Molecular BioSystems

Mastitomics, the integrated omics of bovine milk in an experimental model of Streptococcus uberis mastitis: 1 High abundance proteins, acute phase proteins and peptidomics.

Journal:	*Molecular BioSystems*
Manuscript ID	MB-ART-03-2016-000239.R1
Article Type:	Paper
Date Submitted by the Author:	05-May-2016
Complete List of Authors:	Thomas, Funmi ; Glasgow University, Institute Biodiversity and Animal health Mullen, William; Univeristy of Glasgow, BHF Glasgow Cardiovascular Research Centre Tassi, Riccardo; Moredun Research Institute Ramirez-Torres, Adela; Mosaique Diagnostics GmbH Mudaliar, Manikhandan; University of Glasgow McNeilly, Tom; Moredun Research Institute Zadoks, Ruth; University of Glasgow; Moredun Research Institute Burchmore, Richard; Glasgow university Eckersall, David; Glasgow University,

SCHOLARONE™
Manuscripts

Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 1 High abundance proteins, acute phase proteins and peptidomics

Funmilola Clara Thomas,[a] William Mullen,[b] Ricardo Tassi,[c] Adela Ramírez-Torres,[d] Manikhandan Mudaliar, [a,e] Tom N. McNeilly,[c] Ruth N. Zadoks,[a,c] Richard Burchmore[e] , P. David Eckersall,[a*]

[a]Institute of Biodiversity Animal Health and Comparative Medicine, Bearsden Road, University of Glasgow, Glasgow, United Kingdom.

[b]Institute of Cardiovascular and Medical Sciences, University of Glasgow, University Avenue, Glasgow, United Kingdom.

[c]Moredun Research Institute, Pentlands Science Park/Bush Loan, Penicuik, United Kingdom.

[d] Mosaique Diagnostics GmbH, Rotenburger Str. 20, D-30659 Hannover, Germany.

[e]Glasgow Polyomics, College of Medical, Veterinary and Life Science, University of Glasgow, Glasgow, United Kingdom

*Corresponding author:
P. David Eckersall
Institute of Biodiversity Animal Health and Comparative Medicine,
Bearsden Road,
University of Glasgow,
Glasgow,
G61 1QH
United Kingdom
e-mail: david.eckersall@glasgow.ac.uk

1 **Abstract**
2
3 A peptidomic investigation of milk from an experimental model of *Streptococcus uberis*
4 mastitis in dairy cows has incorporated a study of milk high abundance and acute phase
5 (APP) proteins as well as analysis of low molecular weight peptide biomarkers.
6 Intramammary infection (IMI) with *S. uberis* caused a shift in abundance from caseins, β-
7 lactoglobulin and α-lactalbumin to albumin, lactoferrin and IgG with the increase in
8 lactoferrin occurring last. The APP response of haptoglobin, mammary associated serum
9 amyloid A3 and C-reactive protein occurred between 30-48 hours post challenge with peak
10 concentrations of APPs at 72-96 hours post challenge and declined thereafter at a rate
11 resembling the fall in bacterial count rather than the somatic cell count. A peptide
12 biomarker panel for IMI based on capillary electrophoresis and mass spectrometry was
13 developed. It comprised 77 identified peptides (IMI77) composed mainly of casein derived
14 peptides but also including peptides of glycosylation dependent cell adhesion molecule and
15 serum amyloid A. The panel had a biomarker classification score that increased from 36
16 hour to 81 hour post challenge, significantly differentiating infected from non-infected
17 milk, thus suggesting potential as a peptide biomarker panel of bovine mastitis and
18 specifically that of *S. uberis* origin. The use of omic technology has shown a multifactorial
19 cross system reaction in high and low abundance proteins and their peptide derivatives
20 with changes of over a thousand fold in analyte levels in response to *S. uberis* infection.
21
22
23 **Keywords**; Haptoglobin, mammary associated serum amyloid A, C-reactive protein,
24 bovine mastitis, milk proteins, *Streptococcus uberis,* peptidomics, biomarkers
25

