T. rubrum* were identified from crude extracts without SDS-PAGE separation.

Abbreviations: LC, liquid chromatography; MS, mass spectrometry.

Table 2. Detection of *Trichophyton rubrum* Sub6 in clinical samples by multiple reaction monitoring (MRM)

Mycology positive	Sub6 positive (total sample)
<i>Trichophyton rubrum</i>	98 (108)
<i>Trichophyton rubrum</i> (+ melanine, black pigmentation)	3 (3)
<i>Trichophyton rubrum</i> (africanis)	0 (1)
<i>Trichophyton rubrum</i> (+ <i>Scopulariopsis brevicaulis</i>)	0 (1)
<i>Trichophyton rubrum</i> (+ pyocyanique)	1 (1)
<i>Trichophyton rubrum</i> (Mycelium observed with negative mycology)	3 (4)
	105 (118)
<i>Trichophyton mentagrophytes</i> var. <i>interdigitalis</i>	0 (19)
<i>Trichophyton soudanense</i>	0 (1)
<i>Epidermophyton floccosum</i>	0 (1)
Mycology positive (molds and yeasts)	
<i>Scytalidium dimidiatum</i>	0 (6)
<i>Scytalidium hyalinum</i>	0 (2)
<i>Fusarium</i>	0 (4)
<i>Aspergillus</i>	0 (6)
<i>Scopulariopsis brevicaulis</i>	0 (2)
<i>Acremonium</i> sp	0 (1)
<i>Candida albicans</i>	0 (3)
	0 (24)
Mycology negative	
<i>Psoriasis Onychopathy</i>	0 (2)
<i>Onychogryphosis</i>	0 (1)
Trauma	0 (10)
Others	1 (42)
	1 (55)

Quantifications of Sub6 across 218 clinical samples using an MRM assay including samples infected by *T. rubrum*, negative controls and other nail diseases (for details see Supplementary Table S5).

We developed multiple reaction monitoring (MRM) assays to quantify *T. rubrum* Sub3, Sub4, Sub6, Sub7, DppIV, DppV, Lap1, Lap2, and M14A directly from nail crude protein extracts as described in the Supplementary Material. MRM is a powerful method for sensitive quantitative measurement of target proteins (Castro-Gamero et al., 2014; Keshishian et al., 2007). Sub6 was detected in all infected nail bed samples but not in the control samples (trauma) or in the blank sample (heavy peptides only). Sub7 and DppV were detected in few samples (Supplementary Table S4 online). This may be related to the abundance and/or the stability of these proteins. Lap2 was detected rarely and at very low intensity in agreement with the faint detection of Lap2 using Western blot analysis, suggesting that Lap2 is discreetly produced in onychomycoses (Supplementary Table S2 and Figure S1). No other proteases secreted by the fungus during its growth in vitro in a keratin medium (i.e., Sub3, Sub4, Mep3, Mep4, Lap1, DppIV, and M14A) could be detected in the protein extracts from infected human nails despite the sensitivity of the method and therefore did not appear to be involved during the establishment of

onychomycosis. Quantifications of Sub6, Sub7, and DppV were subsequently performed across 218 clinical samples using MRM assay including negative controls and other nail diseases (Supplementary Table S5 online). Sub6 was detected in almost all samples infected by *T. rubrum* (105 of 118 samples) with one or more peptides, but not in the other samples including nail trauma, psoriasis, and nail beds infected by other pathogens (molds and yeasts) (Table 2). However, a restricted number of samples (13%) identified as positive after mycology culture and/or direct observation were not identified as positive in the MRM assay. Four of 118 of these samples after extraction from nail beds showed a very low concentration of proteins—less than 0.08 mg/ml—which may explain the negative response in the MRM assay. In addition, three samples (mycological culture negative) were identified as positive by MRM and direct mycological examination. The comparison between the clinical evaluation from direct mycological examination and culture results and our mass-spectrometry-based assay showed a good correspondence, as indicated by the high scores of the sensitivity (0.89) and the specificity (0.99) of the assay. Positive and negative predictive values showed important scores: 0.99 and 0.88, respectively. From these results, Sub6 can be considered as a marker of *T. rubrum* nail infection. The identification and quantification of Sub6-specific peptides using MRM were restricted to *T. rubrum* (Table 2). Putative *T. interdigitale* Sub6 could not be identified using this MRM assay because the amino acid sequences of the peptides used for MRM are different in the *T. interdigitale* corresponding peptides (Supplementary Table S6 online).

The gene encoding Sub6 was previously revealed by microarray analysis to be the most upregulated protease gene during skin infection in guinea pigs with the dermatophyte *Arthroderma benhamiae* (Staib et al., 2010). As in the present investigations on onychomycosis, none of the *A. benhamiae* genes encoding specific endo- and exoproteases involved in keratin digestion in vitro was found to

be upregulated during skin infection. In contrast to *T. rubrum*, which is anthropophilic, *A. benhamiae* is zoophilic and causes highly inflammatory cutaneous infections in humans and rodents. As a general conclusion, the proteases secreted in vitro during protein degradation and in vivo during infection are different whatever the dermatophyte species and the tinea.

Sub6 and DppV were first described as the major allergens Tri r 2 and Tri r 4, respectively, in *T. rubrum* (Woodfolk, 2005; Woodfolk et al., 1998). These antigens were found to induce dual immune responses and elicit either immediate hypersensitivity or delayed-type hypersensitivity skin test reactions in different individuals. Exposure to *Trichophyton* proteins may result in bronchial sensitization and symptomatic asthma that can be controlled with systemic antifungal therapy (Ward et al., 1989, 1999; Woodfolk, 2005; Woodfolk et al., 1998). There are also eczematous skin reactions to dermatophytes that are distant of the area of the dermatophytes. These skin reactions, called dermatophytids, are generally reported in patients with tinea pedis, most often with *T. interdigitale* but also with *T. rubrum* (Ilkit et al., 2012; Veien et al., 1994). The dermatophytids go away once the dermatophyte infection has been cured. In cases of eczematous skin reaction of unknown origin, it could be relevant to perform skin test reactions using both Sub6 and DppV antigens. A positive reaction could be indicative of a nondetected dermatophyte infection and suggest further clinical investigations for antifungal treatment.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Nail samples were collected from patients by medical doctors in the Dermatology Department at Saint Louis Hospital (Paris). The samples were analyzed as a part of an internal quality control program according to hospital internal practice and French regulation. Mass spectrometric analyses and database searching for protein were performed (by Jean-William Dupuy) at Plateforme Proteome, Functional Genomic Centre of Bordeaux, University of Bordeaux, Bordeaux, France. MRM assays were developed by Biognosis AG, Wagistrasse, Schlieren, Switzerland. We thank Alexandre Genette and Alexia Seraidaris for their technical assistance, Galderma R&D, Les Templiers, Sophia Antipolis, France.

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SUPPLEMENTARY MATERIAL

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Sub6 (Tri r 2), an onychomycosis marker revealed by proteomics analysis of *Trichophyton rubrum* secreted proteins in patient nail samples

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SUPPLEMENTARY FILES

MATERIAL AND METHODS

Clinical samples.

Protein extraction from beds of infected human nails.

Western blot analysis.

Sample preparation for mass spectrometry analysis.

LC-MS/MS analysis.

Database searching.

Sample preparation for MRM assays.

LC-MRM.

FIGURES AND TABLES

Supplementary Figure S1: Western blot analysis coupled to mass spectrometry analysis.

Supplementary Figure S2a: LC-MRM assays for heavy peptides of selected *T. rubrum* proteins. Black vertical lines represent the predicted retention time after iRT calibration.

Supplementary Figure S2b: Chromatograms for the 6 most intense transitions of the Sub6 peptide ADFSNYGAVVDVYAPGK in sample infected by *T. rubrum*.

Supplementary Table S1: Proteins identified by SDS-PAGE and LC/MS/MS from nail bed samples with *T. rubrum* as an infectious agent.

Supplementary Table S2: Peptides of proteases (*T. rubrum*) identified by mass spectrometry analysis from gels corresponding to positive immuno staining observed in western blot analysis.

Supplementary Table S3: Peptide sequences for LC-MRM assays.

Supplementary Table S4: Overview on light/heavy ratios of target peptides across sample series used in set up MRM assays.

Supplementary Table S5: Overview on light/heavy ratios of target peptides across sample series.

Supplementary Table S6: Alignment of peptide sequences of Subtilisin-like protease 6.

MATERIALS AND METHODS

Clinical samples.

All samples were collected from patients examined for suspected onychomycosis by medical doctors in the Dermatology Department at Saint Louis Hospital (Paris). Our study was performed as a part of an internal quality control program in according to Hospital internal practice and French regulation. Patient consent for experiments was not required because French laws consider human tissue left over from surgery as discarded material. Samples from patients having had topical therapy were not included in this study. Nail samples (N=218) were obtained by scratching the bed of the nail showing hyperkeratosis at the junction of healthy and pathological nail after removing the nail tablet using sterile scalpel blades and tweezers. Samples (nail beds) were collected in a sterile petri dish. Routinely, one portion of each clinical sample was used for direct examination in a dissolving chlorazol black E solution (Feuilhade de Chauvin, 2005). In parallel, another portion of each sample was divided into two test tubes. The first tube contained Sabouraud's agar medium with chloramphenicol (50 µg/mL) and gentalline, and the second tube contained Sabouraud's agar medium with chloramphenicol, gentalline, and cycloheximide (Bio-Rad, Hercules, CA, USA). The cultures were incubated at 27°C. Growing fungi were identified after 21 days of growth by macroscopic and microscopic examination. A third portion of the samples was finally stored at room temperature in a dry box for further protein extraction. Clinical samples were considered as negative in the absence of fungal elements in direct microscopic observation and after three weeks of culture associated with the absence of fungi in the hands and feet. Clinical samples were considered as positive based on a positive direct mycological examination and after three weeks of growth in culture medium. Mould contaminations were based on direct observation and after culture in the absence of

associated dermatophyte. *Candida albicans* were characterised by the observation of pseudo-hyphae and blastopores and from culture.

Protein extraction from beds of infected human nails.

Bed-nails (scratch) were suspended in a 1.5 ml Eppendorf tube with 100 µl or 200 µl, depending on the amount of dry sample of 50 mM bicarbonate ammonium (Sigma, St. Louis, MO, USA) containing 0.1% of Progenta™ Non-ionic Acid Labile Surfactant ALS-400 (Proteabio, Nîmes, France). Extraction was performed at 4°C under stirring (1400 rpm) for 120 min in a thermo mixer (Eppendorf, Hamburg, Germany). Protein extracts were centrifuged at 4°C at 14000 rpm for 10 min. Supernatant were filtered through a 0.22 µm filter for clearing by centrifugation (Ultra free-MC PVDF, Millipore, Billerica, MA, USA). Following the first extraction, pellets were submitted to a second extraction using 100 or 200 µl of buffer H total protein extraction kit Dualsystem with glass beads as recommended by manufacturer (Biotech, Zurich, Switzerland). Samples were homogenised manually using a small potter and then stirred at 4 °C for 120 min as described above. After centrifugation at 4°C for 10 min (12000 rpm), supernatants were collected and stored at -80 °C until used. The protein concentration was determined using the colorimetric method (DC protein assay, Bio-Rad Laboratories, Hercules, CA, USA) and serum albumin as standard.

