A selected reaction monitoring-based analysis of acute phase proteins in interstitial fluids from experimental equine wounds healing by secondary intention

Louise Bundgaard, DVM, PhD[1]; Emøke Bendixen, MSc, PhD[2]; Mette Aa. Sørensen, DVM, PhD[1]; Victoria M. Harman, BSc Hons, MPhil[3]; Robert J. Beynon, BSc Hons, PhD[3]; Lars J. Petersen, MD, PhD, DMSc[4,5]; Stine Jacobsen, DVM, PhD[1]

1. Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark,
2. Department of Molecular Biology and Genetics, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark,
3. Department of Biochemistry, Centre for Proteome Research, Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom,
4. Department of Nuclear Medicine, Clinical Cancer Research, Aalborg University Hospital, Aalborg, Denmark and
5. Department of Clinical Medicine, Aalborg University Hospital, Aalborg, Denmark

Reprint requests:
Stine Jacobsen, Department of Large Animal Sciences, University of Copenhagen, Højbakkegård Alle 5, DK - 2630 Taastrup, Denmark,
Tel: +45 35332620;
Fax: +45 35332858;
Email: stj@sund.ku.dk

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ABSTRACT

In horses, pathological healing with formation of exuberant granulation tissue (EGT) is a particular problem in limb wounds, whereas body wounds tend to heal without complications. Chronic inflammation has been proposed to be central to the pathogenesis of EGT. This study aimed to investigate levels of inflammatory acute phase proteins (APPs) in interstitial fluid from wounds in horses. A novel approach for absolute quantification of proteins, selected reaction monitoring (SRM)-based mass spectrometry in combination with a quantification concatamer (QconCAT), was used for the quantification of five established equine APPs (fibrinogen, serum amyloid A, ceruloplasmin, haptoglobin, and plasminogen) and three proposed equine APPs (prothrombin, α-2-macroglobulin, and α-1-antitrypsin). Wound interstitial fluid was recovered by large pore microdialysis from experimental body and limb wounds from five horses at days 1, 2, 7, and 14 after wounding and healing without (body) and with (limb) the formation of EGT. The QconCAT included proteotypic peptides representing each of the protein targets and was used to direct the design of a gene, which was expressed in *Escherichia coli* in a media supplemented with stable isotopes for metabolically labeling of standard peptides. Co-analysis of wound interstitial fluid samples with the stable isotope-labeled QconCAT tryptic peptides in known amounts enabled quantification of the APPs in absolute terms. The concentrations of fibrinogen, haptoglobin, ceruloplasmin, prothrombin, and α-1-antitrypsin in dialysate from limb wounds were significantly higher than in dialysate from body wounds. This is the first report of simultaneous analysis of a panel of APPs using the QconCAT-SRM technology. The microdialysis technique in combination with the QconCAT-SRM-based approach proved useful for quantification of the investigated proteins in the wound interstitial fluid, and the results indicated that there is a state of sustained inflammation in equine wounds healing with formation of EGT.

Second intention wound healing of equine limb wounds is often complicated by formation of exuberant granulation tissue (EGT), while wounds on the body usually heal uneventfully.[1] The pathogenesis of EGT formation in the horse is still unresolved, but chronic inflammation seems to be implicated.[2] Cytokines, chemokines, and growth factors are the most extensively studied biomolecules in impaired wound healing,[3] whereas the acute phase proteins (APPs) have gained less attention. APPs are a heterogeneous group of proteins,[4] which are used as diagnostic markers in human[5] and veterinary medicine.[4] While APPs are mainly used as markers of systemic inflammation, recent studies in several species have investigated APPs in local microenvironments under normal and inflammatory conditions.[6–8] The aim of this study was to investigate the concentrations of APPs in the local environment of wounds healing with and without formation of EGT.