26 **1 Introduction**

27 Mastitis, mostly caused by bacterial infection of the mammary gland, is the major
28 infectious disease problem in dairy cows, being estimated to cost the global dairy industry
29 €16-26 billion per annum based on a global dairy cow population of 271 million dairy
30 cows (www.dairy.ahdb.org.uk, accessed March 2016) and a cost to farmers of €61-€97 per
31 animal [1]. The early detection of intra-mammary infections (IMI), the main cause of
32 mastitis, would be greatly beneficial in allowing early treatment and prevention of onward
33 transmission of disease. Furthermore early characterisation of the bacterial species
34 causing mastitis would allow more targeted chemotherapy, which may help to reduce
35 inappropriate use of antibiotics [2]. The last decade has shown a major increase in the use of
36 omics technologies in experimental biology and human disease investigations, but, with
37 the exception of genomics, the application of advanced analytical technologies such as
38 proteomics and metabolomics has been limited in studies of animal health and disease.
39 This is undergoing change [3]. This paper is the first of a series of three in which protein and
40 metabolite alteration in the composition of milk during bovine mastitis was investigated
41 with the aim of characterising the molecular biosystem of milk to increase our
42 understanding of the pathology of the disease and to identify potential biomarkers for early
43 detection of IMI.

44 In this series of studies, changes in milk during mastitis were investigated utilising an
45 established experimental model of the disease [4] induced by *Streptococcus uberis* (*S. uberis*)
46 which is one of the most prevalent causes of bovine mastitis in the United Kingdom [5-7] and
47 other countries [6-9]. In the first paper, we focus on high abundance proteins, acute phase
48 proteins [10] and quantitative peptidomics [11]. In the subsequent paper, a label free
49 quantitative proteomic method will be used to monitor changes in higher Mw proteins of
50 milk [12], and in the final paper of the series, the alteration of low Mw metabolites will be
51 described [13]. All investigations used milk samples from an experimental model of *S uberis*
52 mastitis used for the investigation of host immune responses in milk [4] . This has
53 previously revealed changes in concentrations of cytokines such as TNFα and interleukins
54 1-β and 6, which are associated with induction of the acute phase response [14-16], as well as
55 recruitment of lymphocytes (CD4, CD8 and γδ T cells) and polymorphonuclear cells into
56 the milk [4].

57 The high abundance proteins in healthy milk consist largely of the caseins, β-lactoglobin
58 and α-lactoglobin [17] and reduction in these major proteins in milk due to IMI have been
59 documented [18, 19], as well as increases in albumin, lactoferrin and immunoglobulins [20 21].
60 However there has been little investigation of the time course of changes in these high

61 abundance proteins particularly in relation to changes in the low abundance proteins such
62 as acute phase proteins (APP) in milk.

63 Acute phase proteins are serum proteins which increase (or decrease) in concentration by
64 over 25% following stimulation by pro-inflammatory cytokines such as TNFα and IL6,
65 and APP are now recognised as also being elevated in milk during mastitis [22]. Haptoglobin
66 (Hp) and mammary associated serum amyloid A3 (MSAA3), the isoform of SAA
67 synthesised and secreted by the mammary epithelial cells are recognised as milk APP. For
68 example, Pedersen and others [23] studied the early inflammatory responses of the host to an
69 experimental *S. uberis* infection and showed that infection causes a rise in milk Hp and
70 MSAA3. Previous studies in an experimental model of *Staphylococcus aureus (S aureus*)
71 induced mastitis have also demonstrated that measuring APP could be useful in
72 identification of the inflammatory response to the mammary infections [10]. Although
73 several recent studies on APP in milk during mastitis have focussed on Hp and SAA, some
74 investigations have identified a possible value of bovine milk C-reactive protein (CRP) as
75 a biomarker of bovine mastitis[24-27]. However, variation of CRP during the course of an
76 experimental infection has not been previously reported. In addition, APP profiles have
77 been described during the onset of infection, but seldom during resolution of IMI.
78 Knowledge of the change in concentration during resolution of infection is crucial for
79 assessment of the diagnostic specificity of APP as an indicator of IMI.

80 While there have been several proteomic investigations of milk during mastitis [20, 28-30] the
81 lower Mw peptides of milk have had less investigation. Our earlier study of the peptidome
82 of milk during clinical mastitis, caused by *S. aureus* and *Escherichia coli,* indicated that
83 analyses using a peptide biomarker panel could have potential in diagnosis of the disease [11]
84 but the milk peptidome has not been monitored for changes over the course of an
85 experimental infection. Biomarker discovery using a combination of capillary
86 electrophoresis and mass spectroscopy (CE-MS) has enabled the identification of peptide
87 panels which are used in diagnostic procedures for human diseases [31] and have the ability
88 to be applied to diseases of livestock [32].

