Proteolytic signatures define unique thrombin-derived peptides present in human wound fluid in vivo

Saravanan, Rathi; Adav, Sunil S; Choong, Yeu Khai; van der Plas, Mariena J.A.; Petrlova, Jitka; Kjellström, Sven; Sze, Siu Kwan; Schmidtchen, Artur

Published in:
Scientific Reports

DOI:
10.1038/s41598-017-13197-3

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Proteolytic signatures define unique thrombin-derived peptides present in human wound fluid in vivo

Rathi Saravanan1, Sunil S. Adav1,4, Yeu Khai Choong1, Mariena J. A. van der Plas2, Jitka Petrlova2, Sven Kjellström3, Siu Kwan Sze4 & Artur Schmidtchen1,2,5

The disease burden of failing skin repair and non-healing ulcers is extensive. There is an unmet need for new diagnostic approaches to better predict healing activity and wound infection. Uncontrolled and excessive protease activity, of endogenous or bacterial origin, has been described as a major contributor to wound healing impairments. Proteolytic peptide patterns could therefore correlate and “report” healing activity and infection. This work describes a proof of principle delineating a strategy by which peptides from a selected protein, human thrombin, are detected and attributed to proteolytic actions. With a particular focus on thrombin-derived C-terminal peptides (TCP), we show that distinct peptide patterns are generated in vitro by the human S1 peptidases human neutrophil elastase and cathepsin G, and the bacterial M4 peptidases Pseudomonas aeruginosa elastase and Staphylococcus aureus aureolysin, respectively. Corresponding peptide sequences were identified in wound fluids from acute and non-healing ulcers, and notably, one peptide, FYT21 (FYTHVFRLKKWIQKVIDQFGE), was only present in wound fluid from non-healing ulcers colonized by P. aeruginosa and S. aureus. Our result is a proof of principle pointing at the possibility of defining peptide biomarkers reporting distinct proteolytic activities, of potential implication for improved diagnosis of wound healing and infection.

Conditions, such as arterial and venous insufficiency, diabetes, or excessive pressure all predispose to the formation of non-healing ulcers. In the United States alone, about 6.5 million have chronic skin ulcers1. The economic burden on the healthcare system and on society due to failing skin repair and non-healing ulcers is extensive2. In diabetic patients, these ulcers are the leading cause (85%) of all major lower limb amputations, inflicting significant pain and reducing quality of life for patients3. With a growing diabetic population, chronic wounds affect millions of people, with high morbidity and mortality rates, emerging as a serious threat to healthcare systems around the world4. Furthermore, complications of slow or non-healing wounds also affect burn victims. In the United States alone, more than 1.25 million people per year suffer from burns, where local infections cause delayed healing and risk for invasive infections and sepsis. Also notable is that a significant part of hospital antibiotics consumption is related to postoperative prophylaxis, and the costs for postoperative infections are high.

Standard wound diagnostic procedures usually involve clinical evaluations and sometimes bacteriological identification5. Occurrence of multidrug resistant microorganisms has further complicated the wound diagnostic procedures and treatment options. In addition, the management of drug resistant microorganisms is in an urgent need for better treatment options6,7. Early diagnosis paired with prompt and effective therapeutic interventions in case of infection risk may lead to better treatments targeting the causes of wounds, as well as reduction of wound infections. Clearly, there is an unmet need for improved diagnostic approaches in order to better predict healing activity and infection in non-healing wounds.

1Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, Singapore, 636921, Singapore. 2Division of Dermatology, Department of Clinical Sciences, Lund University, Lund, Sweden. 3Centre of Excellence in Biological and Medical Mass Spectrometry "CEBMM5", Biomedical Centre D13, Lund University, Lund, Sweden. 4School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore, 637551, Singapore. 5Wound Healing Centre, Bispebjerg University Hospital, Copenhagen, Denmark. Correspondence and requests for materials should be addressed to R.S. (email: rathi@ntu.edu.sg)
Non-healing ulcers are characterized by the presence of an elevated level of proteases, both endogenous and of bacterial origin, which disrupts the delicate balance between acute inflammation and progression of wound healing. Consequently, these wounds are often characterized by high inflammatory activity, excessive proteolytic processing of matrix molecules and immune mediators and impaired wound closure. Hemostasis is the initial phase of wound healing and commences with the formation of active thrombin which in turn, mediates fibrin clot formation as well as pro-inflammatory and chemotactic effects. We have previously demonstrated that proteolysis of thrombin generates host defense peptides (HDPs), particularly derived from the C-terminal part of the molecule. These thrombin-derived C-terminal peptides (TCPs) bind to bacterial lipopolysaccharide (LPS) and display both anti-microbial and anti-inflammatory activities in vitro, with therapeutic potential against infection and septic shock in vivo. Interestingly, we found that *Pseudomonas aeruginosa* may mimic the formation of such HDPs, leading to generation of anti-inflammatory peptides and circumvention of host responses. In a recent report, we also demonstrated a previously undisclosed role of larger TCPs of about 11 kDa, involving LPS-dependent proteolysis of thrombin. Cleavage of thrombin by human and bacterial proteases were identified, and similar TCPs were found in human wound fluid obtained from sterile as well as infected wounds. Taken together, our study demonstrates a proof of principle of the feasibility for defining peptide biomarkers that "report" on distinct protease activities in vivo.

**Results**

**Degradation of thrombin by endogenous human proteinases in vitro.** Thrombin susceptibility to endogenous human proteinases secreted by neutrophils during wounding was assessed by analyzing the in vitro peptide patterns generated by human neutrophil elastase (HNE) and cathepsin G (HCG). Both HNE and HCG cleaved thrombin completely, generating a large number of fragments (157 and 129 peptides respectively) as evident from the SDS-PAGE and mass spectrometric analysis (Figure S1). Peptide sequence comparisons of the LC-MS/MS data identified distinct TCPs, indicating that HNE and HCG cleave thrombin specifically, with low overlap (Fig. 1 and Figure S4). Furthermore, co-incubation of thrombin with HNE and HCG simultaneously did not yield additional common peptide fragments (Figure S2). Interestingly, the peptide HVF18 (HVFRLLKKWKQKVIDQFGE), a fragment having anti-endotoxic effects was identified in the peptide products of HNE and HCG, both S1 peptidases (Table 1). The results support earlier reports that HNE cleaves thrombin to release bioactive thrombin-derived peptides including HVF18.

**Degradation of thrombin by bacterial proteinases in vitro.** Next, we analysed the thrombin peptides generated by bacterial proteinases such as elastase of the Gram-negative *Pseudomonas aeruginosa*, and aureolysin and V8 proteases of the Gram-positive *Staphylococcus aureus*, two bacteria commonly found in non-healing ulcers and infected burn wounds (Figure S3). As seen in Fig. 2, *P. aeruginosa* elastase (PAE) generates a 21 amino acid TCP, FYT21 (FYTHVFRLLKKWKQKVIDQFGE), reported recently to inhibit host inflammatory responses. Notably, FYT21 was also found to be generated by *S. aureus* aureolysin (ALYS), a metalloproteinase like *P. aeruginosa* elastase (Fig. 2 and Table 1). Peptides generated by V8 were mainly of high molecular weight, in comparison to those generated by PAE and ALYS, possibly reflecting the limited specificity of the protease for anionic...
residues (D/E) at the P1 position (Figure S4). Hence, a limited number of TCPs were identified after V8 digestion (Figure S3). Taken together, similar to the findings with HNE and HCG, the results indicate that bacterial proteases cleave thrombin distinctly, yielding unique peptide patterns (Fig. 3). Notably, the TCPs HVF18 and FYT21 were identified as unique products of human and bacterial protease activity, respectively (Fig. 3 and Table 1).