Simultaneous analysis of a panel of APPs may be more informative than assessment of individual proteins.[9] Selected reaction monitoring (SRM)-based mass spectrometry is a rapidly advancing method that has proved of great

APPs	Acute phase proteins
EGT	Exuberant granulation tissue
SAA	Serum amyloid A
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRM	Selected reaction monitoring
QconCATs	Quantification concatamers

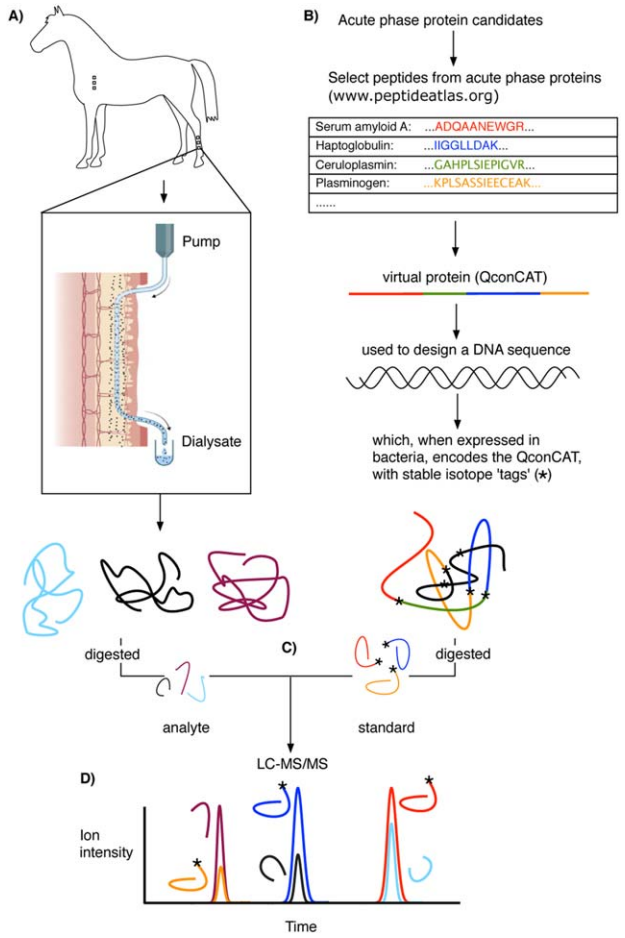

Figure 1. (A) Experimental wounds were created on the shoulder and distal limb of five horses. On sampling days a linear microdialysis probe was inserted into the wound tissue and continuously perfused to sample interstitial fluid dialysate. (B) The equine PeptideAtlas supported selection of protein specific peptides. The peptides were assembled into a virtual protein (QconCAT) used to direct the design of a gene, which was expressed in *Escherichia coli* in a media supplemented with stable isotopes for metabolically labeling of standard peptides. (C) The dialysate and QconCAT were digested, mixed, and analysed by selected reaction monitoring mass spectrometry. (D) The accurately quantified isotope-labeled peptide supported absolute quantification of each protein-specific peptide from the intensity ratio of the labeled and unlabeled peptide. The concentration of each specific protein was calculated by taking into account the molecular weight of each specific full-length protein. The illustration of the microdialysis technology is reprinted from Sorensen et al.,[10] with permission from Elsevier. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

relevance for this purpose (Figure 1). SRM allows simultaneous detection and robust quantification of multiple proteins and circumvents the need of antibodies.[11] SRM methods rely on identification of proteotypic peptides representing the specific proteins of interest, and quantification in absolute terms is possible by coanalysis of stable isotope-labeled reference peptides in known amounts.[12] The quantification concatamers (QconCATs) have become a recognized approach to the production of stable isotope-labeled reference peptides in large numbers. A concatamer of the proteotypic peptides are used to direct the design of a chimeric QconCAT gene, which is expressed in *Escherichia coli* and metabolically labeled with specific amino acids containing stable isotopes. Purification and a subsequent tryptic digestion provide labeled reference peptides.[13]

Here, we describe for the first time a time resolved and quantitative change of equine APPs in wound interstitial fluid collected by microdialysis. QconCAT calibrated SRM was used to compare expression of APPs in wounds healing normally or with formation of EGT.

MATERIALS AND METHODS

Clinical experimental procedure

Details concerning the included animals, protocol for creation of experimental wounds, sampling of blood, and collection of interstitial fluid by microdialysis are described in Sørensen et al., 2014.[14] In short, five healthy geldings each had a total of 12 full-thickness wounds made; three wounds on each shoulder over the deltoid muscle penetrating the superficial part of the fascia and three on the lateral aspect of each metatarsus extending to the periosteum, in a vertical column, 2.5 × 2.5 cm, 3 cm apart. Limb wounds were bandaged during the entire study period to provoke formation of EGT, whereas body wounds were not bandaged. Healing was monitored for 14 days. The protocol was approved by the Danish Animal Experiments Inspectorate, Ministry of Justice (license no. 2010/561 − 1882), and all procedures were carried out according to the Danish Animal Testing Act.