Western blot analysis.

Western blot analysis was performed from pooled nail bed protein sample. Proteins were extracted as described above. Protein extracts were diluted in loading buffer (4% SDS, 20% glycerol and bromophenol blue) containing 10 mM dithiothreitol (Sigma, St. Louis, MO, USA) and heated at 80°C for 10 min. Proteins were loaded on a 4–20% polyacrylamide gel Nupage

(Invitrogen, San Diego, CA, USA). For SDS-PAGE, PageRuler™ Plus Prestained Protein Ladder (Fermentas, Hanover, MD, USA) were used as molecular weight markers. After SDS-PAGE, the proteins were directly transferred onto nitrocellulose with I-blot apparatus (Invitrogen, San Diego, CA, USA). Then the nitrocellulose membrane was briefly washed with water and incubated for 30 min. at room temperature with blocking buffer (LI-COR Bioscience, Lincoln, NE, USA). Primary antibody (Sub3: SZ1452, Sub4: SZ2229, Sub6: SZ2233, DppIV: SZ1910, DppV: SZ1909, Lap1: SZ1511, Lap2: SZ1510, M14A: SZ2661) (Monod *et al*, 2005; Giddey *et al*, 2007; Zaugg *et al*, 2008) diluted 1:3000 in blocking buffer was added to the membrane for 120 min at room temperature. The membrane was washed twice with PBS containing 0.1% of Tween 20 (Sigma, St. Louis, MO, USA) for 15 min and then incubated for 1 h at room temperature with the diluted secondary antibody (1:10000) coupled to a fluorochrome for infrared detection, anti-rabbit-800 (Li-Cor., Inc., Lincoln, NE, USA). The membrane was first washed as previously and then with PBS for 10 min. The blot was scanned with an Odyssey scanner. Recombinant proteins were used as positive controls in western blot analysis.

Sample preparation for mass spectrometry analysis.

Mass spectrometry analysis was performed directly from crude protein extracts. For the protein identification step, sample preparations were performed from a pool of human nails infected by *T. rubrum* and in the same manner using nails with a trauma negative in the mycology analysis. Proteins were digested using trypsin as recommended by the manufacturer (Sigma, St. Louis, MO, USA). Briefly, protein extracts were normalised to approximately 0.2 µg/µl with 100 mM ammonium bicarbonate to 100µl final volume. Samples were reduced with a final concentration of 10 mM dithiothreitol at 56 °C for 60 min. Samples were then alkylated with a final concentration of 20 mM iodoacetamide (Sigma, St. Louis, MO, USA) at room temperature for 45

min and trypsin was added to a final ratio of 1/50 (w/w) enzyme-to-protein. Digestion was performed at 37 °C for 18 h. To remove ALS-400, samples were acidified to pH 2.0 with 1% formic acid, incubated at room temperature for 30 min and spun at 14,000 rpm for 5 min to remove hydrolysed ALS-400. Digested proteins were dried using a speed vacuum and then diluted into 50 µl of 3% acetonitrile containing 0.1% formic acid. Samples were submitted to Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described below. Alternatively, approximately 60 µg of proteins were separated by SDS-PAGE and then submitted to *in gel*-trypsin digestion as described by Shevchenko *et al.* (2006). Eight large bands from SDS-PAGE were obtained to performed *in-gel* digestion.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Online nanoLC-MS/MS analyses were performed using an Ultimate 3000 system (Dionex, Amsterdam, Netherlands) coupled to a nanospray LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Ten microliters of each peptide extract were loaded on a 300 µm ID x 5 mm PepMap C18 precolumn (LC Packings, Dionex, USA) at a flow rate of 30 µL/min. After 5 min of desalting, peptides were separated online on a 75 µm ID x 15 cm C18PepMap™ column (LC Packings, Dionex, USA) with a 5–40% linear gradient of solvent B for 108 min. (solvent A was 0.1% formic acid in 5% ACN and solvent B was 0.1% formic acid in 80% ACN). The separation flow rate was set at 200 nl/min. The mass spectrometer operated in positive ion mode at a 1.8-kV needle voltage and a 42-V capillary voltage. Data were acquired in a data-dependent mode alternating an FTMS scan survey over the range m/z 300–1700 with the resolution set to a value of 60,000 at m/z 400 and six ion trap MS/MS scans with collision-induced dissociation (CID) as activation mode. MS/MS spectra were acquired using a 3- m/z unit ion isolation window and normalised collision energy of 35. Mono-charged ions and unassigned

charge-state ions were rejected from fragmentation. Dynamic exclusion duration was set to 30 sec.

Database searching.

Mascot and Sequest algorithms through Proteome Discoverer 1.3 Software (Thermo Fisher Scientific, Inc.) were used for protein identification in batch mode by searching against a Reference Proteome Set Homo sapiens database from the UniProt website combined to a UniProt database restricted to *Trichophyton rubrum* (74,008 entries, release 2012_03). Two missed enzyme cleavages were allowed. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.8 Da. Oxidation of methionine (dynamic modification) and carbamidomethylation on cysteine (static modification) were searched as variable modifications. Peptide validation was performed using the Percolator algorithm (Käll *et al.*, 2007) and only “high confidence” peptides were retained, corresponding to a 1% false positive rate at the peptide level.

Sample preparation for MRM assays.

50 µg total proteins of each nail bed sample were prepared in 8 M urea (Sigma, St. Louis, MO, USA) and 100 mM ammonium bicarbonate (final concentration). Proteins were reduced by incubation for 1 h at 37 °C with 5mM TCEP (Sigma, St. Louis, MO, USA). Alkylation was carried out using 10 mM iodoacetamide for 30 min in the dark at room temperature. Proteins were digested to peptides using trypsin (sequencing grade, Madison, USA) at a protease:protein ratio of 1:50. Digestion was carried out overnight at 37 °C. Peptides were cleaned up for mass spectrometric analysis using a 96-well C18 spin-plate (The Nest Group, Southborough, MA, USA) according to the manufacturer’s instructions. Peptides were dried down to complete dryness and resuspended in LC solvent A (1% acetonitrile, 0.1% formic acid [FA]).

LC-MRM.

Peptides for LC-MRM assays of Sub3, Sub4, Sub6, Sub7, Lap1, Lap2, DppIV, DppV, and M14A were selected based on LC-MS/MS analysis of *in gel*-digest of purified recombinant proteases and peptides identified in mass spectrometry experiments in our exploratory phase using clinical samples. Signal intensity in LC-MRM during the assay development phase was also considered to select peptides. Synthetic custom-made heavy-labelled reference peptides were produced by Thermo Fisher Scientific (Ulm, Germany). An assay panel was developed to simultaneously measure 37 peptides as light (endogenous) and heavy (reference peptides) forms (Table S3). The light background for each heavy-labelled reference peptide was determined. All reference peptides showed a light background of less than 0.1% and were therefore of high quality for relative quantification. For each protein, the LC-MRM assay for one heavy-labelled reference peptide is shown as an example in Figure S2, panel a. A balanced pool of heavy-labelled reference peptides and Biognosys's iRT-peptides were spiked to each sample (Escher *et al.*, 2012). All peptides were measured in scheduled LC-MRM mode with 3 minute acquisition windows after a calibration run (unscheduled LC-MRM for iRT-peptides only). Resulting chromatograms for light and heavy peptides ideally look like the example shown for the Sub6 peptide ADFSNYGAVVDVYAPGK in Figure S2, panel b. Peptides (calculated amount 0.5µg per sample) were injected to a C18 column (Magic AQ, 3 µm particle size, 200 Å pore size, Michrom [Michrom Bioresources]; 11 cm column length, 75 µm inner diameter, [New Objective, Inc.]) on an Easy nLC nano-liquid chromatography system (Proxeon Biosystems, Odense, Denmark). LC solvents were A: 1% acetonitrile in water with 0.1% FA; B: 3% water in acetonitrile with 0.1% FA. The LC gradient was 5–35% solvent B in 30 minutes followed by 35–100% B in 2 minutes and 100% B for 8 minutes (total gradient length was 40 minutes). A TSQ

Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was equipped with a standard nano-electrospray source. For relative quantification of the peptides across samples, the TSQ Vantage was operated in scheduled MRM mode with an acquisition window length of 3 min. The LC eluent was electrosprayed at 1.9kV, and MRM analysis was performed using a Q1 peak width of 0.7 Da. Collision energies were calculated for the TSQ Vantage by a linear regression according to the vendor's specifications. For the relative quantification of the peptides across the sample series, the six most intense transitions of each assay were measured for the light and heavy version of each peptide. Samples were spiked with a balanced pool of heavy-labelled reference peptides at the same level in all samples. The transition list contained 485 transitions, including the iRT-peptides. Acquisition windows for the scheduled LC-MRM measurement were set to 3 min after a calibration run in unscheduled mode in sample 12 (Escher *et al.*, 2012). Light to heavy ratios for each peptide were determined using SpectroDive™, Biognosys's proprietary software tool based on mProphet (Reiter *et al.*, 2011), and exported to Microsoft Excel for further analysis.

Additionally, an MRM assay was performed using a restricted number of heavy peptides for DppV (TLYVTAEDHATGK; INFVGYGQSTTK; GGVGIWISDAK; GDLYNEAAAK; IYDSLFR; ALVSHDGTFTVGSSK; and VIHIAEVGTNKPVR) and for Sub6 (ADFSNYGAVVDVYAPGK; VLDCDGSNSGVIK; and KPGGTTYDYDPSAGK). NanoHPLC was performed using a nano-capillary system NCS-3500RS UHPLC (Thermo Scientific). NanoPump UPHLC 1000 bar and micropump 500 bar (for online preconcentration) was used with the followed columns: Acclaim PepMap RSLC (Thermo Scientific) 75µm x 15cm, nanoViper C18, 2 µm, 100 Å and as pre-column: Acclaim Pepmap 100 C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm. Autosampler WPS-3000RS, Software Chromeleon 6.80, heated column compartments with two high-pressure switching valves. A nano-electrospray

source was used with uncoated SilicaTips, 10.5 cm length, 360 µm outer diameter, 20 µm inner diameter. For ionisation, 1500 V of liquid junction voltage and 275 °C capillary temperature were used. Parallel reaction monitoring (PRM) analyses were performed using a Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). The method was performed in three steps, one Full MS and two identical tMS² as follows: Full MS event: run time 110 minutes, positive polarity, with 17,500 of resolution, AGC target: 3e6, Max IT: 50 min, Mass range: 400 to 2000 m/z, Spectrum data type: Profile. Targeted-MS² event (*2): run time: 110 minutes, positive polarity, with 17,500 of resolution, AGC target: 5e4, Max IT: 50ms, Isolation window: 1.6 m/z, Fixed first mass: 150 m/z, NCE (collision energy): 27. PRM data of the QExactive were acquired via Xcalibur 2.07 (Thermo Scientific). The most abundant transitions for each peptide were used for quantification. Peak detection and integration were based on two criteria: 1) the same time of retention from heavy peptide and 2) the same relative PRM peak intensity ratios across multiple transitions between light peptide and heavy peptide standards. All data were manually inspected to ensure correct peak detection and accurate integration. The raw data from QExactive were loaded into Skyline 2.6.0 64 bits software (available from MacCoss Lab at the University of Washington) in order to create high-resolution figures of extracted ion chromatograms of multiple transitions monitored for target proteins.