Figure 4 illustrates the obtained cleavage sites by the respective proteases as shown in a 3D model of thrombin (panel A), as well as indicated in the amino acid sequence (panel B). The side chain dispositions of cleavage sites, as highlighted in the 3D model, indicates that the majority of cleavage sites for both bacterial and human proteases are situated on the exterior surface of thrombin.

Thrombin-derived peptides are identified in human wound fluids.

In order to identify TCPs present in human wounds, wound fluids from acute surgical wounds and chronic ulcers co-infected with P. aeruginosa and S. aureus were incubated with thrombin C-terminal antibody-coated dynabeads to pull-down TCPs, followed by mass spectrometry analysis. Comparison of the in vitro generated peptides and the fragments detected in vivo showed the presence of multiple fragments of TCPs in both acute wound fluids (AWF) and chronic wound fluids (CWF) (Fig. 5 and Table 2). The peptides HVF18 and FYT21, products of human and bacterial proteases respectively, were identified in wound fluid from non-healing ulcers, while the peptides FRL16 (FRLKKWIQKVVIDQFGE), LKK14 (LKKWIQKVVIDQFGE), KKW13 (KKWIQKVVIDQFGE) and KWI12 (KWIQKVVIDQFGE), and other truncated fragments were common in wound fluids from both acute wounds and non-healing ulcers (Fig. 5 and Table 2).

**Discussion**

The main finding of this work is the definition of multiple fragments of TCPs which are generated by both human and bacterial proteases. Furthermore, the work shows a proof of principle that it may be technically possible to define peptides “reporting” distinct proteolytic activity patterns not only in vitro, but also in vivo. As protease activity is so fundamentally linked to the healing status of wounds as well as the presence of protease-producing bacteria, the work thus points at the future possibility of using peptides as biomarkers in the development of...
advanced wound and infection diagnostics - based on mass spectrometry technologies, antibody arrays, or other sensor-based technologies.

As mentioned in the introduction, today’s measures in the clinic to assess a wound and define whether it is infected or not, mainly depend on the clinician’s evaluation. In general practice, if there is a suspected infection, a microbiological swab is taken before antibiotics are given to the patient. It is notable that most wound swabs are prone to false positives as most will yield bacterial growth which is not always due to infection. For example, over 90% of non-healing ulcers are colonized by staphylococci - even without infection. The clinical evaluation of infection today is based on obvious signs of infection including redness, heat, swelling, purulent exudate, smell, pain, systemic illness, and the presence of fistulas, “foamy” granulation tissue, or tissue breakdown. The challenge is to detect infection before it reaches this stage.

Alternate methods to swab technique for detecting bacterial infection are based on looking at products from bacteria, or the host, or determining host responses that can report the presence of infection. At current, some developments are directed to the identification of a variety of biochemical by-products. For example, pyocyanin—the blue-green pigment secreted by many P. aeruginosa strains, has been used to detect bacteria21. Urate or uric acid is another metabolite found within wounds, which may be used as a diagnostic marker. The problem here is that bacteria may metabolize urate. Toxins produced by bacteria found in wounds may be used to trigger a sensor output indicating early infection. For example, Zhou et al. developed Trojan-like phospholipid vesicles, which release a fluorescent dye cargo when subjected to bacterial toxins22,23. Of relevance to this study is that HNE and HCG have recently been reported as early stage warning markers for non-healing ulcers24. The enzymatic nature of HNE and HCG has indeed been used to produce a sensor made from a chromophore linked by a short peptide sequence susceptible to cleavage by each of the enzymes. For example, Edwards et al. used cellulose-AP-suc-Ala-Ala-Pro-Ala-pNA substrates25. Rimmer et al. developed a method for binding either