Blood and interstitial fluid (dialysate) were collected at day 1, 2, 7, and 14 days after wounding. Prior to sampling of interstitial fluid the horses were sedated and received a subcutaneous inverted L-block to desensitize the shoulder over the deltoid muscle, and a fibular (peroneal) and tibial nerve block to desensitize the hind limbs from tarsus and distal. Wounds were randomized to be studied at individual study days. Interstitial fluid was sampled by inserting a custom-made, linear microdialysis probe with molecular cut off weight of 2,000 kDa into the wound tissue using a 19 G guide cannula. The probe was continuously perfused with sterile Ringers acetate (2 μL/minute) (Figure 1). Dialysates were collected for 60 minutes starting from two hours after probe insertion. The dialysate was snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

The wound areas were calculated using the Visitrack wound measurement grid (Smith & Nephew, Hørsholm, Denmark) according to the manufacturer's instructions.

Sample processing

Blood samples

Plasma fibrinogen concentration was determined by the Clauss method in an automated coagulometric analyser (ACL 9000, Instrumentation Laboratory, Bedford, MA).

Serum SAA concentrations were determined by a previously described immunoturbidometric method (LZ test SAA; EIKEN Chemical CO, Tokyo, Japan).[15] Total protein in serum was measured on the Advia 1800 Chemistry System (Siemens Healthcare, Erlangen, Germany).

Dialysate samples

The total protein concentrations of the dialysate samples were determined using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA) with bovine serum amyloid as standard, according to the manufacturer's protocol.

Concentrations of fibrinogen, SAA, haptoglobin, ceruloplasmin, plasminogen, prothrombin, α-1-antitrypsin, and α-2-macroglobulin in dialysates were determined by use of the QconCAT-SRM-based method described by Bundgaard et al., 2014[16] (Figure 1). In short, the QconCAT (eAP1) gene was expressed in the vector pET-21a by PolyQuant GmBH (Bad Abbach, Germany) in minimal media containing stable isotope-labeled [$^{13}C_6$]lysine and [$^{13}C_6$]arginine. The bacterial cell pellets were lysed using sonication, and the eAP1, present in inclusion bodies, solubilised in buffers containing 6 M guanidinium hydrochloride and purified by metal affinity chromatography.

A volume of 11 μL dialysate and 48 μL purified eAP1 (two separate digests) were mixed with 25 mM ammonium bicarbonate solution to a final volume of 160 μL. Proteins were denatured with Rapigest™ (Waters, Milford, MA), disulphide bonds were reduced with dithiothreitol and alkylated with iodoacetamide. A volume of 10 μL 2.15 pmol/μL nonlabeled [Glu1]-Fibrinopeptide B (Waters, Milford, MA) was added before tryptic digestion of eAP1 samples to enable quantification in absolute terms. The quality of tryptic digests was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After precipitation with triflouroacetic acid and centrifugation the supernatant fraction was carefully removed for analysis. The tryptic eAP1 peptides were spiked into the sample followed by micropurification (StageTip C18, 20 μL tips, Thermo Scientific), and resuspension in 0.1% formic acid. For each sample a volume of 52 nL dialysate with 7 fmol tryptic eAP1 peptides was processed on an EASY-nLC II (Thermo Scientific) coupled in-line to a Qtrap 5500 (AB SCIEX, Framingham, MA) mass spectrometer, operated under Analyst TF 1.6.1. The Qtrap was run with a scheduled SRM method (60 minutes, 2 seconds cycle time, detection window of 4 minutes).