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FIGURES AND TABLES

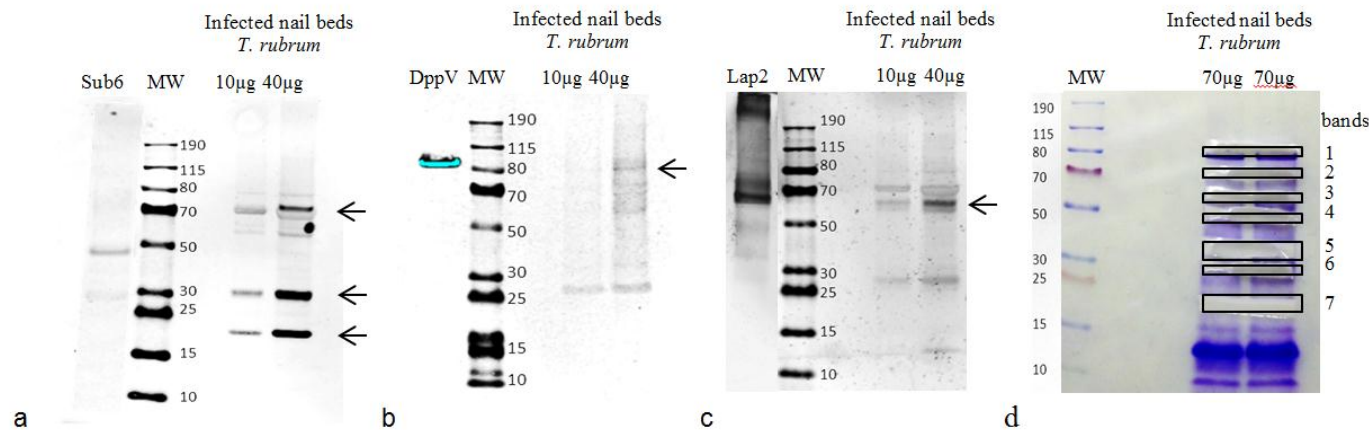


Figure S1: Western blot analysis coupled to mass spectrometry analysis. Recombinant proteins were used as positive controls for western blot analysis. Detection of Sub6 (panel a), DppIV (panel b), Lap2 (panel c). Coomassie blue stained bands (panel d) corresponding to immunoreactive bands were used for MS analysis.

Comment: Sub6, DppV and major proteases known to be secreted by *T. rubrum* during its growth in a keratin medium (Giddey *et al.*, 2007) were tentatively detected by western blot using the same pooled extract for mass spectrometry analysis. Immunolabelling using anti-Sub6 antiserum revealed strong signals (panel a). A positive immunoreactivity at the expected size, corresponding to the mature form of DppV (90 kDa) and Lap2 were also recorded using anti-DppV antiserum and anti-Lap2 antiserum (panel b and c, respectively). A 40 kDa band was faintly detected in the nail bed protein samples using anti-Sub4 antibody but probably related to it cross-reactivity with recombinant Sub3 as well as with recombinant Sub6 (data not shown) confirming the lack of detection by mass spectrometry analysis in the infected nail beds (Table 1). Leucine aminopeptidase (Lap1), Dipeptidyl peptidase (DppIV) and metallocarboxypeptidase (M14A) were not detected by western blot analysis

with antisera, which produced a strong specific signal with recombinant proteins (Monod *et al.*, 2005; Zaugg *et al.*, 2008). These data were in agreement with mass spectrometry analysis. A shotgun protein identification experiment in SDS-PAGE gels was undertaken on the basis of the signals obtained by western blot analyses. Seven gel bands were excised (panel d, bands 1 to 7), and were *in gel*-digested. Surprisingly, Sub6 was identified by mass spectrometry analysis in all seven gel samples (Table 1 and Table S3). The predicted molecular weight of Sub6 was 42.7 kDa, but detection of Sub6 in lower bands (band 7) may reflect degradation of Sub6 product occurring *in vivo* or during sample preparation. Sub6 detection in bands above 42 kDa can be related to protein aggregations. Sub7, DppV and Lap2 were identified by mass spectrometry analysis in bands 5, 1, and 3, respectively (Table 1 and Table S3). The localisation of these three proteases was in accordance with their predicted molecular mass of 40, 90, and 65 kDa, respectively, attesting that they were apparently not degraded in the infected nail beds or during sample preparation. Altogether, the results obtained from western blot and LC-MS/MS analyses confirmed that Sub6 was the major protease secreted by *T. rubrum* in nail beds during onychomycosis.

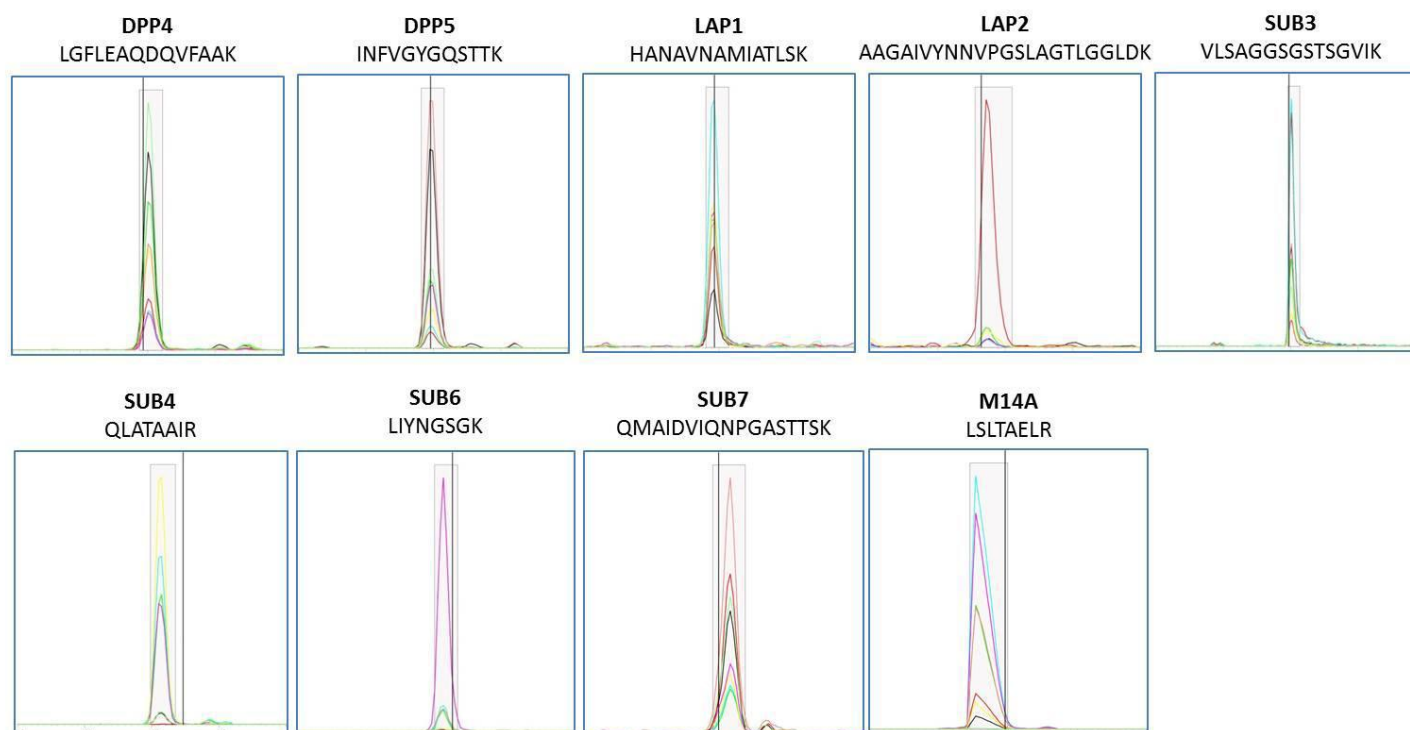


Figure S2a: LC-MRM assays for heavy peptides of selected *T. rubrum* proteins. Black vertical lines represent the predicted retention time after iRT calibration.

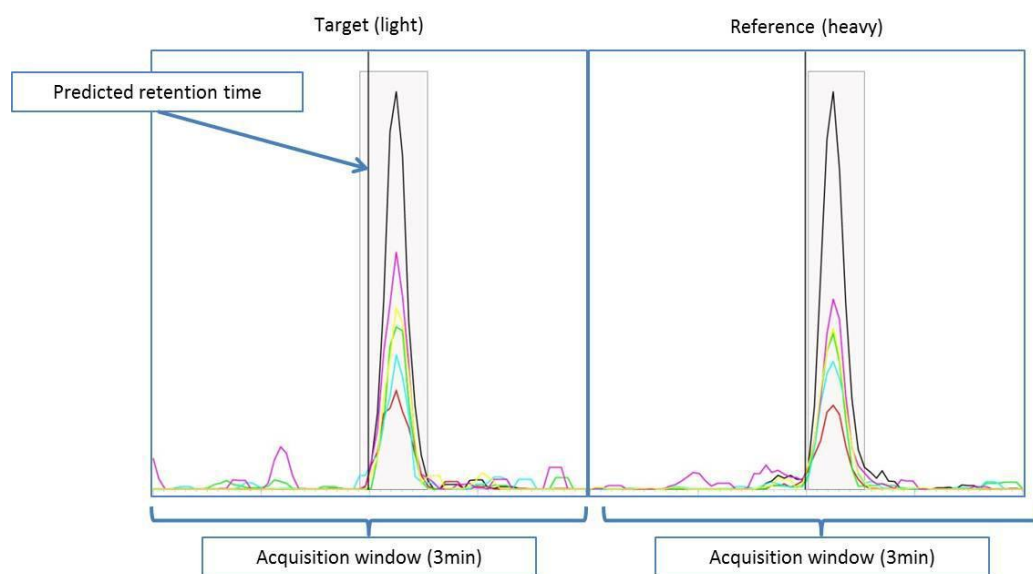


Figure S2b: Chromatograms for the 6 most intense transitions of the Sub6 peptide ADFSNYGAVVDVYAPGK in sample infected by *T. rubrum*. The left panel shows the light channel (the Sub6 peptide present in the sample), the right panel represents the heavy reference peptide. The black vertical line shows the predicted retention time after iRT calibration. Colors represent corresponding transitions, e.g. the black line is the transition monitoring the singly charged fragment ion y11. Transitions must be co-eluting at the same retention time for the light and the heavy peptide and fragmentation patterns (represented by the peak height of the color-coded individual transitions) are very similar. The light grey box represents the peak borders that were selected by mQuest for quantification. Acquisition window length in scheduled MRM was 3min.

