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## Peptidomic Analysis of Rat Plasma: Proteolysis in Hemorrhagic Shock

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### Abstract

It has been previously shown that intestinal proteases translocate into the circulation during hemorrhagic shock and contribute to proteolysis in distal organs. However, consequences of this phenomenon have not previously been investigated using high-throughput approaches. Here, a shotgun label-free quantitative proteomic approach was utilized to compare the peptidome of plasma samples from healthy and hemorrhagic shock rats to verify the possible role of uncontrolled proteolytic activity in shock. Plasma was collected from rats after hemorrhagic shock (HS) consisting of two-hour hypovolemia followed by two-hour reperfusion, and from healthy control (CTRL) rats. A new two-step enrichment method was applied to selectively extract peptides and low molecular weight proteins from plasma, and directly analyze these samples by tandem mass spectrometry. 126 circulating peptides were identified in CTRL and 295 in HS animals. 96 peptides were present in both conditions; of these, 57 increased and 30 decreased in shock. In total, 256 peptides were increased or present only in HS confirming a general increase in proteolytic activity in shock. Analysis of the proteases that potentially generated the identified peptides suggests that the larger relative contribution of to the proteolytic activity in shock is due to chymotryptic-like proteases. These results provide quantitative confirmation that extensive, system-wide proteolysis is part of the complex pathologic phenomena occurring in hemorrhagic shock.

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## Keywords

Hemorrhagic shock; Proteolysis; Serine-Proteases; Matrix Metalloproteases; Peptidomics; Mass spectrometry

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## INTRODUCTION

Circulatory shock is the leading cause of mortality in the intensive care unit [1], and despite decades of basic and clinical research, morbidity and mortality rates remain high. An incomplete understanding of the pathological mechanisms involved in tissue damage, organ dysfunction and failure limits available treatments for shock to source control (e.g. antibiotic therapy) and supportive therapy such as fluids and pressor agents.

The pathways activated in response to an initial insult, for example global ischemia in hemorrhagic shock (HS), suggest that shock involves several common pathologic mechanisms. One specific organ, long suspected as fundamental to the pathogenesis of shock is the intestine, whose failure triggers a widespread inflammatory state, which can ultimately lead to multiple organ failure (MOF) and death. One of the hypotheses that implicate the intestine in the progression of shock is centered around the role of the enteral digestive enzymes [2]. The intestinal mucosal barrier may become severely damaged due to hypoperfusion in hemorrhage and sepsis. As a consequence, its function as a barrier between the intestinal lumen and the interstitial tissue and vasculature may become compromised, and digestive enzymes, such as proteases and lipases may exit the intestinal lumen and reach the systemic circulation, resulting in global cellular and organ dysfunction [2–5]. Digestive proteases contribute to malfunction of important transmembrane receptors, such as the insulin receptor, possibly through cleavage [4], but they are also known to activate matrix metalloproteinases (MMPs) *in vivo*, and could therefore act as mediators of secondary cell and tissue injury. Both serine proteases and MMPs have the potential to cause uncontrolled pathological proteolysis, and this phenomenon may play an important role in the evolution of shock-induced organ failure.

We test here the hypothesis that hemorrhagic shock causes an increase in systemic proteolysis of proteins in the presence of increased enzymatic activity levels. We used high-throughput quantitative mass spectroscopy-based proteomic techniques to analyze the peptidome, in order to precisely identify and quantify the peptides in plasma.

Proteomic approaches have been introduced recently in shock research [6], but interpretation of the data is still limited by the lack of a comprehensive model of the disease. Here we report the results of the analysis of the peptidome of rat plasma collected after hemorrhagic shock (HS) in order to quantify the relative changes in the concentration of circulating peptides relative to their physiologic concentration in the plasma of healthy control rats. A systematic analysis of the circulating peptides, both in terms of their origin and possible generation by proteolytic cleavage, was carried out to obtain information on the proteolytic activity of several serine proteases and MMPs present in the bloodstream. The objective of the study was to garner direct evidence in support of one of the possible mechanistic processes underlying shock-induced organ failure and death, i.e. diffuse proteolysis. These

results may open future directions to identify possible biomarkers in circulatory shock, by analysis of peptides and proteins that appear in the plasma starting from the experimental protocol set up in the present report.

## MATERIALS AND METHODS

### A. Experimental Protocol

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego and conforms to the Guide for the Care and Use of Laboratory Animals, 8th edition, by the National Institutes of Health (2011).

Six male Wistar rats (300–450 g, Harlan Laboratories, Inc., Indianapolis, IN) were randomly assigned to either a control (CTRL, n=3) or a hemorrhagic shock (HS, n=3) group. The animals were housed in the vivarium of the Department of Bioengineering at the University of California San Diego and experiments took place during the morning. The temperature (~20–22 °C) and humidity (~70–75%) in the vivarium are controlled and monitored regularly, and the light/dark cycles (6 hours light/6 hours dark) are standardized according to the facility procedures. Each cage houses at most two rats. Food and water are given at libitum and the health of animals is confirmed by regular veterinary inspection. The well-being of the animals is ensured according to Federal, State and University regulation.

After general anesthesia (ketamine 75 mg/kg + xylazine, 4 mg/kg; i.m.) the right femoral vein was cannulated for blood withdrawal and intravenous supplemental anesthesia (xylazine, 4 mg/kg; ketamine 7.5 mg/kg i.v.) and the right femoral artery for continuous monitoring of arterial blood pressure. Body temperature was measured continuously rectally, and the animals were placed on a heated stage (at 37°C) and covered with a heat-retaining blanket in order to limit the temperature drop occurring during the hypovolemic phase of the experiment. Both HS and CTRL animals were heparinized (porcine heparin from Sagent Pharmaceuticals, Schamburg, IL) following an initial stabilization after the induction of anesthesia (1 unit heparin/cc total blood volume, estimated at 6% of the body weight). Hemorrhage was induced by blood withdrawal from the femoral vein (0.5 cc/min) to a target mean arterial pressure (MAP) of 35 mmHg. Hypovolemia was maintained for two hours, at which point the shed blood was reinfused (0.5 cc/min). The shed blood was maintained at room temperature (~22°C) and gently warmed to 37°C before reinfusion to minimize the temperature gradient.

The animals were monitored for an additional two hours, and euthanized at the end of the observation period, with Beuthanasia-D (120 mg/kg, Merck Animal Health). CTRL animals were monitored under anesthesia and without any further intervention for the same duration of the shock experiment until euthanasia. Death was confirmed by verification of cardiac arrest after thoracotomy to expose the chest cavity.

### B. Plasma collection

Just before euthanasia 1 mL of venous blood was collected in a BD Vacutainer® Plus Plastic K2EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) vial after

the addition of a protease inhibitor solution (a tablet of cOmplete™ Protease Inhibitor Cocktail, Roche, diluted in water to obtain a 10X solution with the plasma sample), and centrifugation at 1300 rpm for 10 min to separate plasma. The supernatant was collected, stored at +4°C and shipped for analysis by mass spectroscopy.

### C. Shotgun analysis and label free quantitation

**C.1 Sample preparation**—500 µL of each plasma sample were diluted with an equal volume of 32% (v/v) acetic acid, transferred to an Amicon Ultra-0.5 mL centrifugal filter (MWCO 10K), and centrifuged at 5000×g at 4 °C for 2 hour to deplete the high molecular weight proteins [7]. After the first centrifugation, 500 µL of 32% (v/v) acetic acid were added to the Amicon Ultra filter device and centrifuged at 5,000 × g for 2 hours. The filtrate was precipitated with two volumes of cold acetonitrile (ACN) containing 0.1% of trifluoroacetic acid (TFA) (stored at 4 °C for at least 1 hours) and centrifuged at 13,200 rpm for 30 minutes [8] to remove residual proteins. The supernatant containing peptides and low molecular weight proteins was collected, dried (Speed Vacuum), dissolved in 1% (v/v) formic acid and desalted (Zip-Tip C18, Millipore) before mass spectrometric (MS) analysis.

**C.2 Liquid chromatography electrospray–tandem MS/MS analysis**—LC-ESI-MS/MS analysis was performed on a Dionex UltiMate 3000 HPLC System with a PicoFrit ProteoPrep C18 column (200 mm, internal diameter of 75 µm) (New Objective, USA). Gradient: 1% ACN in 0.1 % formic acid for 10 min, 1–4 % ACN in 0.1% formic acid for 6 min, 4–30% ACN in 0.1% formic acid for 147 min and 30–50 % ACN in 0.1% formic for 3 min at a flow rate of 0.3 µl/min. The eluate was electrosprayed into an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific). The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap (at resolution 60000, AGC target 1000000) and subsequent collision-induced dissociation (CID) MS/MS in the linear ion trap of the 20 most intense peaks from full scan (normalized collision energy of 35%, 10 ms activation). Isolation window: 3 Da, unassigned charge states: rejected, charge state 1: rejected, charge states 2+, 3+, 4+: not rejected; dynamic exclusion enabled (60 s, exclusion list size: 200). Three technical replicate analyses of each sample were performed. Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

**C.3 Data processing and statistical analysis**—Mass spectra were analyzed using MaxQuant software (version 1.3.0.5). The initial maximum allowed mass deviation was set to 15 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. Enzyme specificity was set as unspecific. N-terminal acetylation, methionine oxidation, and asparagine/glutamine deamidation were set as variable modifications. The spectra were searched by the Andromeda search engine against the rat Uniprot sequence database (release 03.12.2014). Quantification in MaxQuant was performed using the built in XIC-based label free quantification (LFQ) algorithm [9] using fast LFQ. The required false positive rate was set to 5% at the peptide and 5% at the protein level, and the minimum required peptide length was set to 6 amino acids. Statistical analyses were performed using the Perseus software (version 1.4.0.6, [www.biochem.mpg.de/mann/tools/](http://www.biochem.mpg.de/mann/tools/)).

Three biological replicates (3 healthy vs. 3 hemorrhagic shock animals) were analyzed. Three technical replicates were carried out for each biological sample. Only peptides present in at least 2 out of 3 technical replicates were considered as positively identified and quantified in each biological replicate. Then, biological replicates were compared, leading to three groups of peptides: only-HS peptides (199 peptides), i.e. peptides that were found in HS samples in at least 2 out of 3 biological replicates; only-CTRL peptides (30), i.e. peptides that were found in CTRL samples in at least 2 out of 3 biological replicates; and peptides present in both HS and CTRL (96), i.e. identified and quantified in at least 2 out of 3 biological replicates both in HS and CTRL samples).

Peptides were considered increased or decreased if they were present only in either the healthy (CTRL) or hemorrhagic (HS) group or, in the case of the 96 peptides found in both groups, if they showed 50 % fold change differences (> 1.5-fold increase or < 0.67-fold decrease) in HS compared to CTRL, as calculated from the ratio of mean XIC-based label free quantification of the respective biological replicates [10].