The accurately quantified unlabeled [Glu1]-Fibrinopeptide B enabled absolute quantification of the stable isotope-labeled eAP1, which further supported absolute quantification of each protein-specific peptide in fmol/μL digest. The concentration of each specific protein was back-calculated from femtomol signals, of the protein-specific peptides, to μg protein/mL dialysate by taking into account the molecular weight of each specific full length protein.[16]

Data analysis and statistics

The quality of the raw data output from analysis of the dialysate samples was manually verified using Skyline (https://skyline.gs.washington.edu). Due to an expected labeling efficiency of approximately 98% of the QconCATs,[13] a ratio between the stable-isotope-labeled peptide and analyte peptide $< 5\%$ were regarded as below the limit of detection and quantification. The R statistical software version 3.01 (https://www.r-project.org) was used. The library "plyr"[17] was used to calculate means and standard deviation. A repeated-measures mixed linear model with horse as a random factor and wound site (body vs. limb), day and the interaction between site and day as within-subject factors, was used to model the effects of position and time on the protein level. All analyses were performed using the "nlme" library[18] in R (https://cran.r-project.org/web/packages/nlme/nlme.pdf). The level of statistical significance was set at $p < 0.05$. Except for the concentration of plasminogen and haptoglobin, protein concentrations were log-transformed to obtain homogeneously and normally distributed residuals. Graphics were generated by use of the R library "ggplot2" (http://had.co.nz/ggplot2/).

RESULTS

Clinical observations

None of the horses showed signs of lameness or discomfort after the wounding procedure. Granulation tissues were initially healthy and pink in all body and limb wounds, but by day 10–14 the granulation tissues of the limb wounds developed the characteristics of EGT (dark red in color, fragile, and protruding above skin level). Epithelialisation was observed in body wounds from day 7 to 10 and onwards, but was never observed in any of the limb wounds. On day 14 after wounding the limb wounds were reduced to 77% of initial wound size, whereas body wounds were reduced to 44% of initial wound size.

Sample preparation and SRM analysis

SDS-PAGE analysis revealed successful digestion of all dialysate samples. Analysis of the raw data output from the SRM analyses confirmed reliable quantification of the proteins of interest by use of the proteotypic peptides validated by Bundgaard et al., 2014.[16] Two proteotypic peptides were used to determine the concentration in dialysate of fibrinogen, ceruloplasmin, haptoglobin, prothrombin, α-1-antitrypsin, and α-2-macroglobulin. For plasminogen only one proteotypic peptide was suitable for determination of the concentration.

All SRM data from a single sample (horse 1, limb, day 14) were excluded from statistical analysis because all signal intensities were below detection limit. This was most likely due to an unobserved failure in the sample preparation procedure.

Total protein concentration in serum and dialysate

The total protein concentration in serum decreased significantly over the study period ($p = 0.004$). The mean concentration at day 0 was 69.4 g/L with a peak concentration of 72.4 g/L on day 1 and a subsequent decrease to 63.5 g/L on day 14 (Figure 2). The concentration of albumin, which constitutes the main part of total protein, was 33.8 g/L on day 0 with a slight increase to 35.7 g/L on day 1 and a decrease to 32.7 g/L on day 14 (data not shown).

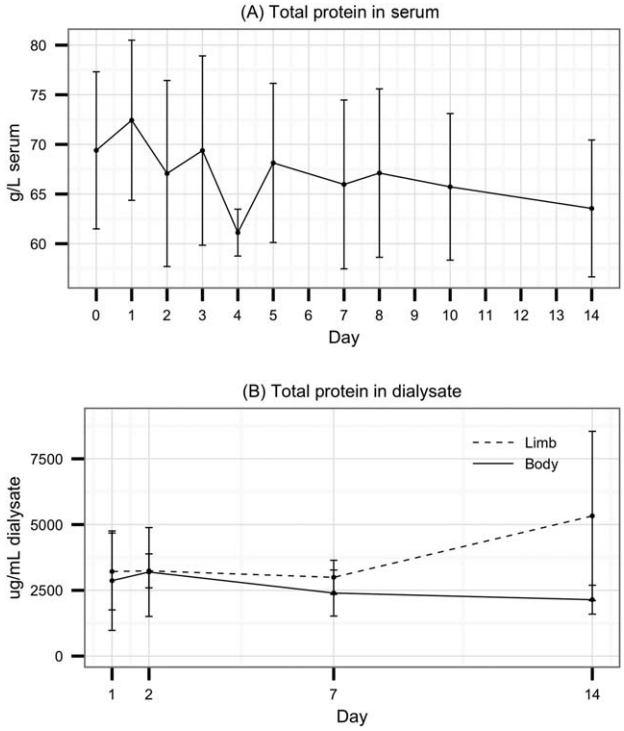

Figure 2. Concentration of (A) total protein in serum (g/L), and in (B) dialysate from equine limb and body wounds (μg/mL). Values are the means ± standard deviation for five horses.