Table S1: Proteins identified by SDS-PAGE and LC/MS/MS from nail bed samples with *Trichophyton rubrum* as an infectious agent.

Accession	Description Name, species, gene name.	Score	Coverage	# Proteins	# Unique Peptides
P02768	Serum albumin OS=Homo sapiens GN=ALB	6385.99	85.06	7	76
P02787	Serotransferrin OS=Homo sapiens GN=TF	1402.29	70.06	5	57
P29508	Serpin B3 OS=Homo sapiens GN=SERPINB3	1069.85	75.13	1	19
P01857	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1	888.71	62.12	1	8
P01859	Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2	787.11	63.19	1	7
P01834	Ig kappa chain C region OS=Homo sapiens GN=IGKC	715.52	94.34	1	12
P01024	Complement C3 OS=Homo sapiens GN=C3	522.84	53.04	2	65
P48594	Serpin B4 OS=Homo sapiens GN=SERPINB4	510.92	58.46	3	9
P01009	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1	437.57	56.7	1	26
P01860	Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3	422.5	46.95	1	5
P01023	Alpha-2-macroglobulin OS=Homo sapiens GN=A2M	366.93	50	3	48
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16	335.49	50.53	13	17
P0CG05	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2	331.62	74.53	4	2
E7EUE8	Uncharacterized protein OS=Homo sapiens GN=KRT6A	314.82	46.73	27	1
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B	304.86	44.86	27	3
P25311	Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1	300.01	61.07	4	19
Q01469	Fatty acid-binding protein, epidermal OS=Homo sapiens GN=FABP5	297.16	79.26	2	18
P01861	Ig gamma-4 chain C region OS=Homo sapiens GN=IGHG4	294.28	54.13	1	4
P04406	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH	275.55	71.04	5	16
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1	244.28	19.83	1	17
B9A064	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens GN=IGLL5	240.06	35.98	2	2
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14	230.02	43.43	13	12
P00738	Haptoglobin OS=Homo sapiens GN=HP	223.69	56.16	1	12
P31151	Protein S100-A7 OS=Homo sapiens GN=S100A7	206.14	71.29	1	8
Q9UW97	Subtilisin-like protease 6 OS=Trichophyton rubrum GN=SUB6	205.96	46.12	1	12
Q04695	Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17	204.43	38.66	13	10
F5H265	Uncharacterized protein OS=Homo sapiens GN=UBC	193.86	96.64	21	15
F5GZC0	Uncharacterized protein OS=Homo sapiens GN=SERPINB7	179.78	55.92	4	18
P01011	Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3	169.19	32.15	1	10

P06702	Protein S100-A9 OS=Homo sapiens GN=S100A9	166.58	85.96	1	11
P02790	Hemopexin OS=Homo sapiens GN=HPX	151.23	56.93	2	19
P02766	Transthyretin OS=Homo sapiens GN=TTR	141.65	69.39	3	11
P00450	Ceruloplasmin OS=Homo sapiens GN=CP	141.38	39.15	3	24
P01876	Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1	139.95	55.81	2	10
P68871	Hemoglobin subunit beta OS=Homo sapiens GN=HBB	138.86	89.8	2	7
P06733	Alpha-enolase OS=Homo sapiens GN=ENO1	137.98	63.59	9	20
P80188	Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2	128.34	66.16	1	12
A2BHY4	Complement component C4B (Chido blood group) OS=Homo sapiens GN=C4B-1	124.1	24.37	19	25
P02774	Vitamin D-binding protein OS=Homo sapiens GN=GC	113.69	30.59	5	10
Q08188	Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3	106.78	45.02	2	21
E7EU87	Uncharacterized protein OS=Homo sapiens GN=KRT5	104.27	27.75	20	6
Q08554	Desmocollin-1 OS=Homo sapiens GN=DSC1	103.25	20.81	1	12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10	101.2	39.38	2	17
F5H1A8	Uncharacterized protein OS=Homo sapiens GN=GSN	101.18	34.91	10	16
C9JM50	Uncharacterized protein OS=Homo sapiens GN=KRT19	100.87	28.57	9	1
P05089	Arginase-1 OS=Homo sapiens GN=ARG1	96.39	69.88	1	16
P01871	Ig mu chain C region OS=Homo sapiens GN=IGHM	93.7	30.31	1	2
P60174	Triosephosphate isomerase OS=Homo sapiens GN=TPI1	87.61	60.84	2	15
Q9UIV8	Serpin B13 OS=Homo sapiens GN=SERPINB13	85.28	45.52	3	10
P60709	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB	75.81	43.73	19	6
P02765	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG	75.63	37.87	3	8
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1	73.22	23.6	1	12
P05109	Protein S100-A8 OS=Homo sapiens GN=S100A8	71.51	60.22	1	13
P31944	Caspase-14 OS=Homo sapiens GN=CASP14	71.18	50.41	1	19
P04220	Ig mu heavy chain disease protein OS=Homo sapiens	65.9	32.74	1	1
P01620	Ig kappa chain V-III region SIE OS=Homo sapiens	64.53	66.06	4	5
Q92820	Gamma-glutamyl hydrolase OS=Homo sapiens GN=GGH	63.75	34.28	2	10
Q9NZT1	Calmodulin-like protein 5 OS=Homo sapiens GN=CALML5	63.15	84.25	1	11
P69905	Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1	63.1	55.63	1	6
B4E1D3	Uncharacterized protein OS=Homo sapiens GN=FGB	60.65	29.32	3	12
P02763	Alpha-1-acid glycoprotein 1 OS=Homo sapiens GN=ORM1	60.14	37.31	1	5
Q8IW75	Serpin A12 OS=Homo sapiens GN=SERPINA12	59.84	31.64	1	8

P02042	Hemoglobin subunit delta OS=Homo sapiens GN=HBD	57.54	63.95	3	3
P51884	Lumican OS=Homo sapiens GN=LUM	56.86	24.26	1	6
Q96P63	Serpin B12 OS=Homo sapiens GN=SERPINB12	56.32	37.53	2	10
P01040	Cystatin-A OS=Homo sapiens GN=CSTA	55.62	75.51	2	7
P00739	Haptoglobin-related protein OS=Homo sapiens GN=HPR	51.45	27.3	1	1
P12035	Keratin. type II cytoskeletal 3 OS=Homo sapiens GN=KRT3	51.03	7.01	7	1
P01610	Ig kappa chain V-I region WEA OS=Homo sapiens	50.64	37.04	3	3
A8K2U0	Alpha-2-macroglobulin-like protein 1 OS=Homo sapiens GN=A2ML1	50.57	9.97	4	10
A6NCP9	Retinol binding protein 4, plasma. isoform CRA_b OS=Homo sapiens GN=RBP4	49.41	62.31	3	8
B4E3A8	Uncharacterized protein OS=Homo sapiens GN=SERPINB1	48.34	45.75	3	12
P17900	Ganglioside GM2 activator OS=Homo sapiens GN=GM2A	47.94	35.75	2	6
P35908	Keratin. type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2	46.8	12.05	11	2
P11021	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5	44	25.08	2	12
Q86SG5	Protein S100-A7A OS=Homo sapiens GN=S100A7A	43.62	58.42	1	3
P19652	Alpha-1-acid glycoprotein 2 OS=Homo sapiens GN=ORM2	42.77	38.81	1	6
F5H308	L-lactate dehydrogenase OS=Homo sapiens GN=LDHA	40.85	26.64	10	5
C9JEU5	Uncharacterized protein OS=Homo sapiens GN=FGG	40.07	22.7	5	8
P05120	Plasminogen activator inhibitor 2 OS=Homo sapiens GN=SERPINB2	38.69	33.98	1	10
B4DKJ3	Uncharacterized protein OS=Homo sapiens GN=COMP	38.09	19.32	3	8
P27482	Calmodulin-like protein 3 OS=Homo sapiens GN=CALML3	37.12	64.43	1	8
E7EN95	Uncharacterized protein OS=Homo sapiens GN=FLNB	35.62	7.22	4	9
P04196	Histidine-rich glycoprotein OS=Homo sapiens GN=HRG	34.82	16.38	1	8
Q14574	Desmocollin-3 OS=Homo sapiens GN=DSC3	34.33	6.7	1	6
F8WE70	Uncharacterized protein OS=Homo sapiens GN=SERPINB13	33.8	36.97	1	1
P01764	Ig heavy chain V-III region VH26 OS=Homo sapiens	30.68	22.22	1	3
P01617	Ig kappa chain V-II region TEW OS=Homo sapiens	30.61	38.94	3	2
P01008	Antithrombin-III OS=Homo sapiens GN=SERPINC1	28.62	27.59	2	7
P35527	Keratin. type I cytoskeletal 9 OS=Homo sapiens GN=KRT9	28.08	13.32	1	5
P80748	Ig lambda chain V-III region LOI OS=Homo sapiens	27.95	37.84	1	3
B4E1H2	Uncharacterized protein OS=Homo sapiens GN=SERPING1	27.73	21.88	4	10
P01625	Ig kappa chain V-IV region Len OS=Homo sapiens	26.53	36.84	2	1
P06727	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4	24.38	19.19	1	6
E7ET08	Uncharacterized protein OS=Homo sapiens GN=HSPA8	23.98	23.89	14	3