Figure 2. Bacterial proteases cleave human thrombin. (a) Western blot analysis of human α-thrombin digested with P. aeruginosa elastase (PAE), S. aureus aureolysin (ALYS) and V8 protease, incubated at 37°C for 3 and 6 hours. Thrombin incubated for 6 hours and the synthetic peptide FYT21 were loaded as control. PAE western blot is shown at exposure time of 20 secs while the V8/ALYS blot is at 8secs. The V8/ALYS western blot at exposure time of 20 secs is provided in the supplementary information S3. (b) A Venn-diagram comparing the LC-MS/MS identified TCPs produced by bacterial proteases PAE (blue), ALYS (brown) and V8 (magenta) in an experimental repeat of three, highlights unique and overlapping peptide sequences (black and orange).
Gram-positive or Gram-negative bacteria to a polymer which alters shape upon binding and has potential as a bacterial sensor. Matrix metalloproteinases (MMPs) are increased in chronic wound fluid (CWF). Gao et al. sensed MMP-2 at concentrations as low as 0.1 ng/mL by spin-coating a gelatin film on top of a pSi resonator. Upon contact with MMP-2, the film was degraded and the generated peptides were able to enter the pores and induced color changes that could be detected by the eye.

Other researchers are focusing on protein patterns, and identification of the total proteome of wounds has been suggested as one method to obtain not only information of the pathogenesis, but also to be able to define...
specific protein biomarkers discriminating between various wound types. For example, Eming et al. demonstrated the differential distribution of tissue repair proteins in exudates of healing wounds and persistent inflammatory tissue damaging mediators in non-healing wounds. Upton's group has established enhanced proteomics workflow to reduce sample complexity by selective depletion of high abundant protein, validating detection of proteins present in low concentrations in chronic wound fluids. Auf dem Keller et al. applied quantitative proteomics strategies to dissect proteolytic pathways in healthy and diseased skin. In a global approach, they analysed wound fluids from a porcine vacuum assisted closure (VAC) wound models and quantitatively assessed the wound proteome and the activity of distinct protease groups along the healing process. They also mapped proteolytic pathways in vivo and established protease-substrate relations that will help to better understand protease action in cutaneous wound repair. Considering the above, it is of note that many approaches are based on detection of products such as pyocyanin, toxins, or proteases. Methods for detection of down-stream products reflecting inflammation are rare. Of note is that auf dem Keller's work involves studies on such protease patterns. However, as their method focuses on “N-terminomics”, low molecular weight peptides are mainly lost during the preparation steps. Notable are also recent developments, where a labelling approach using trimethoxyphenyl phosphonium (TMPP) allows characterization of both N-terminal and internal digestion peptides. Whether the here reported method based on peptide detection will reach a sufficient sensitivity and specificity in order to be of diagnostic use has to be evaluated in future studies. For example, although a recently developed assay, WoundcheckTM was shown to have a good sensitivity for detection of HNE and MMP levels, the predictive potential of protease measurements for various wound pathologies, although showing promising results, needs further clinical studies. From this perspective, it is therefore of note that some peptide fragments were only produced by bacterial enzymes, such as the TCP FYT21. Hence, using peptide markers that are unique for bacteria should enable a high specificity, since such peptides should not be detected at all in sterile wounds. An important factor to address in future studies, of importance for evaluating the diagnostic utility of FYT21 and related fragments, is their stability and half-life in vivo. Of interest is that recent work by Böttger et al. showed peptides with differing cleavage sites exhibited variable proteolytic sensitivities in blood, serum, and plasma. Therefore, future developments aiming at defining peptides as biomarkers must be preceded by a careful analysis of their potential degradation (e.g. using isotope-labelled peptides), particularly in relevant environments, such as wound fluids from acute or chronic wounds.