The total amount of protein extracted from dialysates (Figure 2) was significantly higher in limb wounds than in body wounds throughout the study period ($p < 0.05$), rising from 3.2 to 5.3 mg/mL over the course of the study.

Systemic concentrations of fibrinogen and serum amyloid A

The concentration of fibrinogen in plasma (Figure 3) increased from day 1 postsurgery, with a mean peak level day 5 after surgery, and a subsequent decrease. On day 14 after surgery the concentration was still above the preoperative level. The mean concentration of SAA peaked two days after surgery in serum but with a considerable individual variation (Figure 3).

Fluctuations of acute phase protein levels in dialysate

Fibrinogen concentrations in dialysates changed significantly during the study period ($p < 0.05$) (Figure 3). The mean concentration of fibrinogen in limb wound dialysate was significantly higher than body wound dialysates on day 14 after surgery ($p < 0.001$).

The concentrations of SAA in dialysates were close to the lower limit of detection, and samples with concentration below limit of detection were excluded from the calculations (two samples from day 1, one sample from day 2, three samples from day 7, all samples from day 14). There was a considerable individual variation in the concentration of SAA with no significant change over the study period (Figure 3). SAA concentrations in limb and body wound dialysates did not differ significantly.

Haptoglobin concentrations in dialysates from limb wounds were significantly higher than in dialysates from body wounds on day 14 after surgery ($p < 0.01$) (Figure 4).

For ceruloplasmin, the concentrations differed significantly between sampling days and between limb and body wounds ($p < 0.02$ and $p < 0.03$, respectively; Figure 4).

The concentration of plasminogen in dialysate changed significantly over time ($p < 0.001$), but did not differ between limb and body wound dialysates (Figure 4).

Prothrombin concentrations changed significantly over time ($p < 0.02$) and were significantly higher in limb wound dialysates than in body wound dialysates ($p < 0.005$) (Figure 4).

The concentration of α-1-antitrypsin in dialysate from limb wounds were significantly higher than in dialysates from body wounds ($p < 0.05$; Figure 4).

For α-2-macroglobulin the concentrations changed significantly over time ($p < 0.02$), but did not differ significantly between limb and body wound dialysates (Figure 4).

DISCUSSION

Chronic inflammation has been suggested to be central in the pathogenesis of EGT in the limb wounds of horses.[2] The aim of this study was to assess levels of inflammatory APPs in interstitial fluid sampled from equine limb wounds healing with EGT. This is the first report of simultaneous analysis of a panel of APPs in interstitial fluid from equine wounds.

Microdialysis was used to collect interstitial fluid. A shortcoming of most techniques used to recover interstitial fluid is that sampling causes bleeding and inflammation, and thereby influences the composition of the recovered fluid.[18] The microdialysis technique has previously been described as a minimally invasive technique for successful recovery of interstitial components from wounds,[19] and it was recently reported for the first time in equine wound healing research.[14] The minor trauma induced by implantation of the device is insignificant after 60–100 minutes.[20]

This is the first report of simultaneous analysis of a panel of APPs in wound interstitial fluid using the QconCAT-SRM technology. Immunoassays are the most widely used methods for protein identification and quantification, but the shortcoming of these methods in equine research is the limited access to validated assays and antibodies. The novelty of the SRM technology is the non–antibody-based approach of identification and quantification of a panel of proteins.[11] The current generation of triple quadrupole-based mass spectrometry instruments match the sensitivity and specificity reported when using immunoassays.[21] The development of a single ELISA assay test usually takes over one year, while a SRM assay can be developed and optimized within weeks. Additional advantages of SRM are that it permits multiplexing of assays for several proteins simultaneously[22] and that the sample volume needed is less than for immunoassays (in this study seven proteins were quantified in 11 μL dialysate). Disadvantages of implementing mass spectrometry include the initial high cost of the instrumentation and the complexity of the technology requiring technical expertise to operate and