Proteins were classified as secreted or non-secreted, and the presence of transmembrane topology was searched by combining the results of five different computer programs: Perseus Annotation, SecretomeP [11], SignalP [12], Phobius [13], TMHMM [14].

**C.4 Plasma protease activity**—The role played in the proteolysis observed following shock by some of the most important digestive enzymes (i.e., trypsin, chymotrypsin, elastase), and by several MMPs known for their proteolytic function in pathologic conditions [15, 16] (i.e. MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-14) was determined by matching the C-terminus of the sequences of the peptides identified in the MS-based analysis with the cleavage site specificities of the proteases.

The relative contribution of a specific protease to the overall proteolytic activity was estimated: i) in the case of HS, as the ratio between the number of peptides generated by the protease and found to be present only in HS or in a higher concentration vs. CTRL, and the total number of peptides present in the HS sample; ii) in the case of CTRL, as the ratio between the number of peptides generated by the protease and found to be present only in CTRL or in a higher concentration vs. HS, and the total number of peptides present in the CTRL sample. The data were also analyzed by a Fisher's exact test to detect statistically significant enrichment (increase and/or decrease) in the fraction of peptides potentially generated by a specific protease in HS or CTRL as compared to all peptides detected and quantified.

To cross-validate the estimates of protease activity derived from the peptidomics based approach described above, serine protease activity in the plasma was determined by gelatin zymography, as performed by SDS-PAGE gels containing 80 µg/mL gelatin. Gels were renatured by four 15 min washes with 2.5% Triton X-100 and incubated overnight at 37°C in developing buffer (0.05 M Tris base, 0.2 M NaCl, 4 µM ZnCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>·2H<sub>2</sub>O). Gels were subsequently stained (50% methanol, 10% acetic acid, 40% water, and 0.25% Coomassie blue solution) for one hour before de-staining in buffer (10% methanol, 10% acetic acid, 80% water). A standard protein ladder (Invitrogen) was used to estimate the

molecular weights of the proteases. Gels were digitized and bands were analyzed in ImageJ (<http://imagej.nih.gov/ij/>).

## RESULTS

### A. Peptidomics of plasma in shock

Shotgun label-free quantitative proteomic analysis is widely used to examine differences in global protein/peptide expression. This approach was utilized to compare the peptidome of plasma samples from healthy and hemorrhagic shock rats with the aim to identify and quantify the peptides present in plasma. These data serve to elucidate the possible role of uncontrolled proteolytic activity in shock. Figure 1 provides an overview of the workflow.

Due to the high complexity and wide dynamic range of plasma proteins, we applied a new two-step enrichment method to selectively extract peptides and low molecular weight (LMW) components, which are low abundant in plasma. The process consists of an ultrafiltration step with a 10kDa cut-off filter followed by a precipitation step conducted with a dissociating solution to increase the recovery of LMW molecules. To optimize reproducibility and quantification, each sample was then analyzed directly by LC-ESI MS/MS without any further separation step of the peptides mixture using the so-called shotgun approach. Analyses were performed on three biological replicates (3 CTRL vs 3 HS rats), each one analyzed three times (three technical replicates).

126 and 295 peptides were identified in the CTRL and HS groups, respectively (Figure 2A); among the 96 peptides present in both CTRL and HS, 57 increased (>1.5 fold-change) and 30 decreased (<0.67 fold change) in concentration in HS. Overall, the analysis identified 256 peptides, which were increased or present only in HS while 60 peptides were decreased or present only in the healthy group (CTRL). These peptides are listed in Tables 1 and 2 respectively, clearly showing a significantly increased number of peptides derived from an intrinsic proteolytic activity in HS samples in comparison with CTRL.

Computational identification of secreted and transmembrane proteins by means of prediction tools including Secreome P, SignalP, Phobius in combination with TMHMM allowed determination of the proteins from which the peptides originated (Figure 2B and Tables 3 and 4). The Tables report also the analysis carried out using the reference Plasma Proteome Database (<http://www.plasmaproteomedatabase.org/>), which contains a list of the plasma and serum proteins from healthy individuals. Among the peptides which increased or were present only in HS samples, 73% originated from proteins described as secreted or transmembrane while 23% (60 peptides out of 256) originated from 51 proteins out of 124 (41%) that are classified as non secreted. Moreover, 72% of the proteins have been previously reported in plasma according to the Plasma Proteome Database.

### B. Proteolytic activity

A higher protease activity was found in plasma samples following hemorrhagic shock. In particular, the analyses took into account, as possible effector enzymes for the previously described proteolysis, the main intestinal serine proteases (trypsin-like, chymotrypsin-like and elastase-like enzymes) and the most common MMPS (i.e. MMP-1, MMP-2, MMP-3,

MMP-7, MMP-9, and MMP-14). Both those peptides, which were increased or present only in the HS group, and peptides which were increased or present only in the CTRL group were examined, taking into account the C-terminus of each peptide and the cleavage site specificities of the proteases. In this way, it was possible to elaborate on the possible families of enzymes responsible for the cleavage of the proteins listed in the Tables and the subsequent generation of the related peptides. This analysis showed a huge increase in peptides generated by all proteolytic enzymes examined, in particular a significant increase of peptides possibly generated by chymotryptic-like enzymes: 53% in post-shock plasma compared to 22% in control, (Fisher's exact test p value of 8.06E-06) (Figure 3 and Table 5).

Consistent with the results from the peptidomic analysis, zymography demonstrated a significant increase in serine-protease activity in HS compared to CTRL plasma, with increased activity at a molecular weight compatible with that expected for chymotrypsin (Figure 4).

## DISCUSSION

The main finding of this work is that plasma displays an increase in peptides possibly generated by serine proteases after hemorrhagic shock, linking proteases to the larger presence of circulating peptides. Our data support and quantitatively confirm for the first time the hypothesis of extensive, system-wide proteolytic cleavage as part of the pathologic phenomena occurring in shock.

The importance of proteomics as a tool to shed light on the mechanisms underlying cardiovascular disease, and to propose biomarkers for early diagnosis and monitoring of the responsiveness to therapy has already been recognized [6, 17, 18] but, to our knowledge, no systematic study has utilized a peptidomic approach to investigate systemic proteolysis in the general and highly complex context of the possible pathological mechanisms taking place in shock. Therefore, a high-throughput mass spectroscopy-based approach as the peptidomic one described in this work appeared ideal to quantitatively investigate the problem of enzymatic proteolysis in hemorrhagic shock.

Several studies have examined lymph proteins in hemorrhagic shock [19–22]. The assumption in these studies is that the mesenteric lymph is a vehicle for injurious factors produced or released in the intestine, which are mediators of severe injury to organs distal other than the intestine. However, analysis of the lymph, despite its importance for understanding the generation and early transport of cytotoxic mediators, has the disadvantage of a more limited translational potential, due to the difficulty of collecting gut lymph samples from shock patients. In contrast, further investigations, initially conducted using this mass spectrometry based approach with plasma (a very easily available and abundant source) and involving a suitable larger number of animals and conditions, may lead to the detection of potential biomarkers which could then be routinely quantified using widespread and much less demanding detection techniques, such as antibody-based methods.

A common finding in previous reports is that several proteins are upregulated in shock lymph, especially proteins with inflammatory and pro-coagulation properties. D'Alessandro and coworkers [22] reported that the upregulation of such proteins is accompanied by an impaired homeostasis of the balance between proteases and anti-proteases, especially serine proteases and MMPs, and that this could entail an activation of neutrophils following extracellular matrix metalloproteinase involvement. These observations are consistent with the evidence that digestive enzymes can promote "autodigestion" [2–5]. Hence, we took the next step in this investigation, the analysis of peptides in plasma, with the goal of looking for evidence of proteolytic processes in shock, which could prove impactful in future clinical studies.

Several categories of proteins were affected by the proteolysis detected in our study:

- i. heat shock proteins related to mitochondrial function, which are known to be impaired in shock [23, 24];
- ii. serine protease inhibitors (Inter alpha-trypsin inhibitors, serine protease inhibitor A3N and A3K, plasma protease C1 inhibitor, alpha macroglobulins, etc.), indicating a possible response of the anti-proteases to the increased concentrations of circulating serine proteases in shock, and possibly suggesting that the increased protease activity produces extensive proteolysis because of a failure of the physiologic self-defense against proteases;
- iii. several cytoplasmic (e.g. aspartate aminotransferase) and membrane proteins (e.g.  $\beta_2$  microglobulin), suggesting that the degradation process also occurs inside cells or that cell contents leak into the bloodstream and become substrates for circulating proteases;
- iv. proteins involved in the coagulation cascade, typically involved in the response to hemorrhage, such as fibrinogen, coagulation factor XII, etc.;
- v. proteins involved in the inflammatory response initiated by hemorrhage, and specifically in the innate immune response, such as C-reactive protein, complement proteins, macrophage migration inhibitory factors, thymosin  $\beta_4$ , etc.

Many of the proteins from which the newly found fragments in shock are derived, besides being involved in a system-wide inflammatory and pro-coagulant response, are also typically associated with organ dysfunction, especially in the liver and the heart, and their presence in the plasma of HS patients, if verified, could serve as markers of organ failure and shock progression. The possible relationship between proteolysis and organ failure should be further investigated also on the basis of the result showing that the large majority of the cleaved proteins, from which the peptides increased in HS were generated, are cytoplasmic and membrane proteins. This could indicate cellular damage, which may play a role in organ dysfunction.

The enhanced activity of chymotrypsin-like proteases, estimated from the number of peptides generated by these enzymes with respect to the total number of peptides generated by proteolysis in shock, represents an important finding, which integrates previous data on protease activity in plasma and vital organs [2–5]. Low-throughput, semi-quantitative



techniques such as gel or in situ zymography are unable to discriminate among serine proteases. This intrinsic limitation of these approaches was overcome by the quantitative nature of mass spectroscopy.

It is important to observe that, despite the fact that chymotrypsin-like enzymes appear to be responsible for much of the proteolytic generation of peptides in shock, all the proteolytic enzymes analyzed and reported in Table 5, including elastase-like and MMPs, generate a larger number of peptides in shock than in control animals. However, the overwhelming contribution of chymotrypsin-like enzymes (more than 50% of peptides generated in shock) leads to an apparently limited contribution (i.e., percentage value of peptide generation, Table 5) of other proteases.

An important implication of our findings is that they fit and validate previous reports on the possible effects of digestive enzymes which escape the intestinal lumen because of the damage that gut ischemia induces in the mucosal barrier [2–5] and cause damage in distal organs (“Autodigestion Hypothesis”). The proteolytic activity was not only reported to be increased in plasma, but also in distal organs, and the framework of the Autodigestion Hypothesis first provided a possible explanation for the role of digestive proteases in the organ damage which characterizes circulatory shock. In particular, it was reported that the proteolytic function of these enzymes affect transmembrane receptor integrity, e.g. shock was shown to reduce the density of the extracellular domain of the insulin receptor [25]. The results presented in this manuscript do not include the presence of fragments of the extracellular domain of transmembrane receptors such as the insulin receptor, possibly because of the relative low abundance in comparison to other more abundant plasma proteins. Still, the hypothesis that proteolytic activity takes place and coincides with increased activity of digestive enzymes, such as the chymotrypsin-like enzymes, was quantitatively validated by the findings obtained through the proposed peptidomic approach.