B7Z549	Uncharacterized protein OS=Homo sapiens GN=ITIH1	23.86	13.88	6	5
B7Z5J4	Uncharacterized protein OS=Homo sapiens GN=CPA4	22.98	32.18	4	6
O76013	Keratin, type I cuticular Ha6 OS=Homo sapiens GN=KRT36	22.58	5.57	5	2
P43652	Afamin OS=Homo sapiens GN=	21.91	15.03	1	7
O94919	Endonuclease domain-containing 1 protein OS=Homo sapiens GN=ENDOD1	21.65	20.6	1	5
P00751	Complement factor B OS=Homo sapiens GN=CFB	21.13	13.74	5	7
P01598	Ig kappa chain V-I region EU OS=Homo sapiens	20.75	26.85	1	2
P31947	14-3-3 protein sigma OS=Homo sapiens GN=SFN	20.64	27.42	1	6
Q5T8M8	Actin, alpha 1, skeletal muscle OS=Homo sapiens GN=ACTA1	20.24	22.65	15	1
F8W7G7	Uncharacterized protein OS=Homo sapiens GN=FN1	19.97	4.7	4	6
P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2	19.59	30.3	2	6
P30086	Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens GN=PEBP1	19.31	49.2	2	5
P54652	Heat shock-related 70 kDa protein 2 OS=Homo sapiens GN=HSPA2	19.19	12.52	4	1
P06310	Ig kappa chain V-II region RPMI 6410 OS=Homo sapiens	19.14	15.04	1	1
A6NNY3	Uncharacterized protein OS=Homo sapiens GN=CHI3L2	19.11	25.26	9	6
F2ST98	Secreted serine protease OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_05699	18.22	22	2	4
P61916	Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2	18.21	36.42	3	4
P06312	Ig kappa chain V-IV region (Fragment) OS=Homo sapiens GN=IGKV4-1	17.93	29.75	3	1
Q02487	Desmocollin-2 OS=Homo sapiens GN=DSC2	17.76	14.65	3	7
P00491	Purine nucleoside phosphorylase OS=Homo sapiens GN=PNP	17.48	36.68	1	8
E7ET82	Uncharacterized protein OS=Homo sapiens GN=LYPD3	16.88	16.67	2	3
P08603	Complement factor H OS=Homo sapiens GN=CFH	16.35	5.85	6	6
P04217	Alpha-1B-glycoprotein OS=Homo sapiens GN=A1BG	15.58	12.93	1	4
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P02647	Apolipoprotein A-I OS=Homo sapiens GN=APOA1	15.05	27.72	2	6
A8MW50	L-lactate dehydrogenase OS=Homo sapiens GN=LDHB	14.82	11.64	3	1
P07339	Cathepsin D OS=Homo sapiens GN=CTSD	14.81	16.02	3	4
Q6P4A8	Phospholipase B-like 1 OS=Homo sapiens GN=PLBD1	14.74	14.1	2	6
P10909	Clusterin OS=Homo sapiens GN=CLU	14.52	16.93	2	5
P07108	Acyl-CoA-binding protein OS=Homo sapiens GN=DBI	14.49	54.02	3	4
P05090	Apolipoprotein D OS=Homo sapiens GN=APOD	14.42	19.58	2	3
P00441	Superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD1	14.23	53.9	1	3

Q9UBH0	Interleukin-36 receptor antagonist protein OS=Homo sapiens GN=IL36RN	14.07	39.35	2	4
P14618	Pyruvate kinase isozymes M1/M2 OS=Homo sapiens GN=PKM2	13.92	9.04	4	3
F5H194	Uncharacterized protein OS=Homo sapiens GN=ITIH4	13.86	9.8	5	5
P01621	Ig kappa chain V-III region NG9 (Fragment) OS=Homo sapiens	13.77	47	1	3
A6NDY9	Filamin A OS=Homo sapiens GN=FLNA	13.74	3.15	3	4
P12273	Prolactin-inducible protein OS=Homo sapiens GN=PIP	13.72	43.84	1	4
B8ZZK2	Uncharacterized protein OS=Homo sapiens GN=GGCT	13.69	31.93	2	3
P02750	Leucine-rich alpha-2-glycoprotein OS=Homo sapiens GN=LRG1	13.43	19.31	2	3
Q9NZH8	Interleukin-36 gamma OS=Homo sapiens GN=IL36G	13.22	37.87	1	5
C9JEX1	Uncharacterized protein OS=Homo sapiens GN=KNG1	13.16	18.93	3	4
B7Z4X2	Uncharacterized protein OS=Homo sapiens GN=LTF	12.8	7.66	4	3
O75629	Protein CREG1 OS=Homo sapiens GN=CREG1	12.49	12.27	1	2
P19957	Elafin OS=Homo sapiens GN=PI3	12.39	28.21	1	3
P00915	Carbonic anhydrase 1 OS=Homo sapiens GN=CA1	12.36	18.39	8	3
P01613	Ig kappa chain V-I region Ni OS=Homo sapiens	12.22	30.36	1	2
E7EP11	Uncharacterized protein OS=Homo sapiens GN=HSPA1A	11.94	17.24	8	1
B4E1B7	Uncharacterized protein OS=Homo sapiens GN=SERPINF2	11.68	14.52	4	4
Q5T985	Inter-alpha (Globulin) inhibitor H2 OS=Homo sapiens GN=ITIH2	11.63	5.99	3	3
P01779	Ig heavy chain V-III region TUR OS=Homo sapiens	11.3	26.72	1	2
P01019	Angiotensinogen OS=Homo sapiens GN=AGT	10.08	7.63	2	3
Q562R1	Beta-actin-like protein 2 OS=Homo sapiens GN=ACTBL2	9.76	9.04	1	1
P00747	Plasminogen OS=Homo sapiens GN=PLG	9.75	8.15	1	4
P15924	Desmoplakin OS=Homo sapiens GN=DSP	9.73	1.64	1	3
P35237	Serpin B6 OS=Homo sapiens GN=SERPINB6	9.68	21.81	2	5
C9JFR7	Uncharacterized protein OS=Homo sapiens GN=CYCS	9.66	38.61	2	3
A6NMY6	Putative annexin A2-like protein OS=Homo sapiens GN=ANXA2P2	9.49	8.26	3	3
Q15828	Cystatin-M OS=Homo sapiens GN=CST6	9.43	24.83	1	3
P63104	14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ	9.38	10.61	2	2
E9PIT3	Uncharacterized protein OS=Homo sapiens GN=F2	9.17	8.06	3	3
Q9Y3R4	Sialidase-2 OS=Homo sapiens GN=NEU2	8.95	8.16	1	2
P36955	Pigment epithelium-derived factor OS=Homo sapiens GN=SERPINF1	8.83	11	1	3
P62158	Calmodulin OS=Homo sapiens GN=CALM1	8.65	32.21	4	3
P01605	Ig kappa chain V-I region Lay OS=Homo sapiens	8.63	25	1	1

Q9GZP4	PITH domain-containing protein 1 OS=Homo sapiens GN=PITHD1	8.57	18.01	1	2
F2SH28	Enolase OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_01612	8.34	12.56	1	2
B4DUN2	Malate dehydrogenase OS=Homo sapiens GN=MDH1	8.29	19.59	6	4
F5H7T3	Uncharacterized protein OS=Homo sapiens GN=VCL	8.24	4.27	3	3
Q6NSD8	FGA protein OS=Homo sapiens GN=FGA	7.69	11.42	2	3
P29622	Kallistatin OS=Homo sapiens GN= SERPINA4	7.6	10.77	1	3
P02760	Protein AMBP OS=Homo sapiens GN=AMBP	7.36	8.81	1	2
P05156	Complement factor I OS=Homo sapiens GN=CFI	7.22	7.2	2	4
C9JGI3	Uncharacterized protein OS=Homo sapiens GN=TYMP	7.21	9.66	2	3
E9PHK0	Uncharacterized protein OS=Homo sapiens GN=CLEC3B	7.13	20.63	2	2
P01624	Ig kappa chain V-III region POM OS=Homo sapiens	6.53	24.77	1	1
Q9UW98	Dipeptidyl-peptidase 5 OS=Trichophyton rubrum GN=DPPV	6.48	7.16	1	3
Q8IXD7	Kallikrein 11 isoform 3 OS=Homo sapiens GN=KLK11	6.46	14.91	2	4
F2SUU6	Glucan 1,3-beta-glucosidase OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_06242	6.24	18.16	1	3
F2SLY0	Putative uncharacterized protein OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_02993	6.13	34.25	1	3
P09211	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1	5.97	17.14	1	2
B7Z7A9	Phosphoglycerate kinase OS=Homo sapiens GN=PGK1	5.92	9	2	2
P02652	Apolipoprotein A-II OS=Homo sapiens GN=APOA2	5.76	32	1	2
Q86SR0	Secreted Ly-6/uPAR-related protein 2 OS=Homo sapiens GN=SLURP2]	5.59	27.84	1	2
Q13867	Bleomycin hydrolase OS=Homo sapiens GN=BLMH	5.58	6.81	1	2
E9PFL7	Uncharacterized protein OS=Homo sapiens GN=GDI2	5.5	18.27	7	2
P12830	Cadherin-1 OS=Homo sapiens GN=CDH1	4.93	3.85	2	2
P35858	Insulin-like growth factor-binding protein complex acid labile subunit OS=Homo sapiens GN=IGFALS	4.83	5.29	2	2
B4DPU3	Uncharacterized protein OS=Homo sapiens GN=EEF2	4.73	7.07	2	2
F2SZI9	Beta-glucosidase OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_07959	4.59	7.79	1	2
Q06830	Peroxiredoxin-1 OS=Homo sapiens GN= PRDX1	4.25	9.55	1	1
F2SN23	Superoxide dismutase [Cu-Zn] OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_04335	3.95	25.32	1	2
F2SU38	Hsp70-like protein OS=Trichophyton rubrum (strain ATCC MYA-4607 / CBS 118892) GN=TERG_06505	2.26	6.88	1	1
P04080	Cystatin-B OS=Homo sapiens GN=CSTB	2.26	33.67	1	2
P07360	Complement component C8 gamma chain OS=Homo sapiens GN=C8G	2.21	19.31	1	2

B4DTY5	Uncharacterized protein OS=Homo sapiens GN=GDA	2.15	8.16	4	2
C9JTJ8	Uncharacterized protein OS=Homo sapiens GN=SERPINB8	1.76	16.13	4	2

Table S2: Peptides of proteases (*T. rubrum*) identified by mass spectrometry analysis from gels corresponding to positive immuno staining observed in western blot analysis.

BAND 1 (≈100 kDa)		
Accession	Description	ΣCoverage
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	15,53
	VLDcDGSGSNSGVik	
	mANDVIQSPGEGTTGK	
	KPGGTTYYYDPSAGK	
	GmEWAmR	
	SVmNmSLGGPR	
Q9UW98	Dipeptidyl-peptidase 5 Trichophyton rubrum [DPP5_TRIRU]	11,98
	GGVGIWISDAK	
	TPQGIEGASSPVFSPDGK	
	TLYVTAEDHATGK	
	ALVSHDGTfVgSSK	
	IAYLQmAAK	
	IYDSLFR	
	GGVGIWISDAK	
	INFVGYGQSTTK	
BAND 2 (≈80 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	18,45
	mANDVIQSPGEGTTGK	
	VLDcDGSGSNSGVik	
	SVmNmSLGGPR	
	KPGGTTYYYDPSAGK	
	QGGPGLcDTIKK	
	GmEWAmR	
BAND 3 (≈65 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	15.53
	mANDVIQSPGEGTTGK	
	VLDcDGSGSNSGVik	
	SVmNmSLGGPR	
	KPGGTTYYYDPSAGK	
	KPGGTTYYYDPSAGK	
	GmEWAmR	

Q5QH6	Leucine aminopeptidase 2 Trichophyton rubrum [LAP2_TRIRU]	7.68
	AIAYAIAEYAR	
	NVPAGGLFTGAEVVK	
	ASAmSYSPASK	
BAND 4 (≈60 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	17.96
	GVTAYIIDTGIDIDHEDFQGR	
	mANDVIQSPGEGTTGK	
	VLDcDGSGSNSGVK	
	KPGGTTYYYDPSAGK	
	GmEWAmR	
BAND 5 (≈40 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	11.41
	VLDcDGSGSNSGVK	
	GVTAYIIDTGIDIDHEDFQGR	
	SVmNmSLGGPR	
Q8NID9	Subtilisin-like protease 7 Trichophyton rubrum [SUB7_TRIRU]	
	GADKAVANmSLGGAFSQASNDAAAAIAK	7
	AVANmSLGGAFSQASNDAAAAIAK	
BAND 6 (≈35 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	7.77
	GVTAYIIDTGIDIDHEDFQGR	
	SVmNmSLGGPR	
BAND 7 (≈20 kDa)		
Q9UW97	Subtilisin-like protease 6 Trichophyton rubrum [SUB6_TRIRU]	13.59
	GVTAYIIDTGIDIDHEDFQGR	
	SVmNmSLGGPR	
	mANDVIQSPGEGTTGK	
	ADFSNYGAVVDVYAPGK	
	QGGPGLcDTIK	
	KmANDVIQSPGEGTTGK	

T. rubrum protease peptides identified by mass spectrometry analysis from piece of gels after SDS-PAGE and *in gel*-digestion using trypsin. The analysed bands corresponded to positive immuno stained bands observed by western blot analysis (Figure S1).