It is also of note that P. aeruginosa elastase belongs to a superfamily of zinc-containing metallopeptidases of the M4 (thermolysin) class, also secreted from bacteria such as Enterococcus faecalis (coccolysin) and S. aureus (aureolysin), bacteria very common in chronic wounds. The present findings confirmed the presence of FYT21.
after digestion with *P. aeruginosa* elastase, as previously communicated. Interestingly, we also detected the peptide after digestion with aureolysin, another staphylococcal proteinase of the M4 peptidase group. Hence, it is tempting to speculate that formation of FYT21 could be common for many M4 peptidase-producing bacteria. If so, such peptide markers may reflect bacterial actions more than the presence of bacteria per se, which from a physiological point of view should correlate better to overall bacterial load and actions, and ultimately also, the clinical status of a particular wound. Hypothetically, combining the use of specific, qualitative, peptide markers, such as the ones reported here, with Woundchek™-based analyses of HNE and MMPs or other related more quantitative approaches, could be an attractive strategy in order to attain a higher diagnostic sensitivity and specificity with respect to wound status. These evaluations in turn, could be complemented with sensitive bacterial detection methods, such as the ones mentioned above, which altogether should provide a “multi-dimensional” analysis of wound status, enabling simultaneous determination of bacteria per se, host proteolytic activities, as well as down-stream specific peptides reporting actions of host or bacterial proteases. Clearly, clinical prospective studies on larger patient groups, where bacterial types and loads, and wound proteolysis levels, are determined and correlated with the occurrence of the herein defined peptide patterns, are mandated in order to fully investigate such potential diagnostic possibilities.

From a biological perspective, our findings also illustrate the multi-functionality of thrombin. Being a major enzyme of the coagulation cascade, thrombin acts as a procoagulant converting soluble fibrinogen into insoluble fibrin to initiate wound repair. However, the biological function of thrombin expands beyond blood coagulation, as the enzyme triggers endothelial cell activation, increased adhesion of neutrophils to the endothelium, platelet aggregation and chemotaxis of neutrophils and the induction of cytokine release from epithelial and endothelial cells through proteolytically activated receptors (PAR1, PAR3 or PAR4). Next, intermediate-sized TCPs

<table>
<thead>
<tr>
<th>Sequences</th>
<th>HP</th>
<th>BP</th>
<th>AWF</th>
<th>CWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFYTHVFRKKW</td>
<td>QKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>THYFRLKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THYFRLKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFRLLKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RLKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KKKWQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WQQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YQQKVIDQFGE</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FYTHFRLKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>YTHFRLKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>HFRLLKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>VFRLLKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FRLLKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KKKWQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>QKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>VIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IQKVIDQFGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IQKVIDQF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Sequences of thrombin C-terminal peptides (TCPs) generated by human and bacterial proteases found in human acute wound (AWF) and chronic wound fluids (CWF). *HP: Human protease; BP: bacterial protease, AWF: acute wound fluid; CWF: chronic wound fluid.
truncated forms related to the ELLESYIDGR sequence in our in vitro experiments by HNE and ALYS proteases, which is the principle showing that unique peptide patterns can be used for the definition of protease actions, and that these sequences could provide interesting clues to possible relationships between levels of such peptides in wounds and lesions. Hence, looking beyond TCPs, it is also possible that future detailed analyses of other thrombin cleavage products, such as FYT21 and HVF18, generated by proteolysis of thrombin, can be used for the detection of protease activities, of possible use in future diagnostic developments.

**Materials and Methods**

**Peptides and proteins.** Thrombin was from Innovative Research, USA. The peptides FYT21 (FYTHVFRKKWQKVIDQFGE) and HVF18 (HVFRLKKWQKVIDQFGE), were synthesized by Bioprobe (San Diego, CA). The purity of these peptides (>95%) was confirmed with MALDI-TOF MS. The endopeptidases, human neutrophil elastase and cathepsin G were from Calbiochem, USA. V8 Glu-C endoprotease was obtained from Roche, aureolysin from BioCol, and P. aeruginosa elastase (PAE) was purified as previously described with some modifications.