The choice of the experimental hemorrhagic shock model, and the related advantages and possible pitfalls of this model deserve to be discussed as well. This study used the *Fixed-Pressure Hemorrhage* (Wiggers) model, which is one of the standard models for experimental hypovolemic shock. The predicted mortality rate for this type of model exceeds 75% [4], and animals that do not survive consistently demonstrate multiorgan failure as measured by gross tissue examination, histology, and certain cellular and tissue markers (e.g., Troponin I, lung wet/dry ratios).

As detailed by Fülöp and colleagues [26], this model has the advantage of being highly standardized, controlled and reproducible, and as such is optimal for the study of the pathophysiology and proteomics/peptidomics of shock. In particular: i) the controlled procedure for blood withdrawal and return (executed at the same rate of 0.5 cc/min, as explained in the Materials and Methods) is completely reproducible; ii) the objective control of the hemodynamic state of the animal during hypovolemia is ensured by the maintenance of blood pressure around the target value of 35 mmHg; iii) the monitoring of conditions such as body temperature (decreased following bleeding, constant throughout the hypovolemic phase, and increased again to physiologic levels upon reperfusion) is conveniently repeatable. All these elements are consistently observed in our experience with this HS

model [3–5, 25]. Of particular benefit for proteomics/peptidomics studies is this particular model minimizes trauma to the animals (e.g., a laparotomy was not done), mitigating the potential for confounders to the analysis of hemorrhage per se.

One of the main technical requirements of the *Fixed-Pressure Hemorrhage* model (and of every experiment where the animal is cannulated) is the use of anticoagulation to prevent the clotting of catheters and ensure the ability to withdraw, add, and store whole blood during the ischemic period. We used systemic heparinization both in the shock and control groups, which prevented any bias between the shock and the control groups. However, there could be a potential limitation of our results, in that heparin may have potentially interfered with proteolytic mechanisms, specifically the clotting cascade. Heparin increases (up to 600-fold [27, 28]) the inhibitory activity of antithrombin, a serpin (serine protease inhibitor) which binds, upon activation by heparin [27–30], to thrombin, trypsin and other coagulation factors. Further, proteases other than circulating serine proteases have been shown to be inhibited more effectively in the presence of heparin [30]. This may have led to an underestimate of the amount of proteolytic activity and subsequent peptide generation occurring in shocked animals compared to controls; thus our observations are most likely conservative in nature.

A second limitation of the experimental model is the necessity for storage of the drawn blood during hypovolemia. In order to maintain mean arterial pressure around the target level (i.e., ~ 35 mmHg in this experiment), it may be necessary to draw and/or reinfuse small aliquots of blood during the two-hour hypovolemic phase, and this requires that the shed blood is readily available at body temperature. Previous reports from our laboratory obtained both in hemorrhagic and non- hemorrhagic shock models, which do not entail ex vivo storage of blood for a pre-defined amount of time (e.g., splanchnic arterial occlusion shock or septic (peritonitis) shock or by endotoxin administration) have consistently shown that protease levels and activity are increased in shock independent of the model studied [4, 31–33]. Still, the possible effect of the storage of the shed (heparinized) blood at room temperature on excess proteolytic activity should be limited. Zimmerman and coworkers demonstrated that the temperature (including the range of room temperatures) and time of storage do not affect significantly blood proteins, which are broadly stable at the peptide level [34]. Additionally, heparin also has a protective effect on the stability of whole blood proteins at room temperatures, as reported by Henriksen et al. [35].

## Conclusions and Perspectives

In summary, the main conclusions of this study are:

- a quantitative confirmation that massive proteolysis occurs on the organism scale as a fundamental degrading phenomenon induced by shock;
- serine proteases, whose plasma activity is increased in shock, are largely responsible for the observed proteolysis;
- the experimental conditions of sample treatment and analysis suitable for the future identification of novel biomarkers of hemorrhagic shock in plasma has been set up.

One of the limitations of our study is the presentation of a “static” picture of the effect of shock at the end of the hypovolemia and reperfusion experiment. However, the possibility of collecting multiple blood samples in the rat is strongly limited by the sensitivity of the animal to blood withdrawal, especially during the hypovolemic phase, and a dynamic description of the phenomena reported in this manuscript during a similar hemorrhagic shock protocol requires the use of larger animals. Another possible limitation concerns the low number of animals included in the study. However, the use of technical replicates, which ensures robustness to the results obtained from each biological replicate, and the detection of a large increase in proteolytically produced peptides in all HS samples compared to CTRL, allows us to conclude that the present data fully support the direct experimental evidence of a general proteolytic activity in shock.

The role of the peptides, which are generated in shock, should be the subject of future analyses, given their potential role in shock. For example, vasoactive intestinal peptides, which are generated in the gut and promote cardiovascular responses (e.g., vasodilation and blood pressure reduction) are an example of peptides with patho-physiological relevance, to be further investigated, particularly in circulatory shock. If peptides present only in plasma from hemorrhagic shock animals, but not in control plasma, have possible vasoactive effects, they could become important pathogenic factors to study in the context of organ dysfunction induced by shock as well as new therapeutic targets.

The results presented in this manuscript serve as a starting point towards validation in large animals and clinical studies, in which an appropriate design should also include the assessment of the “dynamic” effects of shock on the peptidome and proteome (i.e., several blood samples over time from the same subject). Such studies should also propose the test of protease blockade as a cornerstone of an innovative therapeutic approach to protein degradation and peptide formation in circulatory shock.