Table S3: Peptide sequences for LC-MRM assays.

Protein	Peptide sequence	Number of positive signals	Protein	Peptide sequence	Number of positive signals
DppIV	LYDSMYTER	0	M14A	VHIANMK	0
	LGFLAQDQVFAAK	0		GFVLPADQIIPSGEETLAGTVAMLK	0
	TITSNDALLEK	0		VLSAGGSGSTSGVIK	0
DppV	IYDSLFR	0	Sub3	AALNLSLGGFSQASNDVTR	0
	GDLYNEAAAK	0		LLYNGSGR	0
	LSVAEGVGLFNVLQEK	2		DYVFDDAGR	0
	VIHIAEVGTNKPVR	1	Sub4	QLATAAIR	0
	ALVSHDGTFTVGSSK	1		NANIVAVK	0
	GGVGIWISDAK	0		LIYNGSGK	0
	INFVGYGQSTTK	0	Sub6	AIPIVLAALSTVNGAR	0
	TLYVTAEDHATGK	1		KPGGTTYYYDPSAGK	9
	VSFGIITDNVNANLTK	0		MANDVIQSPGEGTTGK	10
Lap1	LIVGFVTELAKE	0	Sub7	VLDCDGSNSGVIK	8
	FLIELAPGETR	0		ADFSNYGAVVDVYAPGK	6
	HANAVNAMIAATLSK	0		SVMNMSLGGPR	0
	QEQVHLWSHAEALNANGK	0		VQINAIEQQDNVPSWGLAR	0
Lap2	GDTVANINK	0		AIPLALTAASVINGAEILETR	0
	AAGAIVYNNVPGSLAGTLGGLDK	4		QMAIDVIQNPGASTTSK	3
M14A	LSLTAELEK	0			

Heavy-labelled reference peptides integrated in the LC-MRM assay panel. The numbers of positive signals are indicated, in total, ten nail bed samples infected by *T. rubrum* were analysed. Nails with trauma were negative. Corresponding light/heavy ratios are illustrated in Figure S2.

Table S4: Overview on light/heavy ratios of target peptides across sample series used in set up MRM assays.

Sample No.	Mycology	Sub6 MANDVIQSP GEGTTGK	Sub6 VLDCDGS SNSGVIK	Sub6 KPGGTTY YDPSAGK	Sub6 ADFSNYGAVV DVIYAPGK	Sub7 QMAIDVIQN PGASTTSK	DppV LSVAEGVG LFNVLQEK	DppV IYDSLFR	DppV VIHIAEVG TNKPVQR	DppV TLYVTAE DHATGK	DppV ALVSHDG TFVGSSK	Lap2 AAGAIYNNVPG SLAGTLGGLDK
1	<i>T. rubrum</i>	0.008	0.364	0.058	0.013	ND	ND	ND	ND	ND	ND	ND
2	<i>T. rubrum</i>	0.062	2.491	0.248	ND	ND	ND	ND	ND	ND	ND	0.011
3	<i>T. rubrum</i>	0.003	0.023	ND	ND	ND	ND	ND	ND	ND	ND	0.001
4	<i>T. rubrum</i>	0.065	3.181	0.336	0.050	0.055	0.053	ND	0.012	0.015	0.378	ND
5	<i>T. rubrum</i>	0.005	ND	0.032	ND	ND	ND	ND	ND	ND	ND	ND
6	<i>T. rubrum</i>	0.012	ND	0.053	ND	ND	ND	ND	ND	ND	ND	ND
7	<i>T. rubrum</i>	0.052	3.145	0.671	0.217	0.001	ND	ND	ND	ND	ND	0.005
8	<i>T. rubrum</i>	0.116	3.196	0.392	0.056	ND	ND	ND	ND	ND	ND	ND
9	<i>T. rubrum</i>	0.049	1.553	0.555	0.456	ND	ND	ND	ND	ND	ND	ND
10	<i>T. rubrum</i>	0.134	6.128	1.575	0.603	0.075	0.086	ND	ND	ND	ND	0.008
33	trauma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
34	trauma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
--	blank	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Samples used in set up MRM assays and corresponding values light/heavy ratios. ND= peptides not detected. Nails with trauma were negative in mycology test. Sub6 was detected in all infected nail samples but not in the controls samples (trauma) or in the blank sample (heavy peptides only). Sub6 levels were highest in sample 10 and overall profiles for light/heavy ratios of three different Sub6 peptides were in good accordance. Sub6 was detected faintly in samples 3 and 5 but clearly distinguishable from background. Sub7 was detected with the peptide QMAIDVIQNPGASTTSK in few samples as well as for DppV using the assay for peptide LSVAEGVGLFNVLQEK in infected samples by *T. rubrum*. Other DppV peptides were detected (VIHIAEVGTNKPVR, TLYVTAEDHATGK, and ALVSHDGTFVGSSK) but at very low levels. Lap2 was rarely detected at very low intensity using the peptide AAGAIYNNVPGSLAGTLGGLDK. However, the signal was clearly distinguishable from measurements in blank samples and the two negative control samples. All other proteins (DppIV, Lap1, Sub3, Sub5, and M14A) were not detected in the protein extracts from infected human nails and were not retained for further MRM assays.

Table S5: Overview on light/heavy ratios of target peptides across sample series.

Sample No.	Mycology	SUB6 MANDVIQSPGEGTTGK	SUB6 VLDCDGSNSGVIK	SUB6 KPGGTTYYYDPSAGK	SUB6 ADFSNYGAVVDVYAPGK	SUB7 QMAIDVIQNPASTTSK	DPP5 LSVAEGVGLFNVLQEK	DPP5 IYDSLFR
	blank	ND	ND	ND	ND	ND	ND	ND
1	<i>T. rubrum</i>	0.0225	0.2061	ND	ND	ND	ND	ND
2	<i>T. rubrum</i>	0.3761	2.9031	0.4571	0.0886	ND	ND	ND
3	<i>T. rubrum</i>	ND	ND	ND	ND	ND	ND	ND
4	<i>T. rubrum</i>	0.1633	1.6697	ND	0.0451	0.1469	ND	0.0013
5	<i>T. rubrum</i>	ND	ND	ND	ND	ND	ND	ND
6	<i>T. rubrum</i>	0.0243	0.3472	ND	ND	ND	ND	ND
7	<i>T. rubrum</i>	0.3301	2.4270	0.4009	0.0739	ND	ND	0.0015
8	<i>T. rubrum</i>	0.4306	3.2942	0.5324	0.0963	ND	ND	ND
9	<i>T. rubrum</i>	0.0869	0.9862	ND	0.0213	ND	ND	ND
10	<i>T. rubrum</i>	0.2641	3.2168	0.5778	0.1123	0.1494	ND	0.0032
11	<i>T. rubrum</i>	0.0264	0.2209	0.0520	0.0094	ND	ND	ND
12	<i>T. rubrum</i>	0.0663	0.5262	0.0913	0.0179	ND	ND	ND
13	<i>T. rubrum</i>	0.0540	0.5677	0.0924	0.0188	ND	ND	ND
14	<i>T. rubrum</i>	0.0075	0.1137	ND	0.0030	ND	ND	ND
15	<i>T. rubrum</i>	0.0782	0.8144	ND	0.0136	ND	ND	ND
16	<i>T. rubrum</i>	0.0215	0.1389	ND	0.0046	ND	ND	ND
17	<i>T. rubrum</i>	0.0832	0.8010	0.1227	0.0294	ND	ND	ND
18	<i>T. rubrum</i>	0.3032	1.8497	0.3503	0.0596	ND	ND	ND
19	<i>T. rubrum</i>	0.0450	0.3197	ND	0.0083	ND	ND	ND
20	<i>T. rubrum</i>	0.0879	1.0017	0.1932	0.0225	ND	ND	0.0002
21	<i>T. rubrum</i>	0.1756	1.2318	0.2119	0.0284	ND	ND	0.0005
22	<i>T. rubrum</i>	0.1322	1.0604	0.1943	0.0284	ND	ND	ND
23	<i>T. rubrum</i>	0.0245	0.2570	ND	0.0102	ND	ND	ND
24	<i>T. rubrum</i>	0.0287	0.2070	0.0327	0.0074	0.0140	0.0016	0.0008
25	<i>T. rubrum</i>	0.0319	0.2363	0.0530	0.0076	ND	ND	ND
26	<i>T. rubrum</i>	ND	0.0136	ND	ND	ND	0.0050	0.0023
27	<i>T. rubrum</i>	0.1095	1.1303	0.1685	0.0346	0.0159	ND	ND