**Biological materials.** Wound fluids from non-healing venous ulcers co-infected with *P. aeruginosa* and *S. aureus* were collected under a Tegaderm dressing for 2 hours as reported earlier. Bacteria were identified by routine bacteriological methods from swabs from the wound surface. The patients were not treated with antibiotics. Sterile wound fluids were obtained from surgical drainages after mastectomy. Collection was for 24 to 48 hours after surgery. Wound fluids were centrifuged, aliquoted and stored at −20 °C. The use of human wound fluids was approved by the Ethics Committee at Lund University (LU 708-01 and LU 509-01). All experimental methods were performed in compliance to the guidelines and regulations of the Ethics Committee at Lund University, Lund Sweden with written informed consent from all participants.

**Digestion by human proteases in vitro.** Thrombin (4 μg–15 μg) was incubated with either human neutrophil elastase (HNE) or human neutrophil cathepsin G (HCG) at an enzyme-substrate ratio of 1:30 (w/w) in 10 mM Tris, pH 7.4 (volume of 10 μl) at 37 °C for variable time points (30, 60 and 180 minutes). The enzyme activity was blocked after the incubation by heating the samples at 95 °C for 3 minutes and further analysed by Tricine SDS-PAGE. The in vitro digestion analysis was performed in triplicates.

**In vitro digestion by bacterial proteases.** Thrombin (4 μg–15 μg) was incubated at 37°C with either V8 protease (0.2 μg/μg of thrombin), aureolysin (0.2 μg/μg of thrombin) or PAE (4 μU/μg of thrombin) separately in 10 mM NH₄HCO₃ (pH 7.8), 10 mM Tris containing 5 mM CaCl₂ (pH 7.8) and 10 mM Tris (pH 7.4) respectively in a total volume of 10 μl for 3 hours and 6 hours. The enzyme activity was blocked by heating the sample at 95 °C for 3 minutes and analysed by Tricine SDS-PAGE. Digestion analysis was performed in triplicates.

**SDS-PAGE and silver staining.** Seven μl of Novex sample buffer was added to each sample, and heated at 95°C for 10 minutes. The peptides, FYT21 and HVF18 were loaded for comparison. Five μl diluted samples were analysed by SDS-PAGE using a 10–20% Novex Tricine pre-cast gel run at 90 V, for 2 hours. The gel was stained with a Pierce Silver Stain Kit according to the manufacturer’s protocol.

**Immunoblotting.** Immediately following SDS-gel electrophoresis, the gels were assembled into the blot module (Invitrogen) based on the manufacturer's protocol. The transfer was performed at 25 V for 90 minutes on ice. After the transfer, the PVDF membranes were blocked with 5% milk for an hour, followed by overnight incubation of polyclonal antibodies recognizing the peptide VFR17 (VFRLKKWQKVIDQFGE) to a 1:5000 dilution at 4 °C. The membranes were washed 3 times for 10 minutes and incubated with HRP-conjugated secondary antibodies (Dako) at 1:5000 dilutions for an hour. The membranes were washed 3 times for 10 minutes. The C-terminal thrombin fragments were detected by SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, USA) for 5 minutes and thereafter imaged using a gel documentation system (Gel Doc XR + System).