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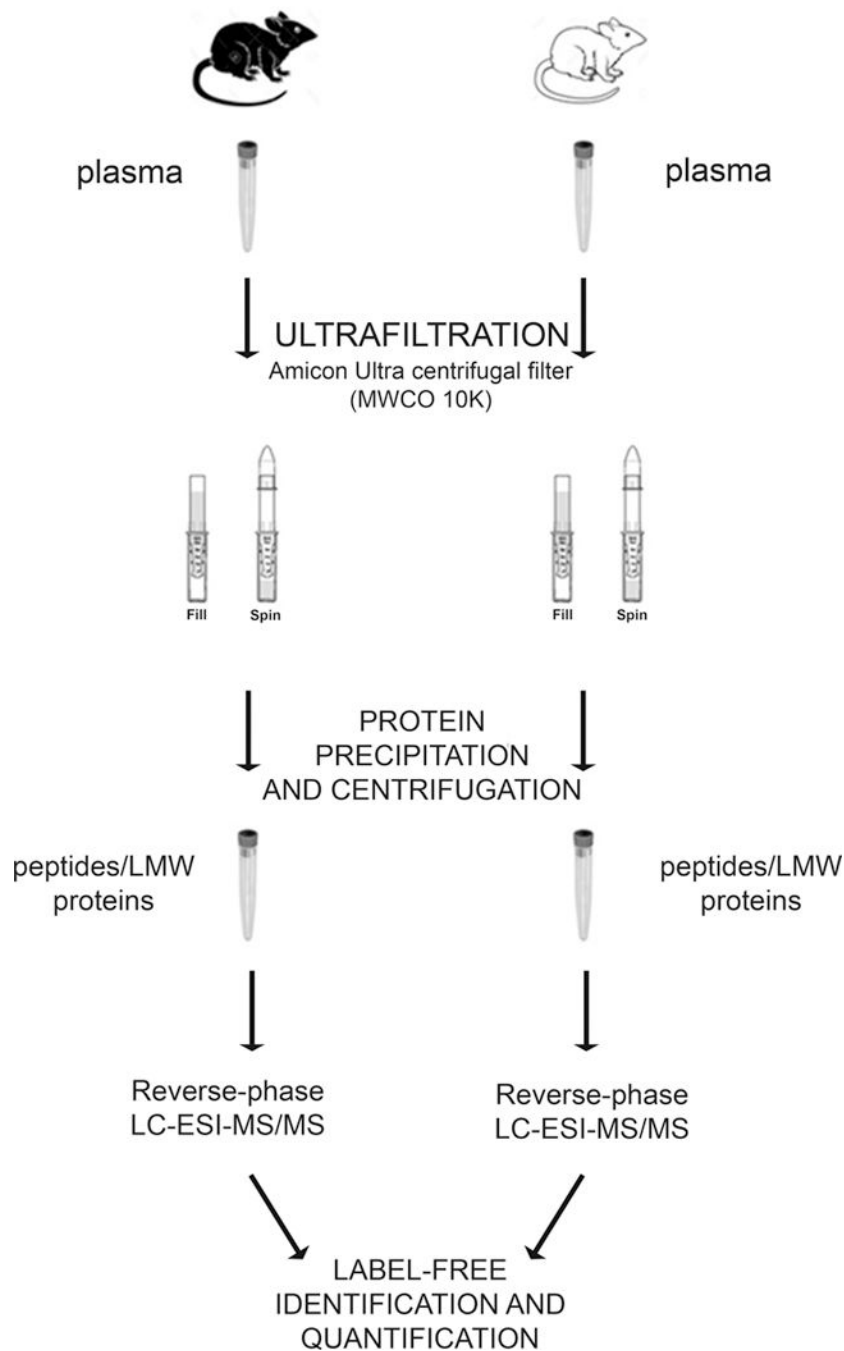
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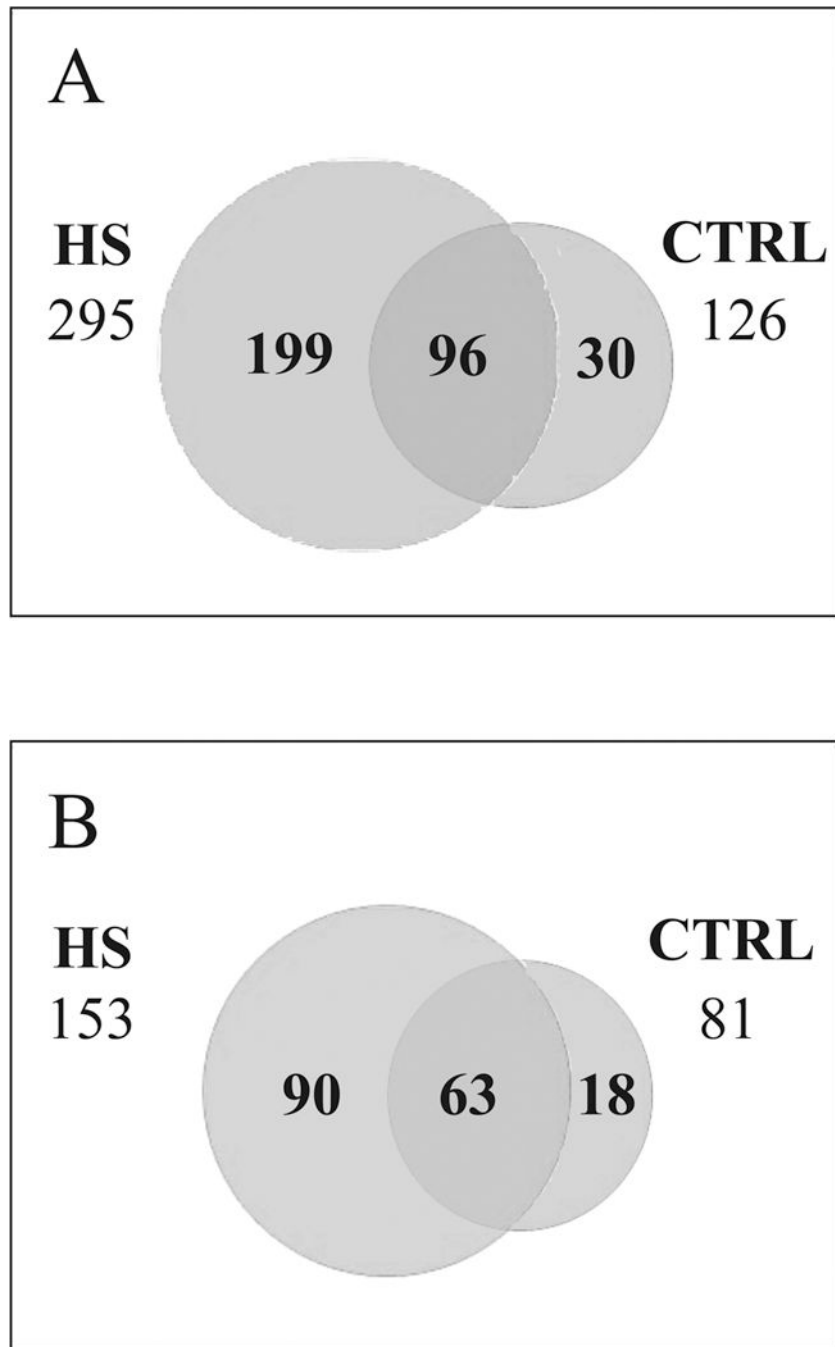
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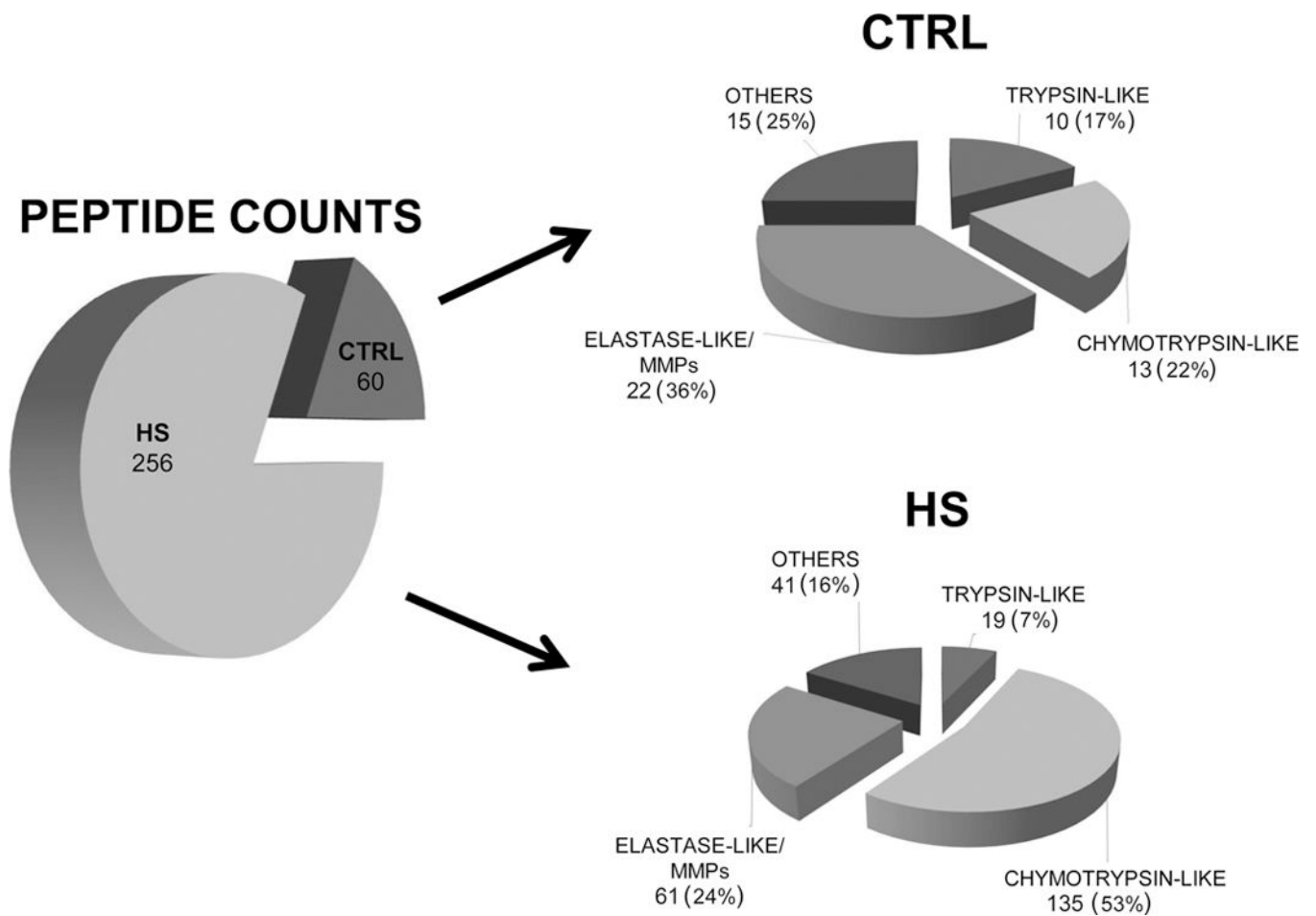


**Figure 1. Peptidome sample preparation workflow for mass spectrometric analysis**

A new two-step enrichment method to selectively extract peptides and LMW components in plasma samples from healthy and hemorrhagic-shock rats was applied. The process consists of an ultrafiltration step with a 10kDa cut-off filter followed by a precipitation step conducted with a dissociating solution before LC-ESI MS/MS analysis



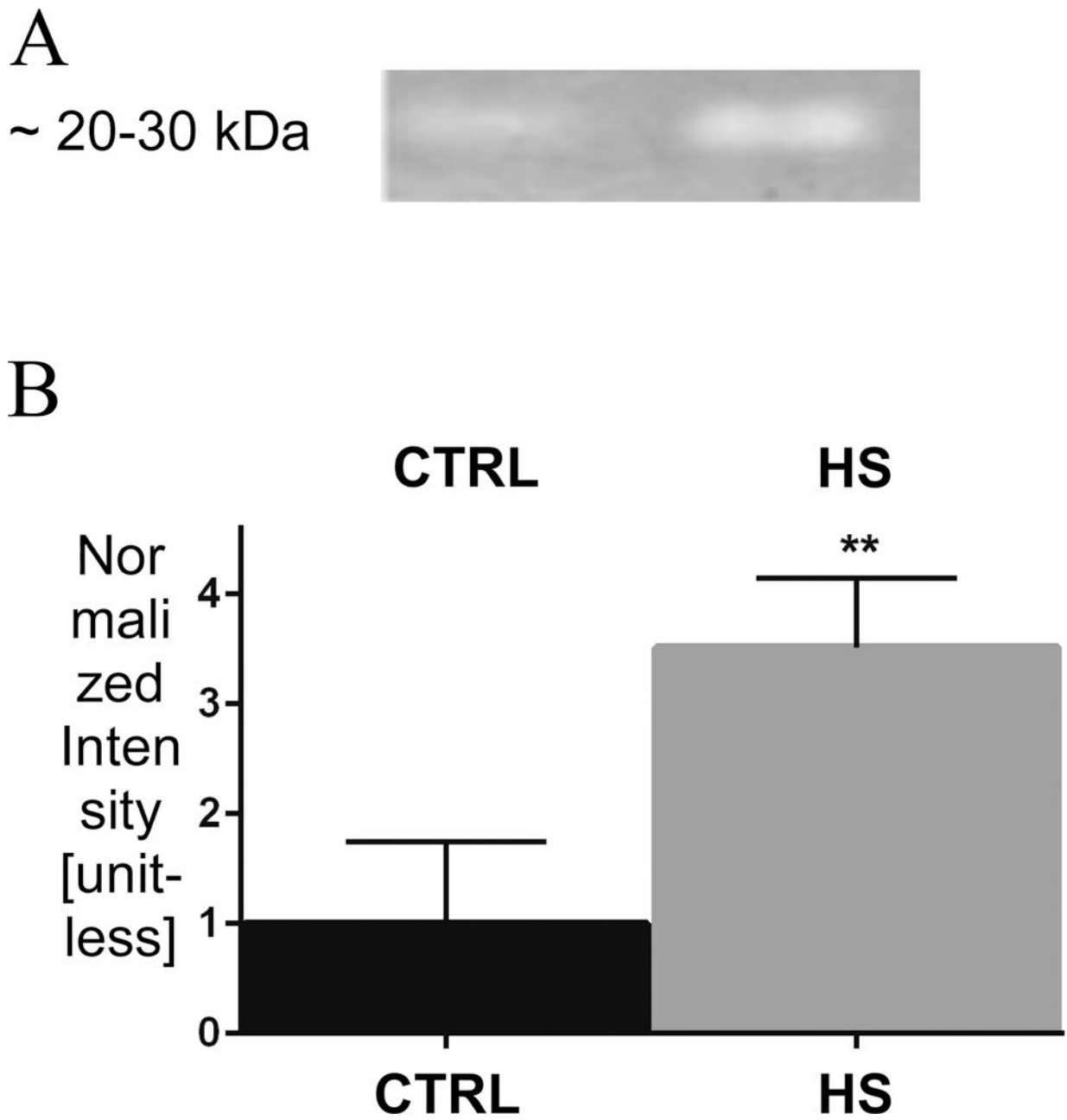
**Figure 2.**  
A) Venn diagram of the peptides identified in hemorrhagic (HS) and healthy (CTRL) rats. B)  
Venn diagram of the proteins identified in hemorrhagic (HS) and healthy (CTRL) rats



**Figure 3. Pie chart showing the number and the % of peptides in plasma samples of CTRL and HS**

The analysis was carried out both on peptides increased and present only in HS group (Table 1) and peptides decreased in HS and present only in healthy group (Table 2) focusing on the cleavage site specificities of the main serine proteases potentially derived from the intestine (trypsin-like, chymotrypsin-like and elastase-like enzymes) and on the most common MMPs: (i.e. MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-14)





**Figure 4. Plasma serine protease activity by gelatin zymography (n = 3 rats/group)**  
(A) Serine protease activity band (~ 20–30 kDa) and relevant statistics (B) \*\*p < 0.01 CTRL vs. HS.