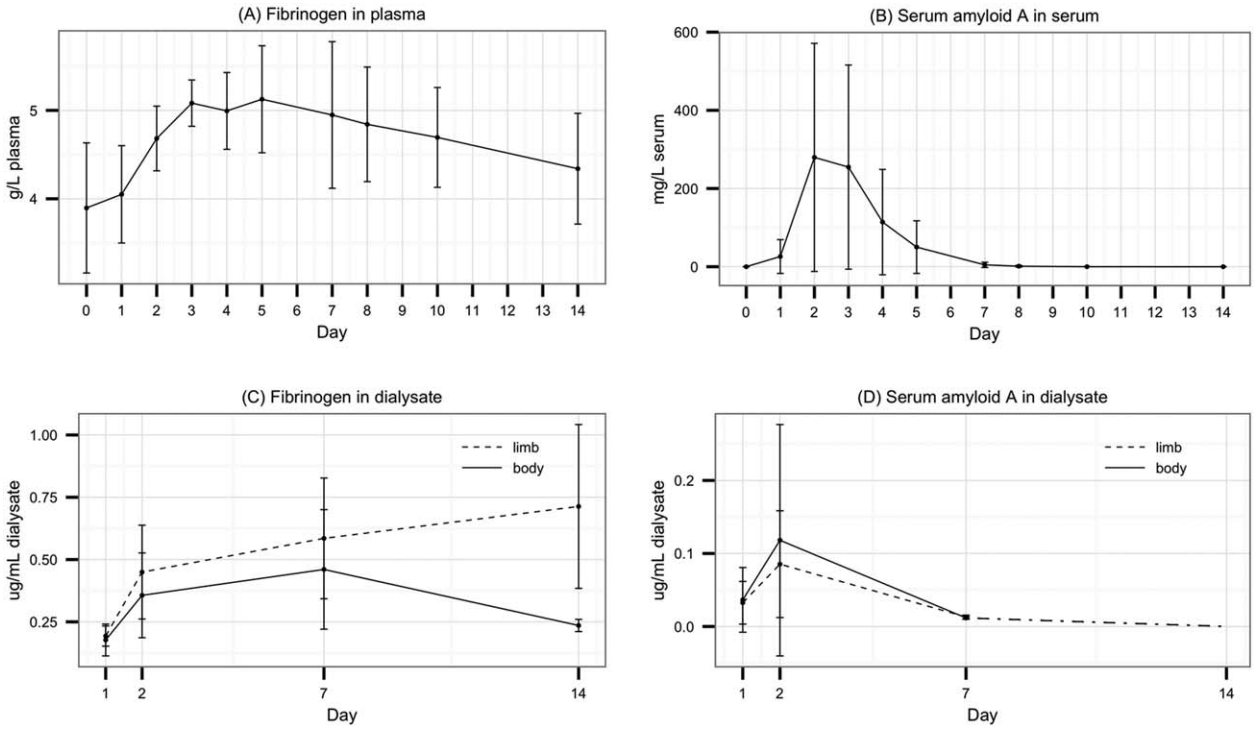

Figure 3. The concentration of (A) fibrinogen in plasma (g/L), (B) serum amyloid A in serum (mg/L), and (C) fibrinogen and (D) serum amyloid A in dialysate from equine limb and body wounds (μg/mL). Values are the means ± standard deviation for five horses. Fibrinogen concentrations in dialysates changed significantly during the study period ($p < 0.05$). The mean concentration of fibrinogen in limb wound dialysate was significantly higher than body wound dialysates on day 14 after surgery ($p < 0.001$). Serum amyloid A concentrations in dialysates did not differ significantly between limb and body wounds.

maintain the instruments. However, once established, the cost of sample preparation and analysis by SRM is usually lower than for immunoassays.[21] The use of the QconCAT approach becomes cost-effective compared to synthetic stable isotope-labeled peptides when quantification is required for more than five different proteins simultaneousy.[23] In human clinical practice the SRM-technology is already a viable option for measurement of relevant analytes and biomarkers (e.g., endocrine hormones, metabolic disorders in newborns, therapeutic drug monitoring) and it is expected that new instruments will enable testing to be performed by the general medical technologist.[21,22]

The QconCAT-SRM approach is still not widely implemented for use in farm animal research, but has been successfully used to quantify the host response to mastitis in cows[23] and protein expression during chicken muscle development.[24] In this study, a previously validated QconCAT-based SRM method[16] was succesfully used for the quantification of fibrinogen, SAA, ceruloplasmin, haptoglobin, plasminogen, prothrombin, α-1-antitrypsin, and α-2-macroglobulin. APP concentrations in dialysates were subject to individual variation similar to previous report on concentrations in blood,[7,25] which is apparent from the large standard deviations (Figures 3 and 4). Previous studies using the QconCAT approach found robust reproducibility.[24]

Here, microdialysis in combination with SRM analyses used to study the wound healing process is reported for the first time and aids a novel approach for investigating a local "time-resolved" progress of wound healing.