28	<i>T. rubrum</i>	0.2185	0.6152	0.1247	0.0079	ND	ND	ND
29	<i>T. rubrum</i>	0.4307	2.9728	0.5871	0.0934	ND	ND	0.0023
30	<i>T. rubrum</i>	0.0626	0.4711	ND	0.0171	ND	ND	0.0004
31	<i>T. rubrum</i> + melanine	0.0040	0.0386	ND	0.0018	ND	ND	ND
32	<i>T. rubrum</i> + melanine	0.0509	0.5624	0.0837	0.0125	ND	ND	ND
33	trauma	ND	ND	ND	ND	ND	ND	ND
34	trauma	ND	ND	ND	ND	ND	ND	ND
35	trauma	ND	0.0006	ND	ND	ND	ND	ND
36	trauma	ND	ND	ND	0.0003	ND	ND	ND
37	trauma	ND	0.0250	ND	ND	ND	ND	ND
38	trauma	ND	ND	ND	ND	ND	ND	ND
39	trauma	ND	ND	ND	ND	ND	ND	ND
40	trauma	ND	ND	ND	ND	ND	ND	ND
41	trauma	ND	ND	ND	0.0006	ND	ND	ND
42	trauma	ND	ND	ND	ND	ND	ND	ND
43	<i>Trichophyton mentagrophytes var.interdigitalis</i>	ND	ND	ND	ND	ND	ND	ND
44	<i>Trichophyton mentagrophytes var.interdigitalis</i>	ND	ND	ND	ND	ND	ND	ND
45	<i>Trichophyton mentagrophytes var.interdigitalis</i>	ND	ND	ND	ND	ND	ND	ND
46	<i>Trichophyton mentagrophytes var.interdigitalis</i>	ND	ND	ND	ND	ND	ND	ND
47	<i>Trichophyton mentagrophytes var.interdigitalis</i>	ND	ND	ND	ND	ND	ND	0.0015
48	<i>Scytalidium dimidiatum</i>	ND	0.0033	ND	ND	ND	ND	ND
49	<i>Scytalidium dimidiatum</i>	ND	ND	ND	ND	ND	ND	ND
50	<i>Scytalidium dimidiatum</i>	ND	ND	ND	ND	ND	ND	ND
51	<i>Scytalidium dimidiatum</i>	ND	ND	ND	ND	ND	ND	ND
52	<i>Fusarium sp.</i>	ND	ND	ND	ND	ND	ND	ND
53	<i>Fusarium sp.</i>	ND	0.0010	ND	ND	ND	ND	ND
54	<i>Aspergillus</i>	ND	ND	ND	ND	ND	ND	ND

55	Psoriasis	ND	0.0027	ND	ND	ND	ND	ND
56	Psoriasis	ND	ND	ND	ND	ND	ND	ND
57	Onychogryphosis	ND	ND	ND	ND	ND	ND	ND
58	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
59	<i>T. rubrum</i>	NP	6.3081	1.8336	ND	NP	NP	NP
60	<i>T. rubrum</i>	NP	4.5106	0.8434	ND	NP	NP	NP
61	<i>T. rubrum</i>	NP	1.7852	0.3992	13.4	NP	NP	NP
62	<i>T. rubrum</i>	NP	242.5	ND	515.9	NP	NP	NP
63	<i>T. rubrum</i>	NP	56.93	8.7881	0.0583	NP	NP	NP
64	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
65	<i>T. rubrum</i>	NP	5.1991	0.8852	ND	NP	NP	NP
66	<i>T. rubrum</i>	NP	2.1929	ND	21.62	NP	NP	NP
67	<i>T. rubrum</i>	NP	6.213	7.5604	ND	NP	NP	NP
68	<i>T. rubrum</i>	NP	1.2329	0.2094	ND	NP	NP	NP
69	<i>T. rubrum</i>	NP	11.02	ND	172.01	NP	NP	NP
70	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
71	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
72	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
73	<i>T. rubrum</i> black pigmentation	NP	4.6167	0.5482	18.59	NP	NP	NP
74	negative	NP	ND	ND	ND	NP	NP	NP
75	negative	NP	ND	ND	ND	NP	NP	NP
76	negative	NP	ND	ND	ND	NP	NP	NP
77	negative	NP	ND	ND	ND	NP	NP	NP
78	negative	NP	ND	ND	ND	NP	NP	NP
79	<i>Acremonium</i> sp.	NP	ND	ND	ND	NP	NP	NP
80	<i>Fusarium</i> sp.	NP	ND	ND	ND	NP	NP	NP
81	<i>Trichophyton</i> <i>mentagrophytes</i> <i>var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
82	<i>Trichophyton</i> <i>mentagrophytes</i> <i>var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
83	<i>Trichophyton</i> <i>mentagrophytes</i>	NP	ND	ND	ND	NP	NP	NP

	<i>var.interdigitalis</i>							
84	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
85	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
86	direct obs.= dematophyte filaments; culture = steril	NP	ND	ND	ND	NP	NP	NP
87	direct obs.= dematophyte filaments; culture = steril	NP	0.0651	0.0209	0.2155	NP	NP	NP
88	<i>T. rubrum</i>	NP	10.87	2.8137	24.84	NP	NP	NP
89	<i>T. rubrum</i>	NP	0.0273	0.0046	0.079	NP	NP	NP
90	<i>T. rubrum</i>	NP	ND	ND	0.0483	NP	NP	NP
91	<i>T. rubrum</i>	NP	0.2684	0.0233	1.8036	NP	NP	NP
92	<i>T. rubrum</i>	NP	0.9857	0.2138	9.322	NP	NP	NP
93	<i>T. rubrum</i>	NP	2.1803	0.1798	12.82	NP	NP	NP
94	<i>T. rubrum</i>	NP	0.6454	0.0717	9.3634	NP	NP	NP
95	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
96	<i>T. rubrum</i>	NP	8.8487	0.9895	54.53	NP	NP	NP
97	<i>T. rubrum</i>	NP	2.5721	0.3753	13.23	NP	NP	NP
98	<i>T. rubrum</i>	NP	0.0574	0.0143	1.4206	NP	NP	NP
99	<i>T. rubrum</i>	NP	5.2788	0.8653	1.2918	NP	NP	NP
100	<i>T. rubrum</i>	NP	0.0133	0.0049	0.1193	NP	NP	NP
101	<i>T. rubrum</i>	NP	0.087	0.0331	1.0571	NP	NP	NP
102	<i>T. rubrum</i>	NP	17.23	6.4123	ND	NP	NP	NP
103	negative	NP	ND	ND	ND	NP	NP	NP
104	negative	NP	ND	ND	ND	NP	NP	NP
105	negative	NP	ND	ND	ND	NP	NP	NP
106	negative	NP	ND	ND	ND	NP	NP	NP
107	negative	NP	ND	ND	ND	NP	NP	NP
108	negative	NP	ND	ND	ND	NP	NP	NP
109	negative	NP	ND	ND	ND	NP	NP	NP
110	negative	NP	ND	ND	ND	NP	NP	NP
111	negative	NP	ND	ND	ND	NP	NP	NP

112	negative	NP	ND	ND	ND	NP	NP	NP
113	negative	NP	ND	ND	ND	NP	NP	NP
114	negative	NP	ND	ND	ND	NP	NP	NP
115	negative	NP	ND	ND	ND	NP	NP	NP
116	negative	NP	ND	ND	ND	NP	NP	NP
117	negative	NP	ND	ND	ND	NP	NP	NP
118	negative	NP	ND	ND	ND	NP	NP	NP
119	negative	NP	ND	ND	ND	NP	NP	NP
120	negative	NP	ND	ND	ND	NP	NP	NP
121	negative	NP	ND	ND	ND	NP	NP	NP
122	negative	NP	ND	ND	ND	NP	NP	NP
123	negative	NP	ND	ND	ND	NP	NP	NP
124	negative	NP	ND	ND	ND	NP	NP	NP
125	negative	NP	ND	ND	ND	NP	NP	NP
126	negative	NP	ND	ND	ND	NP	NP	NP
127	negative	NP	0.8987	0.132	2.4312	NP	NP	NP
128	negative	NP	ND	ND	ND	NP	NP	NP
129	negative	NP	ND	ND	ND	NP	NP	NP
130	<i>Candida albicans</i>	NP	ND	ND	ND	NP	NP	NP
131	direct obs.= dematophyte filaments; culture = steril	NP	1.767	0.0214	0.3514	NP	NP	NP
132	direct obs.= dematophyte filaments; culture = steril	NP	0.0578	0.006	0.0743	NP	NP	NP
133	<i>T. rubrum</i>	NP	0.6578	0.0157	0.2423	NP	NP	NP
134	<i>T. rubrum</i>	NP	0.0354	0.2554	4.2529	NP	NP	NP
135	<i>T. rubrum</i>	NP	45.86	0.7951	11.70	NP	NP	NP
136	<i>T. rubrum</i>	NP	27.95	0.0537	17.72	NP	NP	NP
137	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
138	<i>T. rubrum</i>	NP	5.041	0.5972	21.71	NP	NP	NP
139	<i>T. rubrum</i>	NP	2.7298	0.5554	12.41	NP	NP	NP
140	<i>T. rubrum</i>	NP	21.603	6.9036	231.13	NP	NP	NP
141	<i>T. rubrum</i>	NP	3.4252	0.6664	13.3329	NP	NP	NP

142	<i>T. rubrum</i>	NP	0.8401	0.0682	3.3177	NP	NP	NP
143	<i>T. rubrum</i>	NP	13.34	0.0422	8.9115	NP	NP	NP
144	<i>T. rubrum</i>	NP	4.7624	0.579	13.53	NP	NP	NP
145	<i>T. rubrum</i>	NP	0.8579	0.1039	2.1419	NP	NP	NP
146	<i>T. rubrum</i>	NP	0.3631	0.025	0.7015	NP	NP	NP
147	<i>T. rubrum</i>	NP	0.0689	0.0133	0.3481	NP	NP	NP
148	<i>T. rubrum</i>	NP	0.5078	0.3125	4.3156	NP	NP	NP
149	<i>T. rubrum</i>	NP	9.3903	1.2283	47.3716	NP	NP	NP
150	<i>T. rubrum</i>	NP	8.3974	0.1152	1.2643	NP	NP	NP
151	<i>T. rubrum</i>	NP	0.1001	0.0317	1.0231	NP	NP	NP
152	<i>T. rubrum</i>	NP	1.7143	0.2915	5.8643	NP	NP	NP
153	<i>T. rubrum</i>	NP	0.1639	0.0402	0.911	NP	NP	NP
154	<i>T. rubrum</i>	NP	1.5984	0.4726	7.5221	NP	NP	NP
155	<i>T. rubrum</i>	NP	1.4248	0.1338	5.2936	NP	NP	NP
156	<i>T. rubrum</i>	NP	15.12	ND	ND	NP	NP	NP
157	<i>T. rubrum</i>	NP	0.6378	1.5422	0.0316	NP	NP	NP
158	<i>Scopulariopsis brevicauli</i>	NP	ND	ND	ND	NP	NP	NP
159	<i>Scopulariopsis brevicauli</i>	NP	ND	ND	ND	NP	NP	NP
160	<i>Fusarium</i>	NP	ND	ND	ND	NP	NP	NP
161	<i>Candida albicans</i>	NP	ND	ND	ND	NP	NP	NP
162	<i>Candida albicans</i>	NP	ND	ND	ND	NP	NP	NP
163	<i>Aspergillus sp.</i>	NP	ND	ND	ND	NP	NP	NP
164	<i>Aspergillus sp.</i>	NP	ND	ND	ND	NP	NP	NP
165	<i>Aspergillus sp.</i>	NP	ND	ND	ND	NP	NP	NP
166	<i>Aspergillus sp.</i>	NP	ND	ND	ND	NP	NP	NP
167	<i>Aspergillus sp.</i>	NP	ND	ND	ND	NP	NP	NP
168	negative	NP	ND	ND	ND	NP	NP	NP
169	negative	NP	ND	ND	ND	NP	NP	NP
170	negative	NP	ND	ND	ND	NP	NP	NP
171	negative	NP	ND	ND	ND	NP	NP	NP
172	negative	NP	ND	ND	ND	NP	NP	NP