**Table 1**  
**List of the peptides increased or present only in HS**

Peptides were considered increased if they were present only in either the healthy (CTRL) or hemorrhagic (HS) group or if they showed significant fold change differences between the two groups (> 1.5-fold increase or < 0.67-fold decrease in HS compared to CTRL) (ratio mean LFQ HS/mean LFQ CTRL). The specificity of proteases responsible for the generation of the peptides data set is indicated in the table (column: Selected Proteolytic Enzymes).

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
LGEQHFQGLVL	80.28	P02770	Alb	chymotrypsin-like
IQKTPQIQVY	28.79	P07151	B2m	chymotrypsin-like
SLEIPGSSDPNVIPDGFSSF	28.62	M0RB00	C4	chymotrypsin-like
LTTNPQGDITLDVDF	25.83	Q6IRS6	Fetub	chymotrypsin-like
ASTVVRPSFSLGNETLKVPLAL	24.55	Q510D7	Pepd	chymotrypsin-like
ATGKPRYVVLVPSELY	23.63	Q63041	A1m	chymotrypsin-like
EVTSDQVANVMWDY	19.5	P02651	Apoa4	chymotrypsin-like
LNGNSKYMVL	18.79	Q03626	Mug1	chymotrypsin-like
EAHKSEIAHRFKDLGEQHFQGLVL	17.84	P02770	Alb	chymotrypsin-like
AAPRPPAISVSVSAPAF	16.83	D4A7U1	Zyx	chymotrypsin-like
LQLTGGHEAF	15.61	B2GVB9	Fermt3	chymotrypsin-like
MSKQAFVFPVGSATAY	15.36	P48199	Crp	chymotrypsin-like
DAGLTNNLKPVAEEF	15.34	Q7TMC7	Tf	chymotrypsin-like
QKASLEHMVSMQY	12.56	D4ACQ4	RGD15608	chymotrypsin-like
DEPQSQWDRVKDFATVY	11.55	P04639	Apoa1	chymotrypsin-like
LLFGNSKY	11.35	Q6IE52	Mug2	chymotrypsin-like
KIILDPSGSMNIY	11.14	Q7TP05	Cfb	chymotrypsin-like
MDLPGQQPVSEQAQQLPPLAL	10.72	Q68FT8	Serpinf2	chymotrypsin-like
HEDMSKQAFVFPVGSATAY	7.88	P48199	Crp	chymotrypsin-like
HPSSPPVVDITKGVLGKY	7.37	P10959	Ces1c	chymotrypsin-like
SFSYKPRAPSAEVEVTAYVL	7.23	Q63041	A1m	chymotrypsin-like
DVQMTQSPSY	7.14	P01681		chymotrypsin-like
QAAETDVQTLFSQY	7.04	P04638	Apoa2	chymotrypsin-like
PVTSVDAAFRGPDSVF	7.03	P20059	Hpx	chymotrypsin-like
PNHFRPEGLPEKY	6.32	F7EHL9	Hps5	chymotrypsin-like
SDKPDMAEIEKFDKSKL	5.03	P62329	Tmsb4x	chymotrypsin-like
EDVPAADLSDQVPDITDSETRIL	4.71	M0RBF1	C3	chymotrypsin-like
LNGNSKYMVLVPSQL	3.42	Q03626	Mug1	chymotrypsin-like
DLPGQQPVSEQAQQLPPLAL	2.54	Q68FT8	Serpinf2	chymotrypsin-like
SDKPDMAEIEKF	1.94	P62329	Tmsb4x	chymotrypsin-like
KESTLHLVL	1.61	F1LML2	Ubc	chymotrypsin-like
AADISQWAGPLSL		P31044	Pebp1	chymotrypsin-like
AALQERLDNVSHTPSSY		Q5U2Z3	Nap114	chymotrypsin-like
AAPAKGENLSL		P27867	Sord	chymotrypsin-like

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
AAVVLENGVLSRKLSDFGQETSY		P04176	Pah	chymotrypsin-like
AEFYGSLEHPQTHY		Q7TMC7	Tf	chymotrypsin-like
AETDVQTLFSQY		P04638	Apoa2	chymotrypsin-like
AEVTGLSPGVTYLKFVF		P04937	Fn1	chymotrypsin-like
AGQAFRKFLPLF		P26772	Hspe1	chymotrypsin-like
AGQAFRKFLPLFDRVL		P26772	Hspe1	chymotrypsin-like
AGVLSRDAPDIESIL		O89000	Dpyd	chymotrypsin-like
AHKSEIAHRFKDLGEQHFGLVL		P02770	Alb	chymotrypsin-like
AKLLGLTL		P55159	Pon1	chymotrypsin-like
ALKNVPRSEVLAWNPDNLADY		Q920L0	Lcp2	chymotrypsin-like
ALKPLAPLLRGYHVVL		D4AA35	Asmtl	chymotrypsin-like
ALSMPNLGLKEEDKEPLIEL		G3V8C4	Clic4	chymotrypsin-like
APPSFFAQVPQAPPVLVFKL		P13221	Got1	chymotrypsin-like
ARGSVSDEEMMELREAF		Q5X138	Lcp1	chymotrypsin-like
ASINTDFTLSL		P05545	Serpina3k	chymotrypsin-like
ASSDIQVKELEKRASGQAFEL		P13668	Stmn1	chymotrypsin-like
ASTVRPSFSLGNETLKVPLALF		Q5I0D7	Pepd	chymotrypsin-like
ASVLTAPRLMEPIY		P05197	Eef2	chymotrypsin-like
ATGKPRYVVLVPSSEL		Q63041	A1m	chymotrypsin-like
ATTFKQDSPGQSSGFVY		P18757	Cth	chymotrypsin-like
ATTVSTQRGPVY		A2RUW1	Tollip	chymotrypsin-like
AVYSLSKSY		Q03626	Mug1	chymotrypsin-like
DEPQSQWDRVKDF		P04639	Apoa1	chymotrypsin-like
DGILGRDTPLEDQGGKGRQLHSLTL		P05545	Serpina3k	chymotrypsin-like
DIISNILHNF		D3ZY96	Ngp	chymotrypsin-like
DIVLTQSPVL		FILYU4		chymotrypsin-like
EAHKSEIAHRFKDLGEQHFGL		P02770	Alb	chymotrypsin-like
ELVEAYQEQAAGLLDGGVDILL		G3V8A4	Mtr	chymotrypsin-like
EMLKGMIMSGMNV AHL		D3ZH80	LOC689343	chymotrypsin-like
EMQQQELAQMRQRDANL		M0R4D8	Taf4a	chymotrypsin-like
ETLKDDTEKLGKQLNTEQNIL		Q5FVG2	Epb4115	chymotrypsin-like
FAQVPQAPPVLVFKL		P13221	Got1	chymotrypsin-like
FGSPLGKDLLFKDSAFGL		Q7TMC7	Tf	chymotrypsin-like
FIGGDAGDAFDGYDFGDDPSDKF		P02680	Fgg	chymotrypsin-like
FILKHTGPGILSMANAGPNTNGSQF		M0RCZ9	Ppia	chymotrypsin-like
FQVAEKPTVDGGVWSIL		Q5EBC0	Itih4	chymotrypsin-like
FRVGPESDKYRLTY		P02680	Fgg	chymotrypsin-like
FRVGPESDKYRLTYAY		P02680	Fgg	chymotrypsin-like
FTKTPKFFKPAMPFDL		M0RBF1	C3	chymotrypsin-like
FTNIHGRGGGALLGDRWIL		D4A1T6	C1r	chymotrypsin-like
FVLSPEQINAVY		P48199	Crp	chymotrypsin-like
FYARGNFEAQQRGSGGVW		F7EHL9	Hps5	chymotrypsin-like

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
GFGDLKTPAGLQVLNDYLADKSY		Q7TPK5	Eef1b2l	chymotrypsin-like
HKSEIAHRFKDLGEQHFHGLVL		P02770	Alb	chymotrypsin-like
IAHRFKDLGEQHFHGLVL		P02770	Alb	chymotrypsin-like
IAVDYLNKHLQGFQRIL		P24090	Ahsg	chymotrypsin-like
IIGGRNAELGLFPWQAL		Q8CHN8-2	Masp1	chymotrypsin-like
IVEGWDAEKGIAPWQVML		G3V843	F2	chymotrypsin-like
KPRLLLFSPSVVNLGTPLSVGVQL		M0RB00	C4	chymotrypsin-like
LDTKSYWKALGISPFHEY		P02767	Ttr	chymotrypsin-like
LELLDHVL		G3V852	Tln1	chymotrypsin-like
LGAPQEADASEEGVQRALDF		P14841	Cst3	chymotrypsin-like
LQIGATTQQAQKLKGEEVAF		P10959	Ces1c	chymotrypsin-like
LQKPEAELSPSL		P14046	A1i3	chymotrypsin-like
LQKPEAELSPSLIYDLPGMQDSNF		P14046	A1i3	chymotrypsin-like
LRSEPLDIKFNKPFILL		P31211	Serpina6	chymotrypsin-like
MEGPAGYLRRASVAQLTQELGTAF		P12928-2	Pklr	chymotrypsin-like
MEMDKRIY		P49911	Anp32a	chymotrypsin-like
MEVKPKLY		P14942	Gsta4	chymotrypsin-like
MFRNQYDNDVTVWSPQGRHQIEY		P18420	Psm1	chymotrypsin-like
MIVSETQSPLF		P17475	Serpina1	chymotrypsin-like
MMDQARSAFSNLF		G3V679	Tfrc	chymotrypsin-like
MNAAAEAEFNIL		Q80Z29	Nampt	chymotrypsin-like
MQKDASSGFLPSFQHF		P18757	Cth	chymotrypsin-like
PKPDSEAGTAFIQTQQLHAAMADTF		P11980	Pkm2	chymotrypsin-like
PYEIKKVF		Q5RKI0	Wdr1	chymotrypsin-like
RKLQPNLYVVAELFTGSEDL		D4AEH9	Agl	chymotrypsin-like
RLLWESGSL		M0RBF1	C3	chymotrypsin-like
RVELDTKSYWKALGISPFHEY		P02767	Ttr	chymotrypsin-like
SEIAHRFKDLGEQHFHGLVL		P02770	Alb	chymotrypsin-like
SEKKQPVLDGLLEEDDEF		D3ZHW9	Shfm1	chymotrypsin-like
SFSYKPRAPSAEVMETAY		Q63041	A1m	chymotrypsin-like
SKPDNPGEDAPAEDMARY		P07808	Npy	chymotrypsin-like
SLLDEFYKL		Q5M9G3	Caprin1	chymotrypsin-like
SLLFGNSKY		Q6IE52	Mug2	chymotrypsin-like
SLPEGVVDGIEIY		Q63416	Itih3	chymotrypsin-like
SMTDLLSAEDIKKAIGAF		P02625	Pvalb	chymotrypsin-like
SPMYSIITPNVLRLESEETF		M0RBF1	C3	chymotrypsin-like
SPTVFRLLWESGSL		M0RBF1	C3	chymotrypsin-like
SPTVFRLLWESGSL		M0RBF1	C3	chymotrypsin-like
SQADFKAEEVVKRLKTQPTDEEML		P11030	Dbi	chymotrypsin-like
SSGNAKIGHPPAPSF		Q63716	Prdx1	chymotrypsin-like
STNESSNSHRGLAPTNDVFAFNL		P31211	Serpina6	chymotrypsin-like
SYDRAITVFSFDGHLFQVEY		F1LSQ6	Psm1	chymotrypsin-like