The concentrations of fibrinogen, haptoglobin, ceruloplasmin, prothrombin, and α-1-antitrypsin were significantly higher in limb wound dialysate than body wounds dialysate during the study period. This difference in concentration of proteins in the dialysate indicated a difference in the healing process between limb and body wounds. Previous investigations of APPs in wounds primarily report results from studies in humans. In one study, concentrations of the APP C-reactive protein were found to decrease in wound exudates when wounds progressed from the non-healing phase to the healing phase, and this change was suggested to reflect a local decrease in inflammation.[26] In a recent study on effluent from combat wounds the concentration of ceruloplasmin and α-1 -antichymotrypsin were included in a panel of biomarkers to determine the risk of wound dehiscence.[27] In a study on wound fluid from chronic leg ulcers in humans a number of APPs and their degradation products were suggested as potential molecules to evaluate inflammation and wound healing activity.[8] In other disease processes local accumulation of APPs have been used to assess the local inflammatory response in horses.[6,28] The APP composition in these compartments has been shown to reflect local inflammation and to be independent of the systemic APP response.

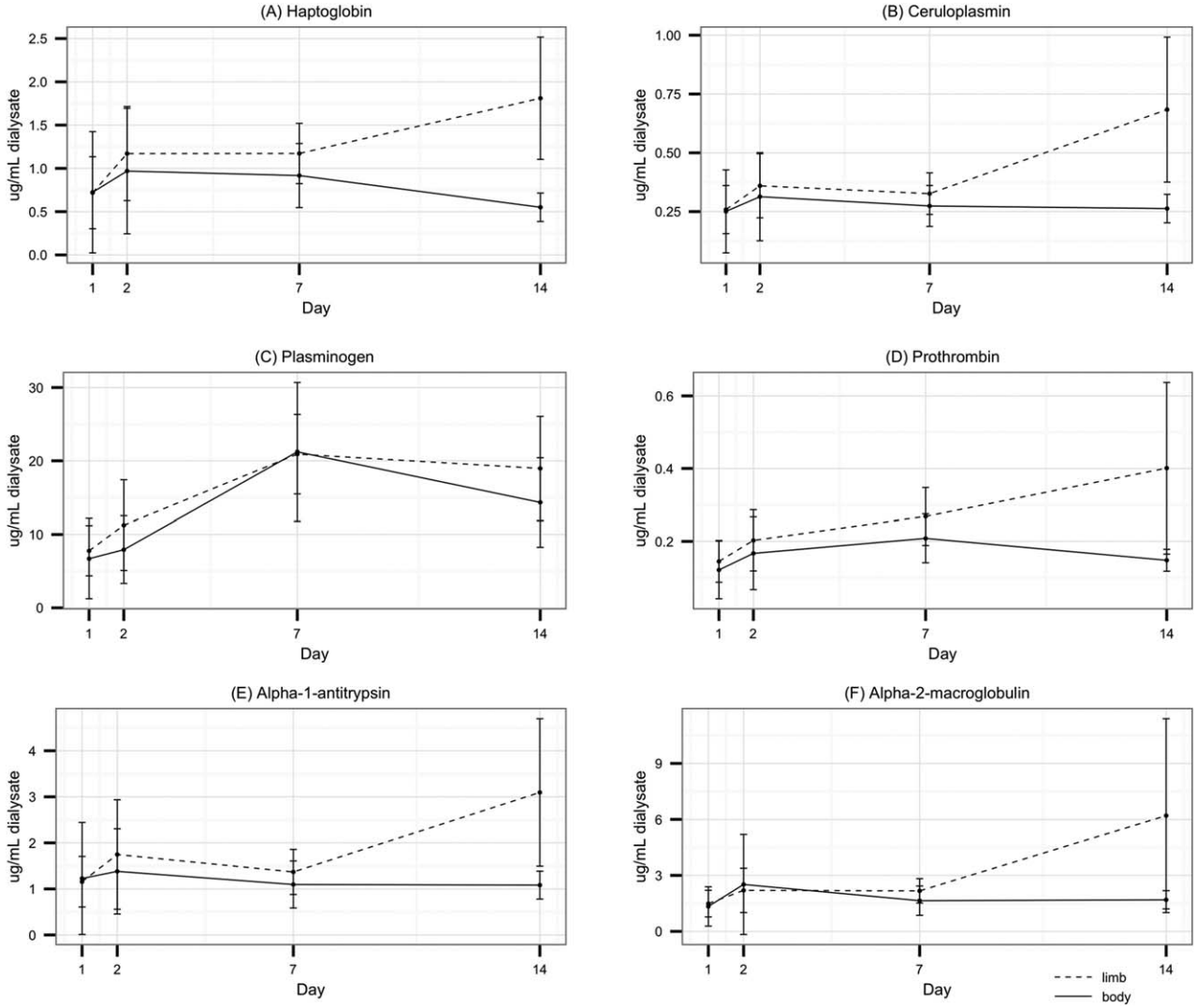

Figure 4. The concentration of (A) haptoglobin, (B) ceruloplasmin, (C) plasminogen, (D) prothrombin, (E) α-1-antitrypsin, and (F) α-2-macroglobulin in dialysate from equine limb and body wounds (μg/mL). Values are the means ± standard deviation for five horses. The concentrations differed significantly between sampling days for ceruloplasmin ($p < 0.02$), plasminogen ($p < 0.001$), prothrombin ($p < 0.02$), and α-2-macroglobulin ($p < 0.02$). The concentrations differed significantly between limb and body wounds for ceruloplasmin ($p < 0.03$), prothrombin ($p < 0.02$), and α-1-antitrypsin ($p < 0.05$). The haptoglobin concentrations in dialysates from limb wounds were significantly higher than in dialysates from body wounds on day 14 after surgery ($p < 0.01$).