173	negative	NP	ND	ND	ND	NP	NP	NP
174	negative	NP	ND	ND	ND	NP	NP	NP
175	negative	NP	ND	ND	ND	NP	NP	NP
176	negative	NP	ND	ND	ND	NP	NP	NP
177	negative	NP	ND	ND	ND	NP	NP	NP
178	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
179	<i>T. rubrum</i>	NP	7.2025	1.0581	35.91	NP	NP	NP
180	<i>T. rubrum</i>	NP	0.1364	0.0574	0.9945	NP	NP	NP
181	<i>T. rubrum</i>	NP	30.05	6.9603	ND	NP	NP	NP
182	<i>T. rubrum</i>	NP	0.0393	0.0273	0.5179	NP	NP	NP
183	<i>T. rubrum</i>	NP	10.68	0.6809	33.88	NP	NP	NP
184	<i>T. rubrum</i>	NP	0.906	0.0749	1.6222	NP	NP	NP
185	<i>T. rubrum</i>	NP	112.06	0.463	ND	NP	NP	NP
186	<i>T. rubrum</i>	NP	54.17	ND	ND	NP	NP	NP
187	<i>T. rubrum</i>	NP	0.1888	0.0672	1.4326	NP	NP	NP
188	<i>T. rubrum</i>	NP	45.599	6.8234	ND	NP	NP	NP
189	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
190	<i>T. rubrum</i>	NP	1.7151	0.1081	3.9304	NP	NP	NP
191	<i>T. rubrum</i>	NP	16.36	4.7772	20.62	NP	NP	NP
192	<i>T. rubrum</i>	NP	2.4296	0.5619	12.28	NP	NP	NP
193	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
194	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
195	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
196	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
197	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
198	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP

199	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
200	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
201	<i>Trichophyton mentagrophytes var.interdigitalis</i>	NP	ND	ND	ND	NP	NP	NP
202	<i>T. rubrum + Scopulanopsis brevicaulis</i>	NP	ND	ND	ND	NP	NP	NP
203	<i>T. rubrum africanis</i>	NP	ND	ND	ND	NP	NP	NP
204	<i>T. soudanense</i>	NP	ND	ND	ND	NP	NP	NP
205	<i>T. rubrum</i> (+pyocyanique	NP	5.9002	0.7553	45.89	NP	NP	NP
206	<i>T. rubrum</i>	NP	1115.02	25.03	1836.42	NP	NP	NP
207	<i>T. rubrum</i>	NP	ND	ND	ND	NP	NP	NP
208	<i>T. rubrum</i>	NP	3.51	ND	ND	NP	NP	NP
209	<i>T. rubrum</i>	NP	5.7153	0.9342	23.11	NP	NP	NP
210	<i>T. rubrum</i>	NP	0.742	3.0494	0.0311	NP	NP	NP
211	<i>T. rubrum</i>	NP	26.76	5.1811	145.95	NP	NP	NP
212	<i>Scytalidium dimidiatum</i>	NP	ND	ND	ND	NP	NP	NP
213	<i>Scytalidium hyalinium</i>	NP	ND	ND	ND	NP	NP	NP
214	<i>Epidermophyton floccosum</i>	NP	ND	ND	ND	NP	NP	NP
215	<i>Scytalidium dimidiatum</i>	NP	ND	ND	ND	NP	NP	NP
216	<i>Scytalidium hyalinium</i>	NP	ND	ND	ND	NP	NP	NP
217	<i>T. rubrum</i>	NP	82.15	1.9745	ND	NP	NP	NP
218	<i>T. rubrum</i>	NP	17.76	1.5164	74.15	NP	NP	NP

NP: not performed. ND: not detected. All nail with trauma or psoriasis as well with Onychogryphosis were negative by direct mycological examination and culture assays. Sub6 MRM assay was performed using the following peptides: VLDCDGSNSGVIK, KPGGTTYYYDPSAGK and ADFSNYGAVVDVYAPGK for all samples. For sample 1 to 57 Sub6 was also detected using the peptide: MANDVIQSPGEGTTGK. Sample 25 was unclear. In addition, non-significant ratios were observed for Sub6 peptides in the samples 35, 36, 37, 41, 48, 53 and 55 (manual validation). DppV and Sub7 were not analysed in sample 58 to 218.

Table S6: Alignment of peptide sequences of Subtilisin-like protease 6.

MANDVIQSPGEGTTGK	404	Q9UW97	SUB6_TRIRU	<i>Trichophyton rubrum</i>
MANDVIQSPGEGTTGK	404	A1XIH1	SUB6_TRISD	<i>Trichophyton soudanense</i>
MAHDAIQSPGEGTTGK	404	A0A059J8X0	A0A059J8X0_9EURO	<i>Trichophyton interdigitale</i> (MR816)
MATDAIQSPGEDTTSK	404	D4D5H3	SUB6_TRIVH	<i>Trichophyton verrucosum</i> (strain HKI 0517)
MANDAIQSPGEDTTSK	404	Q5VJ72	SUB6_TRIVC	<i>Trichophyton verrucosum</i>
MAHDAIQRPGEGETTSK	404	Q3ZEJ8	SUB6_TRITO	<i>Trichophyton tonsurans</i>
MAHDAIQRPGEGETTSK	404	A1XIH0	SUB6_TRIEQ	<i>Trichophyton equinum</i>
MANDAIQSPGEDTTSK	404	Q64K31	SUB6_ARTBE	<i>Arthroderma benhamiae</i>
MANDAIQSPGEDTTSK	404	D4ALV9	SUB6_ARTBC	<i>Arthroderma benhamiae</i> (strain ATCC MYA-4681)
MARPVIKNPGQGTTNK	398	C5FQI3	SUB6_ARTOC	<i>Arthroderma otae</i> (strain ATCC MYA-4605)
MATDAIKNPGEGETTGK	400	E4V4J8	SUB6_ARTGP	<i>Arthroderma gypseum</i> (strain ATCC MYA-4604)
VLDCDGSNSGSGVIK	238	Q9UW97	SUB6_TRIRU	<i>Trichophyton rubrum</i>
VLDCDGSNSGSGVIK	238	A1XIH1	SUB6_TRISD	<i>Trichophyton soudanense</i>
VLDCEGSGSNSGSGVIK	238	A0A059J8X0	A0A059J8X0_9EURO	<i>Trichophyton interdigitale</i> (MR816)
VLDCEGSGSNSGSGVIK	238	D4D5H3	SUB6_TRIVH	<i>Trichophyton verrucosum</i> (strain HKI 0517)
VLDCEGSGSNSGSGVIK	238	Q5VJ72	SUB6_TRIVC	<i>Trichophyton verrucosum</i>
VLDCEGSGSNSGSGVIK	238	Q3ZEJ8	SUB6_TRITO	<i>Trichophyton tonsurans</i>
VLDCEGSGSNSGSGVIK	238	A1XIH0	SUB6_TRIEQ	<i>Trichophyton equinum</i>
VLDCEGSGSNSGSGVIK	238	Q64K31	SUB6_ARTBE	<i>Arthroderma benhamiae</i>
VLDCEGSGSNSGSGVIK	238	D4ALV9	SUB6_ARTBC	<i>Arthroderma benhamiae</i> (strain ATCC MYA-4681)
VLDCEGSGSNSGSGVIK	233	C5FQI3	SUB6_ARTOC	<i>Arthroderma otae</i> (strain ATCC MYA-4605)
VLDCEGSGSNSGSGVIM	234	E4V4J8	SUB6_ARTGP	<i>Arthroderma gypseum</i> (strain ATCC MYA-4604)
KPGGTTYYYDPSAGK	159	Q9UW97	SUB6_TRIRU	<i>Trichophyton rubrum</i>
KPGGTTYYYDPSAGK	159	A1XIH1	SUB6_TRISD	<i>Trichophyton soudanense</i>
KPGGTTYYYDPSAGK	159	D4D5H3	SUB6_TRIVH	<i>Trichophyton verrucosum</i> (strain HKI 0517)
KPGGTTYYYDPSAGK	159	Q5VJ72	SUB6_TRIVC	<i>Trichophyton verrucosum</i>
QAGGTTYYYDSSAGK	159	A0A059J8X0	A0A059J8X0_9EURO	<i>Trichophyton interdigitale</i> (MR816)
KAGGTTYYYDSSAGK	159	Q3ZEJ8	SUB6_TRITO	<i>Trichophyton tonsurans</i>
KAGGTTYYYDSSAGK	159	A1XIH0	SUB6_TRIEQ	<i>Trichophyton equinum</i>
QAGGTTYYYDPSAGK	159	Q64K31	SUB6_ARTBE	<i>Arthroderma benhamiae</i>

QAGGTTYYYDPSAGK 159 D4ALV9 SUB6_ARTBC *Arthroderma benhamiae* (strain ATCC MYA-4681)
 EPGGTTYFYDESAGE 154 C5FQI3 SUB6_ARTOC *Arthroderma otae* (strain ATCC MYA-605)
 EAGGSTYYYDDTAGK 155 E4V4J8 SUB6_ARTGP *Arthroderma gypseum* (strain ATCC MYA-4604)

ADFSNYGAVVDVYAPGK 339 Q9UW97 SUB6_TRIRU *Trichophyton rubrum*
ADFSNYGAVVDVYAPGK 339 A1XIH1 SUB6_TRISD *Trichophyton soudanense*
 ADFSNYGAVVDIYAPGN 335 E4V4J8 SUB6_ARTGP *Arthroderma gypseum* (strain ATCC MYA-4604)
 ADFSNYGQLVDVYAPGK 339 A0A059J8X0 A0A059J8X0_9EURO *Trichophyton interdigitale* (MR816)
 ANFSNFGSVVDVYAPGK 339 D4D5H3 SUB6_TRIVH *Trichophyton verrucosum* (strain HKI 0517)
 ANFSNFGSVVDVYAPGK 339 Q5VJ72 SUB6_TRIVC *Arthroderma benhamiae* (strain ATCC MYA-4681)
 ADFSNYGQLVDVYAPGK 339 Q3ZEJ8 SUB6_TRITO *Trichophyton tonsurans*
 ADFSNYGQLVDVYAPGK 339 A1XIH0 SUB6_TRIEQ *Trichophyton equinum*
 ASFSNYGSAVDVYAPGK 339 Q64K31 SUB6_ARTBE *Arthroderma benhamiae*
 ASFSNYGSAVDVYAPGK 339 D4ALV9 SUB6_ARTBC *Arthroderma benhamiae* (strain ATCC MYA-4681)
 ASFSNWGQAVDVVFAPGM 333 C5FQI3 SUB6_ARTOC *Arthroderma otae* (strain ATCC MYA-605)

Peptide in bold indicates identity with *Trichophyton rubrum*. Sequence alignment was performed using Program CLUSTALO 113480 (date of job execution Aug 4. 2014).