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
TDVANYLDWIQEHTAF		D3ZTE0	F12	chymotrypsin-like
TVFRLLWESGSL		M0RBF1	C3	chymotrypsin-like
VDIFEPQGISRLDAQASF		B2RYM3	Itih1	chymotrypsin-like
VDLPGGLHLQSFPLSVEPALGIY		Q63041	A1m	chymotrypsin-like
VRLLWESGSL		M0RBF1	C3	chymotrypsin-like
VSLEGFTQPVAVF		P10959	Ces1c	chymotrypsin-like
VTGLSPGVTYLTKVF		P04937	Fn1	chymotrypsin-like
VVFTANDSGHRHYTIAALLSPYSY		P02767	Ttr	chymotrypsin-like
VVLSAPAVESELSPRGGEF		Q03626	Mug1	chymotrypsin-like
VYDAGLTPNNLKPVAAEF		Q7TMC7	Tf	chymotrypsin-like
WEFWQDEPQSQWDRVKDF		P04639	Apoa1	chymotrypsin-like
WTKTLPQKIQLKGSQSKHAEL		D3ZE31	Ces2i	chymotrypsin-like
YASVLTAQPRLMPEIY		P05197	Eef2	chymotrypsin-like
YAVGGRSHKPLDMSKVF		M0RB00	C4	chymotrypsin-like
YSIITPNVLRLESEETFIL		M0RBF1	C3	chymotrypsin-like
YSLAPQIKVIAPWRMPEF		P09034	Ass1	chymotrypsin-like
YVRPGGGFVFNQFL		P23764	Gpx3	chymotrypsin-like
YVGRPLVSQYNV	13.72	Q9WUW3	Cfi	elastase-like
FDWISYYVGRPLVSQYNV		Q9WUW3	Cfi	
FRLGNMIV		P02091	Hbb	
MATPVVTKTAWKLQEIV		Q4VFZ4	Katnb1	
FAIEEYSAPFSSDSEQGNA	6.87	Q63041	A1m	elastase-like/MMPs
YPSKPDNPGEDAPAEDMA	4.16	P07808	Npy	elastase-like/MMPs
FLSRLMSPEEKAPAAA	1.92	P04638	Apoa2	elastase-like/MMPs
AAGTLYTYPENWRAFKALIA		Q68FR6	Eef1g	elastase-like/MMPs
AEVVFTANDSGHRHYTIA		P02767	Ttr	elastase-like/MMPs
AGQAQRKFLPLDFRVLVERSA		P26772	Hspe1	elastase-like/MMPs
ASDPILYRPVA		P11980	Pkm2	elastase-like/MMPs
DAGLTPNNLKPVA		Q7TMC7	Tf	elastase-like/MMPs
FDKFTWSSLMMSQVVNPA		P31211	Serpina6	elastase-like/MMPs
INYYDMNAANVGWNGSTFA		D3ZE63	LOC686548	elastase-like/MMPs
MDFLSRLMSPEEKAPAAA		P04638	Apoa2	elastase-like/MMPs
MPLFFRKRKRPSEEARKRLEYQMCLA		D3ZI42	Lrsam1	elastase-like/MMPs
PSKPDNPGEDAPAEDMA		P07808	Npy	elastase-like/MMPs
SETAPAETTAPAPVEKSPA		D3ZBN0	Hist1h1b	elastase-like/MMPs
SFSYKPRAPSAEVEMTA		Q63041	A1m	elastase-like/MMPs
VVFTANDSGHRHYTIA		P02767	Ttr	elastase-like/MMPs
VVYPWTQRYFDSFGDLSSA		P02091	Hbb	elastase-like/MMPs
YETDEFAIEEYSAPFSSDSEQGNA		Q63041	A1m	elastase-like/MMPs
KPRLLLFSPSVVNLGTPLSVG	34.99	M0RB00	C4	elastase-like/MMPs
EAHKSEIAHRFKDLGEQHFKG		P02770	Alb	elastase-like/MMPs
FGIDKDAIVQAVKGLVTKG		G3V826	Tkt	elastase-like/MMPs

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
HPSSPPVVDTTKGGVLGKYVSLEG		P10959	Ces1c	elastase-like/MMPs
LLLFSFSVVNLGTPLSVG		M0RB00	C4	elastase-like/MMPs
LVQKDTVVKPVIVEPEG		Q63041	A1m	elastase-like/MMPs
VPETGRKDTVVKVLIVEPEG		Q03626	Mug1	elastase-like/MMPs
VQKDTVVKPVIVEPEG		Q63041	A1m	elastase-like/MMPs
SDKPDMAEIEKFDKS	34.7	P62329	Tmsb4x	MMPs
VQEQQEQVQPKPLES	18.24	P02651	Apoa4	MMPs
NPLPSKETIEQEQAGES	1.83	P62329	Tmsb4x	MMPs
AAVSKIAWHVIRNS		Q9EQV9	Cpb2	MMPs
EALAAVSKIAWHVIRNS		Q9EQV9	Cpb2	MMPs
EKNPLPSKETIEQEQAGES		P62329	Tmsb4x	MMPs
EQVQEQQVQPKPLES		P02651	Apoa4	MMPs
ETQEKPLPSKETIEQEQAGES		P62329	Tmsb4x	MMPs
KNPLPSKETIEQEQAGES		P62329	Tmsb4x	MMPs
QVQEQQEQVQPKPLES		P02651	Apoa4	MMPs
TVFRLWES		M0RBF1	C3	MMPs
ALLSPYSYSTTAVVSNPQN	1.77	P02767	Ttr	MMPs
FYIMPVMNVGDYDTWKKNRMWRKN		Q9EQV9	Cpb2	MMPs
LNETHGDEPFQYKN		P09606	Glul	MMPs
SFFNLTKEMVKDVLRGQN		B1WC01	Kif20a	MMPs
SFSYKPRAPSAEVE	5.16	Q63041	A1m	MMPs
AALKDQLIVNLLKE		P04642	Ldha	MMPs
AMQKIFAREILDSRGNPTVE		P15429	Eno3	MMPs
ASKRALVILAKGAEEME		O88767	Park7	MMPs
DFESEFVYMLNQCFKFLQMKRETE		F1LW73		MMPs
DGLFSFFKE		F7EHL9	Hps5	MMPs
DVGSYQEKVDVVLGPIQLQSPSKE		Q8CIZ5	Dmbt1	MMPs
EHICMSEHMCSEHMCLSEHMCMSSE		M0R8P2		MMPs
MWASCCNWFCLDGQPE		Q8CIN9	Rffl	MMPs
QSLLNQMMGKKQTLQRPTME		F1M3B2	Maml2	MMPs
RVELDTKSYWKALGISPFHEYAE		P02767	Ttr	MMPs
SDKPDMAEIEKFDKSKLKKTE		P62329	Tmsb4x	MMPs
SDKPDMAEIEKFDKSKLKKTETQE		P62329	Tmsb4x	MMPs
TSQIRQNYSTEVE		Q7TP54	Fam65b	MMPs
VDYSVWDHIE		Q63692	Cdc37	MMPs
VQKDTVVKPVIVEPE		Q63041	A1m	MMPs
SETQSPLFVGKVIDPTR	20.81	P17475	Serpina1	trypsin-like
SDKPDMAEIEKFDKSK	3.33	P62329	Tmsb4x	trypsin-like
MIVESETQSPLFVGKVIDPTR	3.29	P17475	Serpina1	trypsin-like
DSEVTSHSSQDPLVVQEGSR	3.15	Q6P734	Serpig1	trypsin-like
EDVPAADLSDQVPDTSSETR	1.94	M0RBF1	C3	trypsin-like
SFSQVTPAQMDLVFQR	1.91	Q64602	Aadat	trypsin-like

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
SETAAPAAPAPAEKTPIK	1.51	P15865	Hist1h1c	trypsin-like
DLEAMRMQTEMELRMFRQNEFMYHK		D4A3D2	Smyd1	trypsin-like
IISDTETAIPFLAKIFNPK		P09006	Serpina3n	trypsin-like
LASVSTVLTSKYR		P01946	Hba1	trypsin-like
LTSVPRYSMWFNQIMK		G3V8K8	Proz	trypsin-like
SDKPDMAEIEKFDKSKLK		P62329	Tmsb4x	trypsin-like
SETAAPAAPAPVEKTPVK		M0R7B4	LOC684828	trypsin-like
SPTVFRLWESGSLLR		M0RBF1	C3	trypsin-like
SSPTVFRLWESGSLLR		M0RBF1	C3	trypsin-like
TVFRLWESGSLLR		M0RBF1	C3	trypsin-like
VFRLWESGSLLR		M0RBF1	C3	trypsin-like
VLLAYLTSASSRPT		Q63041	A1m	trypsin-like
YPSKPDNPGEDAPAEDMAR		P07808	Npy	trypsin-like
FVKRQFMNKSLSGPGQ	31.94	B2GV73	Arpc3	
VITDNNGQSVFFMGKVTNPM	14.32	P05545	Serpina3k	
WTELLAKNPPQTEHTEHT	12.73	P10959	Ces1c	
MIVESETQSPLFVGKVID	7.35	P17475	Serpina1	
VLLAYLTSASSRPT	6.74	Q63041	A1m	
SDKPDMAEIEKFDKSKLKKT	6.56	P62329	Tmsb4x	
YQPVTMVTSQGGVVTQAIPQ	6.14	A0A096MJK	LOC100364346	
LAYLTSASSRPT	3.51	Q63041	A1m	
GEDAAQAEKFQHPNTD	2.79	Q66H98	Sdpr	
AQASLGEYLFERLTLKHD		Q7TP54	Fam65b	
ASGVAVSDGVIKVFND		M0R6D6	Cfl1	
DEPQSQWDRVKDFATVYVD		P04639	Apoa1	
EEVVSESFASGP		P02783	Svs4	
EQLNRNRRRQNEIMAKIQAAIIQI		Q9R095-2	Spef2	
FAQVPQAPPVLVFKLIAD		P13221	Got1	
FLFRDGDILGKYVD		P26772	Hspe1	
GEDAAQAEKFQHPNT		Q66H98	Sdpr	
GGGEMNPFETKVKTRIT		P04812	Svs5	
GGWVLVRDLLIPSNMMLYIVANGMI		Q2TGI4	Zdhhc25	
GMLNQQLTPVAGMMGGYP		D3ZT52	Pbrm1	
INDFHRAI		FILSG1	Kcnt1	
IQEVNTINQSLTTPMNLQIDPTIQ		Q4FZU2	Krt6a	
LAFQMYYQMAKYQAP		M0RAT6	Tmed8	
LGRFLESANMFFMVNQKVII		E9PTA3	LOC502618	
LVITDNNGQSVFFMGKVTNPM		P05545	Serpina3k	
MDAGLTFKTNQGLQTDQRED		M0RBF1	C3	
MIVESETQSPLFVGKVIDPT		P17475	Serpina1	
MKLTSEKLPKNPFSLSQYAAKQ		G3V9B8	Lrp2bp	
MRDKNRKPVVMKIKPDEIQNGQT		D3ZZW3	Entpd4	

Sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
MTAYVLLAYLTSASSRPT		Q63041	A1m	
SDKPDMAEIEKFDKSKLKKTET		P62329	Tmsb4x	
SDKPDMAEIEKFDKSKLKKTETQ		P62329	Tmsb4x	
SPMYSIITPNVLRLESEET		M0RBF1	C3	
TQLKRKENEIELSLQLREQQAT		D3Z8G3	Rpgrip11	
TRAGMERWRDRLALVT		G3V978	Dhrs11	
VAQASLGEYLFERLTLKHD		Q7TP54	Fam65b	
VKVLDAVRGSPAVD		P02767	Ttr	
VVLARLTAQPAPSPED		Q03626	Mug1	
WKQQVMTTVQNMQHESAQLQEELH		D4ABD7	Trip11	
YPSKPDNPGEDAPAED		P07808	Npy	
YVLLAYLTSASSRPT		Q63041	A1m	

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**Table 2**  
**List of the peptides decreased or present only in CTRL**

Peptides were considered increased if they were present only in either the healthy (CTRL) or hemorrhagic (HS) group or if they showed significant fold change differences between the two groups (> 1.5-fold increase or < 0.67-fold decrease in HS compared to CTRL) (ratio mean LFQ HS/mean LFQ CTRL). The specificity of proteases responsible for the generation of the peptides data set is indicated in the table (column: Selected Proteolytic Enzymes).

sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
SGNFIDQTRVLNLGPITRQGVQA	0.66	P27590	Umod	elastase-like/MMPs
TVIDQNRDGIIDKEDLRDT	0.66	P04466	Mylpf	
ELEAMSRYTSPVNPVAV	0.55	D3ZS52	LOC10036304	elastase-like
TAPKIPEGEKVDFFDDIQK	0.45	P09739;3	Tnnt3	trypsin-like
MEKEEETREL	0.35	M0R9D5	Ahnak	chymotrypsin-like
GTTSEFIEAGGDIR	0.31	P06399;Q	Fga	trypsin-like
PRLLLLLLLLLPSLAWG	0.26	B0BN16	Igfbp1	elastase-like/MMPs
DISNADRLGSSEVEQVQ	0.25	P00564	Ckm	
GQGLEWIGRIGPGSGDTNY	0.24	M0RBX3		chymotrypsin-like
WESGPEDQLTTPPLEP	0.24	P06759	Apoc3	
ELEEAEERADIAESQVN	0.22	F1LRV9;G	Myh4;Myh8;M	elastase-like/MMPs
HELEEAERADIAESQVN	0.22	F1LRV9;G	Myh4;Myh8;M	elastase-like/MMPs
TTALQPWHGAESKTDDSA	0.21	Q99MH3	Hamp	chymotrypsin-like
TTALQPWHGAESKTDDSA	0.21	Q99MH3	Hamp	elastase-like/MMPs
TTALQPWHGAES	0.19	Q99MH3	Hamp	elastase-like/MMPs
FEVTNLNMKSGMSLKIKGKIHN	0.19	Q9Z144	Lgals2	elastase-like/MMPs
MFRLLTACWSRQKATEGKQ	0.19	Q6AXT3	Arl13a	
ATTDSKVDLSIAR	0.16	P14480	Fgb	trypsin-like
TGTTSEFIEAGGDIR	0.16	P06399;Q	Fga	trypsin-like
SIASITVPLENQ	0.16	P02650	Apoe	
PTIETTFSDFDLLDALS	0.15	P08934;M	Kng1	elastase-like/MMPs
MLRLGALRLRGLALRS	0.15	Q5RKL4;Q	Dmgdh	elastase-like/MMPs
ETDAIQRTTEEEEA	0.14	G3V885;P	Myh6;Myh7;M	elastase-like/MMPs
ADTGTTFSEFIEAGGDIRGPRIVE	0.14	P06399;Q	Fga	elastase-like/MMPs
GFMGVMNMRKQRTLCDVI	0.11	Q5XHZ6	Klhl7	
ADTGTTFSEFIEAGGD	0.1	P06399;Q	Fga	
ADTGTTFSEFIEAGGDIR	0.09	P06399;Q	Fga	trypsin-like
DTGTTFSEFIEAGGDIR	0.08	P06399;Q	Fga	trypsin-like
TRQTTALQPWHGAES	0.06	Q99MH3	Hamp	elastase-like/MMPs
MGTAVLRKQLVLLL	0.05	B2RYM1	Angptl6	chymotrypsin-like
DIENPSSHVPEF		P06399;Q	Fga	chymotrypsin-like
EEAEQSNVNLAKF		F1LRV9;F1	Myh4;Myh8	chymotrypsin-like
EIQYRNNGVSSKQLMVF		P32232-2	Cbs	chymotrypsin-like
KENTHLLTL		Q921A3	Ubd	chymotrypsin-like

sequence	ratio HS/CTRL	Proteins	Genes	Selected Proteolytic enzymes
MMNFLRRRLSDSSFIANLPNGY		Q63537;Q	Syn2	chymotrypsin-like
MSPASLTFFCIGL		M0R8A3;	Gp6	chymotrypsin-like
TMMGKQEESQAGIIPQL		O88658;O	Kif1b	chymotrypsin-like
VGPIQSLQM		D3ZTA8	Rbm33	chymotrypsin-like
ARNKMLTGVVGLVL		M0RDH5		chymotrypsin-like
TKYETDAIQRTEELEEA		G3V885;P	Myh6;Myh7;M	elastase-like/MMPs
ADTGTTFEFIEAGGDIRG		P06399;Q	Fga	elastase-like/MMPs
SQDAIMDAIAGQAQAQG		A0A096M	Ldb3	elastase-like/MMPs
EGELEVDQLPGQS		P02650	ApoE	elastase-like/MMPs
AGSVADSDAVVKLDDGHLN		B4F7A3	Lgals1	elastase-like/MMPs
KNKMTKEQYIKMNRGIN		D4A631;Q	Arfgef1;Arfgef	elastase-like/MMPs
LEEAEERADIAESQVN		F1LRV9;G	Myh4;Myh8;M	elastase-like/MMPs
VIDLFGNEHDNFTKNLEN		D4A3H4	RGD1307621	elastase-like/MMPs
SQMPVLGKSSLRNVE		E9PSU2	Donson	elastase-like/MMPs
DAGAGIALNDFVK		M0R6I3;M	RGD1560797;	trypsin-like
DIDPEDEEELGPR		P31394;Q	Proc	trypsin-like
DLEEATLQHEATAATLR		F1LRV9;G	Myh4;Myh13	trypsin-like
TDSKVDLSIAR		P14480	Fgb	trypsin-like
DGPEGPKGRGGPNGDPGPL		Q9JI03;G3	Col5a1	
EHVQYMAEVIERLSNEPL		D3ZVJ0	Gmn	
MEHTGQYLHLVFLMT		Q5HZW9	Evi2a	
QQQQQQMQQLQQQ		F1LXK8	LOC100362634	
SRPSSKTSHRQQEEVKAPQMSQ		D3ZK09	Scyl3	
TAPKIPGEKVDFFDIQ		P09739-3;	Tnnt3	
TEEDDPGSSALLDVTVEH		G3V8D4	Apoc2	
VELGNDATDIEDD		P06866-2;	Hp	

**Table 3**  
**Proteins increased or present only in HS from which the peptides in Table 1 originate**

List of the proteins predicted to be secreted/transmembrane or non-secreted by Perseus Annotation, Secreome P, SignalP, Phobius and TMHMM software or previously reported in plasma according to the Plasma Proteome Database.

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma proteome DB
Alpha-1-inhibitor 3	P14046	AI13	x		
Alpha-1-macroglobulin	Q63041	AI1m	x		
Amylo-1, 6-glucosidase	D4AEH9	Agl		x	x
Alpha-2-HS-glycoprotein	P24090	Ahsg	x		x
Serum albumin	P02770	Alb	x		x
Acidic leucine-rich nuclear phosphoprotein 32 family	P49911	Anp32a		x	x
Apolipoprotein A-I	P04639	Apoa1	x		x
Apolipoprotein A-II	P04638	Apoa2	x		x
Apolipoprotein A-IV	P02651	Apoa4	x		x
Actin-related protein 2/3 complex subunit 3	B2GV73	Arpc3	x		x
Protein Asmtl	D4AA35	Asmtl	x		x
Argininosuccinate synthase	P09034	Ass1		x	x
Beta-2-microglobulin	P07151	B2m	x		x
Protein Clr	D4AIT6	Clr		x	x
Complement C3	M0RBF1	C3	x		x
Complement C4	M0RB00	C4	x		x
Caprin-1	Q5M9G3	Caprin1		x	
Hsp90 co-chaperone Cdc37	Q63692	Cdc37		x	x
Carboxylesterase 1C	P10959	Ces1c	x		
Protein Ces2a	D3ZE31	Ces2i	x		
Da1-24	Q7TP05	Cfb	x		x
Complement factor I	Q9WUW3	Cfi	x		x
Cofilin-1	M0R6D6	CH1	x		x
Chloride intracellular channel protein 4	G3V8C4	Clc4	x		x
Carboxypeptidase B2	Q9EQV9	Cpb2	x		x
C-reactive protein	P48199	Crp	x		x
Cystatin-C	P14841	Cst3	x		x