**LC-MS/MS and data analysis.** After in vitro enzymatic digestion of thrombin, the reaction was stopped by acidifying the samples. The peptides were then dried and reconstituted in 0.1% formic acid (FA). Peptides were separated and analysed on a Dionex Ultimate 3000 RSLC nanoLC system coupled to a Q-Exactive tandem mass spectrometer (Thermo Fisher, MA). Five μl of sample was injected into an acclique peptide trap column via the auto-sampler of the Dionex RSLC nanoLC system. Flow rate was at 300 nL/min, and mobile phase A (0.1% FA in 5% acetonitrile) and mobile phase B (0.1% Fa in acetonitrile) were used to establish a 60 minute gradient. Peptides were analysed on a Dionex EASY-spray column (PepMap® C18, 3um, 100 A) using an EASY nanoprospect source. An electrospray potential was set at 1.5 kV. A full MS scan (350–1600 m/z range) was acquired at a resolution of 70000 at m/z 200, with a maximum ion accumulation time of 100 ms. Dynamic exclusion was set to
30 seconds. Resolution for MS/MS spectra was set to 35,000 at m/z 200. The AGC setting was 1E6 for the full MS scan and 2E5 for the MS2 scan. The 10 most intense ions above a 1000 count threshold were chosen for HCD fragmentation, with a maximum ion accumulation time of 120 milliseconds. An isolation width of 2 Da was used for the MS2 scan. Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 28. The underfill ratio was defined as 0.1%. Raw data files were converted into the mascot generic file format using Proteome Discoverer version 1.4 (Thermo Electron, Bremen, Germany) with the MS2 spectrum processor for de-isotoping the MS/MS spectra. The concatenated target-decoy UniProt human database (total sequences 92867, total residues 36953354, downloaded on 25 July 2016) was used for data searches. Database search was performed with non-enzyme option using an in-house Mascot server (version 2.4.1, Matrix Science, Boston, MA). Oxidation (M) and deamidation (N and Q) were kept as variable modifications. A total of three samples with two technical replicates (3 × 2) for each in vitro digest were analysed by LC-MS/MS. The identified peptides were then sorted on the peptide score and hits with <30 were omitted from analyses. Peptide hits consistently identified in all experimental and technical replicates were considered for qualitative comparison between in vitro and in vivo samples.

**Pull-down of thrombin C-terminal peptides from wound fluids.** Dynabeads M-280 sheep anti-rabbit IgG (Novex Life Technologies) were used according to the manufacturer’s instructions and as per the methods described earlier. Briefly, 100 μl of dynabeads pre-coated with sheep anti rabbit IgG antibodies were coupled with rabbit polyclonal antibodies (50 μl) recognizing the peptide sequence VFR17 (VFRKKWQKVIDQGE) (Innovagen, Lund, Sweden) by incubation overnight at 4°C. The coated dynabeads were subsequently washed according to the manufacturer’s protocol and incubated with 100 μl of AWF and/or CWF for 1 hour at RT, which was followed by elution with 20 μl of citric acid monohydrate (100 mM, pH 2).

**Mass spectrometry analysis of wound fluid.** Acute (AWF) and chronic wound fluids (CWF) from two patients each (n = 2 for AWF and CWF) were utilised in this study. Wound fluid samples were acidified and analysed by online nanoflow liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS experiments were performed on an EASY-nLC system (Thermo Scientific) connected to a LTQ orbitrap Velos Pro (Thermo Scientific) through a nanoelectrospray ion source and analysed as described earlier.

**Data availability.** All data generated or analysed during this study are included in this published article (and its Supplementary Information files). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD006992.

**References**


**Acknowledgements**

This research was supported by the Lee Kong Chian School of Medicine, Nanyang Technological University Start-Up Grant, Singapore Ministry of Education under its Singapore Ministry of Education Academic Research Fund Tier 1 (2015-T1-001-082), the Swedish Research Council (project 2012–1883), the Knut and Alice Wallenberg Foundation, and the Swedish Foundation for Strategic Research. RS was supported by a Lee Kong Chian School of Medicine Postdoctoral Fellowship, Nanyang Technological University.

**Author Contributions**

A.S., R.S., S.A., M.P., J.P., S.K. designed the research; R.S., S.A., Y.K.C., M.P., J.P. and S.K. performed the experiments and analysed the results; R.S. and A.S. wrote the manuscript. S.S. supported with mass spectrometry facility. All authors reviewed the manuscript; A.S. and R.S. arranged funding for this work.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-13197-3.
**Competing Interests:** The authors declare that they have no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

[Open Access] This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit [http://creativecommons.org/licenses/by/4.0/](http://creativecommons.org/licenses/by/4.0/).

© The Author(s) 2017