The relative changes in fibrinogen concentration observed in dialysate in limb wounds did not parallel the changes measured in plasma, thus suggesting that they reflected local inflammation. In contrast to total protein concentrations in body wounds, which followed the changes in serum total protein, total protein concentrations in limb wound dialysates were significantly higher than in body wound dialysates. Others have shown that total protein levels in granulation tissue debrided from slowly healing granulating wounds were higher than in normal skin.[29] A technical explanation for the observed variance in total protein in this study could be a difference in microdialysis efficiency between body and limb wounds.[30] This explanation seems, however, implausible, as this would have led to consistent differences in total protein levels between limb and body wounds throughout the study period. Different approaches have been used to determine the recovery efficiency of large molecules, but as no golden standard exist,[30] no attempt was made at standardizing recovery in the present study.

The observations suggest that APPs accumulate locally, which supports the assumption that wounds healing with EGT suffer from chronic inflammation. The greater concentration of total protein, fibrinogen, haptoglobin, ceruloplasmin, prothrombin, and α-1-antitrypsin in limb wound dialysates might have been a consequence of increased accumulation due to passive or selective transportation of proteins into the wound bed, reduced clearance due to

poor lymphatic drainage[31] a reduced degradation, or as a result of local synthesis and release of proteins in the wound bed. Synthesis of some APPs has been demonstrated in cells related to wound healing, for example, α-2-macroglobulin in human skin fibroblasts[32] and haptoglobin in keratinocytes from normal and diseased skin.[33]

In conclusion, this study successfully demonstrated how QconCAT-derived isotope-labeled peptides in combination with SRM analysis can be used to absolutely quantify APPs in a small volume of wound interstitial fluid sampled by the minimally invasive microdialysis technology. The expected differences in protein concentrations between body and limb wound dialysates were observed, and the results suggested a nonresolved inflammatory response in the wounds healing with formation of EGT, but a prospective investigation in large scale will be needed to verify this observation.

The combination of technologies used in this study might be a promising approach for assesment of the protein composition in the wound microenvironment in future investigations to obtain valuable information about the pathophysiology of wound healing.

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