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma	proteome DB
Cystathionine gamma-lyase	P18757	Cth		x		x
Protein Dhrs11	G3V978	Dhrs11	x			
Deleted in malignant brain tumors 1 protein	Q8CIZ5	Dmbl1	x			x
Dihydropyrimidine dehydrogenase [NADP(+)]	O89000	Dpyd		x		x
Ac2-067	Q7TPK5	Eef1b2l	x			x
Elongation factor 1-gamma	Q68FR6	Eef1g		x		x
Elongation factor 2	P05197	Eef2		x		x
Beta-enolase	P15429	Eno3		x		x
Ectonucleoside triphosphate diphosphohydrolase 4	D3ZZW3	Entpd4	x			x
Band 4.1-like protein 5	Q5FVG2	Epb4115		x		x
Coagulation factor XII	D3ZTE0	F12	x			x
Prothrombin	G3V843	F2	x			x
Ferritin	Q7TP54	Fam65b		x		
Fermt3 protein	B2GVB9	Fermt3		x		x
Fetuin-B	Q6IRS6	Fetub	x			x
Fibrinogen gamma chain	P02680	Fgg	x			x
Fibronectin;Anastellin	P04937	Fn1	x			x
Aspartate aminotransferase	P13221	Got1		x		x
Glutathione peroxidase 3	P23764	Gpx3	x			x
Glutathione S-transf. alpha-4	P14942	Gsta4		x		
Hemoglobin subunit alpha-1/2	P01946	Hba1	x			x
Hemoglobin subunit beta-1	P02091	Hbb		x		x
Histone H1.5	D3ZBN0	Hist1h1b		x		x
Serum amyloid A protein	F7EHL9	Hps5	x			x
Hemopexin	P20059	Hpx	x			x
10 kDa heat shock protein	P26772	Hspe1	x			x
Itih1	B2RYM3	Itih1	x			x
Inter-alpha-trypsin inhibitor heavy chain H3	Q63416	Itih3	x			x
Inter alpha-trypsin inhibitor, heavy chain 4	Q5EBC0	Itih4	x			x
Katanin p80 WD40 repeat-containing subunit B1	Q4VFF4	Katmb1		x		x
Potassium channel subfamily T member 1	FILSG1	Kent1	x			x

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma	proteome DB
Kinesin-like protein	B1WC01	Kif20a		x		x
Keratin, type II cytoskeletal 6A	Q4FZU2	Krt6a		x		x
Lymphocyte cytosolic protein 1	Q5X138	Lcp1	x			x
Lymphocyte cytosolic protein 2	Q920L0	Lcp2		x		
Protein Pknox2	A0A096MJK1	LOC100364346		x		
Protein LOC502618	E9PTA3	LOC502618	x			
Protein LOC684828	M0R7B4	LOC684828		x		
Macrophage migration inhibitory factor	D3ZE63	LOC686548	x			x
Pyruvate kinase	D3ZH80	LOC689343		x		
LRP2-binding protein	G3V9B8	Lrp2bp	x			
Protein Lrsam1	D3ZI42	Lrsam1		x		x
Protein Maml2	F1M3B2	Maml2		x		
Mannan-binding lectin serine protease 1	Q8CHN8-2	Masp1	x			x
Methionine synthase	G3V8A4	Mtr		x		x
Murinoglobulin-1	Q03626	Mug1	x			
Murinoglobulin-2	Q6IE52	Mug2	x			
Nicotinamide phosphoribosyltransferase	Q80Z29	Nampt		x		x
Nucleosome assembly protein 1-like 4	Q5U2Z3	Nap1l4		x		x
Neutrophilic granule protein (P	D3ZY96	Ngp	x			
Pro-neuropeptide Y	P07808	Npy	x			x
Phenylalanine-4-hydroxylase	P04176	Pah		x		x
Protein DJ-1	O88767	Park7		x		x
Protein Pbrm1	D3ZT52	Pbrm1		x		x
Phosphatidylethanolamine-binding protein 1	P31044	Pebp1	x			x
Xaa-Pro dipeptidase	Q5I0D7	Pepd		x		x
Pyruvate kinase PKLR	P12928-2	Pklr		x		x
Pyruvate kinase M1/M2	P11980	Pkm2		x		
Serum paraoxonase/arylesterase 1	P55159	Pon1	x			x
Pept.-prolyl cis-trans isom/A	P10111	Ppia		x		x
Peroxi redoxin-1	Q63716	Prdx1	x			x
Protein Proz	G3V8K8	Proz	x			x

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma proteome DB
Proteas. Sub. alpha type-1	P18420	Psmal		x	x
Proteas. sub. alpha type	FILSQ6	PsmA7		x	x
Parvalbumin alpha	P02625	Pvalb		x	
E3 ubiq.-prot. ligase rffiflylin	Q8CIN9	Rffl	x		
Protein Zc3h12b	D4ACQ4	RGD1560891		x	
Protein Rpgrip11	D3Z8G3	Rpgrip11		x	
Alpha-1-antitrypsinase	P17475	Serpina1	x		x
Ser. Prot. inhibitor A3K	P05545	Serpina3k	x		
Ser. Prot. inhibitor A3N	P09006	Serpina3n	x		
Corticosteroid-binding globulin	P31211	Serpina6	x		x
Protein Serpinf2	Q68FT8	Serpinf2	x		x
Plasma protease C1 inhib.	Q6P734	Serping1	x		x
Protein Shfm1	D3ZHW9	Shfm1		x	
Protein Smyd1	D4A3D2	Smyd1		x	x
Sorbitol dehydrogenase	P27867	Sord	x		x
Stathmin	Q99R095-2	Spef2		x	
Sem. ves. Secr. protein 4	P13668	Stmn1	x		x
Sem. ves. Secr. prot. 5	P02783	Svs4	x		
Serotransferrin	P04812	Svs5	x		
Transferrin rec. protein 1	Q7TMC7	Tf	x		x
Transketolase	G3V679	Tfrc	x		x
Protein Tln1	G3V826	Tkt		x	x
Protein Tmed8	G3V852	Tln1		x	x
Thymosin beta-4	M0RAT6	Tmed8		x	
Toll-interacting protein	P62329	Tmsb4x	x		x
Protein Trip11	A2RUW1	Tollip		x	x
Transthyretin	D4ABD7	Trip11		x	x
WD repeat-cont. protein 1	P02767	Tr	x		x
Palmitoyltransferase	Q5RK10	Wdr1	x		x
Protein Zyx	Q2TGI4	Zdhhc25	x		
	D4A7U1	Zyx	x		x

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma proteome DB
Uncharacterized protein	FILW73		x		
Uncharacterized protein	FILYU4		x		
Uncharacterized protein	M0R4D8	Taf4a		x	
Ig kappa chain V region S211	P01681		x		

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**Table 4**  
**Proteins decreased or present only in CTRL from which the peptides in Table 2 originate**

List of the proteins predicted to be secreted/transmembrane or non-secreted by Perseus Annotation, Secreome P, SignalP, Phobius and TMHMM software or previously reported in plasma according to the Plasma Proteome Database.

Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma proteome DB
Protein Ahnak	M0R9D5	Ahnak		x	x
Angiopoietin-like 6	B2RYM1	Angptl6	x		x
Apolipoprotein C-II	G3Y8D4	Apoc2	x		x
Apolipoprotein C-III	P06759	Apoc3	x		x
Apolipoprotein E	P02650	Apoe	x		x
Brefeldin A-inhibited guanine nucleotide-exchange protein 1	D4A631	Arfgef1		x	x
ADP-ribosylation factor-like protein	Q6AXT3	Arh13a	x		
Cystathionine beta-synthase	P32232-2	Cbs	x		x
Creatine kinase M-type	P00564	Ckm		x	x
Collagen alpha-1(V) chain	Q9JH03	Col5a1	x		x
Dimethylglycine dehydrogenase	Q5RKL4	Dmgdh		x	x
Protein Donson	E9PSU2	Donson	x		x
Ecotropic viral integration site 2A	Q5HZW9	Evi2a	x		x
Fibrinogen alpha chain	P06399	Fga	x		x
Fibrinogen beta chain	P14480	Fgb	x		x
Geminin (Predicted)	D3ZWJ0	Gmn	x		x
Protein Gp6	M0R8A3	Gp6	x		
Hepcidin	Q99MH3	Hamp	x		
Haptoglobin	P06866-2	Hp	x		x
Insulin-like growth factor binding protein-like 1	B0BN16	Igfbp1	x		
Kinesin-like protein KIF1B	O88658	Kif1b		x	x
Kelch-like protein 7	Q5XHZ6	Klh7		x	
Kininogen-1	P08934	Kng1	x		x
Protein Ldb3	A0A096MKD	Ldb3		x	x
Galectin-2	Q9Z144	Lgals2	x		
Galectin	B4F7A3	Lgalsl		x	x
Histone-lysine N-methyltransferase	FILXK8	OC100362634		x	



Protein names	Protein IDs	Genes	transmembrane/secreted	non secreted	plasma	proteome DB
Myosin-4	FILRV9	Myh4		x		x
Myosin regulatory light chain 2	P04466	Mylpf		x		x
Vitamin K-dependent protein C	P31394	Proc	x			
Protein Rbm33	D3ZTA8	Rbm33		x		
Uncharacterized protein	D4A3H4	RGD1307621	x			
Protein Scyl3	D3ZK09	Scyl3	x			
Synapsin-2	Q63537	Syn2		x		
Troponin T	P09739-3	Tnni3		x		
Ubiquitin D	Q921A3	Ubd	x			
Uromodulin	P27590	Umod	x			x
	M0RKD5		x			
Uncharacterized protein	M0RBX3		x			

**Table 5****Plasma protease activity**

The contribution of a specific protease was calculated as the relative proportion between the number of peptides increased and present only in HS and on peptides decreased in HS and present only in CTRL and the total number of peptides present in the HS or CTRL sample.

<b>HS</b>				
<b>Digestive protease</b>	<b>Cleavage site C-term</b>	<b>peptide count (tot 256)</b>	<b>%</b>	<b>Total %</b>
Trypsin-like	K (Lys)	7	2.7	<b>7</b>
	R (Arg)	12	4.7	
Chymotrypsin-like	W (Trp) not before P	1	0.4	<b>53</b>
	Y (Tyr) not before P	39	15.2	
	F (Phe) not before P	37	14.5	
	L (Leu) not before P	58	22.7	
	M (Met) not before P	/		
Elastase-like and Metalloprotease (MMPs)	A (Ala)	18	7.0	<b>24</b>
	G (Gly)	8	3.1	
	V (Val)	4	1.6	
	S (Ser)	11	4.3	
	N (Asn)	4	1.6	
	E (Glu)	16	6.3	
<b>CTRL</b>				
<b>Digestive protease</b>	<b>Cleavage site C-term</b>	<b>peptide count (tot 60)</b>	<b>%</b>	<b>Total %</b>
Trypsin-like	K (Lys)	2	3.3	<b>17</b>
	R (Arg)	8	13.3	
Chymotrypsin-like	W (Trp) not before P	/		<b>22</b>
	Y (Tyr) not before P	2	3.3	
	F (Phe) not before P	3	5.0	
	L (Leu) not before P	7	11.7	
	M (Met) not before P	1	1.7	
Elastase-like and Metalloprotease (MMPs)	A (Ala)	4	6.7	<b>36</b>
	G (Gly)	3	5.0	
	V (Val)	1	1.7	
	S (Ser)	5	8.3	
	N (Asn)	7	11.7	
	E (Glu)	2	3.3	