89 Therefore the aim of this study is to identify the effects of *S uberis* mastitis on the
90 molecular pathophysiology of (a) high abundance milk proteins, (b) the APP in the low
91 abundance milk proteins and (c) low Mw peptides (<25kD) in milk during IMI. The
92 research described here is the first of three linked mastitomic studies [11,12] which along with
93 clinical and immunological data of the same sample sets[4] aims to contribute to an
94 integrated systems biology approach to increase our understanding of bovine mastitis.

95

96 **2 Methods**

97 **2.1 Experimental challenge model of *S. uberis* mastitis**

98 Milk samples were obtained from an intramammary challenge study of a single udder
99 quarter from each of six healthy Holstein cows using a putative host adapted strain of *S.*
100 *uberis*, strain FSL Z1–048. Full details of the procedure and the results of clinical evalua-
101 tion of infected cows as well as laboratory investigation of these milk samples such as for
102 microbiology, somatic cell count (SCC), cytokines and lymphocyte ratios have been previ-
103 ously reported [4]. The milk samples were stored at -20°C in the period between the analyses
104 reported in Tassi et al [4] and the investigation described here. Samples were obtained at 19
105 time points from each challenged quarter comprising 0, 6, 12, 18, 24, 30, 36, 42, 48, 57, 72,
106 81, 96, 120, 144, 168, 192, 240 and 312 hours (h) post challenge (PC) and at 7 time points
107 including 0, 12, 36, 57, 96, 192 and 312 h PC, from the control quarters (n=1 per cow) that
108 were infused with 2 ml sterile phosphate buffered saline (PBS). The timings were designed
109 for collection at every 6 hours for the first 2 days; from 2 to 11 d PC, milk samples were
110 collected twice a day; and from 11 to 13 d PC once a day. Skimmed milk was prepared by
111 centrifuging 50 ml of milk at 2,800 x g at 4°C for 20 minutes (min). The fat layer was dis-
112 carded and the supernatant was transferred to a new 50 ml Falcon tube. Centrifugation was
113 repeated and the supernatant was stored at −20°C.. All animal experiments were conduct-
114 ed at the Moredun Research Institute (Penicuik,UK) with approval of the Institute's Exper-
115 iments and Ethical Review Committee in accordance with the Animals (Scientific Proce-
116 dures) Act 1986 [4].

117

118 **2.2 High abundance milk proteins: One Dimensional electrophoresis**

119 Prior to gel electrophoresis, protein concentration was determined using the Bradford pro-
120 tein assay with bovine serum albumin as standard (BSA; Sigma-Aldrich, USA). Sodium
121 dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 4-
122 15% gradient polyacrylamide gels in a Criterion electrophoresis system (BioRad Ltd,
123 Hemel Hempstead , UK) as previously described [33]. Samples of milk taken at each time
124 point were separated by SDS-PAGE. The identity of protein in the SDS-PAGE bands was
125 determined in a reference gel by analysis of milk from a healthy cow and a cow with mas-
126 titis run under the identical conditions, followed by LC-MS/MS. Protein bands were ex-
127 cised and processed [33] prior to analysis at Glasgow Polyomics on a nanoflow uHPLC sys-
128 tem (Thermo RSLCnano) and electrospray ionisation (ESI) mass spectrometry (MS) on an
129 Amazon ion trap MS/MS (Bruker Daltonics). MS data were processed using Data Analysis
130 software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.1.06).

131 Protein identifications were assigned using the Mascot search engine to interrogate protein
132 sequences in the NCBI databases restricting the search to *Bos taurus* proteins.
133

134 **2.3 Acute phase protein assays**

135 Milk samples from all 19 time points (for challenged quarters; 7 time points for control
136 quarters) were assayed for bovine Hp, MSAA3 and CRP. An in-house ELISA for bovine
137 Hp using purified polyclonal rabbit anti-bovine Hp IgG (Life Diagnostics Inc, West
138 Chester, Pennsylvania, USA) was carried out as described previously [27]. Commercial
139 ELISAs for SAA (Tridelta Development Ltd, Dublin Ireland) and bovine CRP (Life
140 Diagnostics Inc, West Chester, Pennsylvania, USA) were used to quantify these proteins in
141 milk from the *S. uberis* experimental model of mastitis as described previously [27]
142

143 **2.5 Peptidome Analysis: Sample preparation, CE-MS setup and data processing**

144 Samples were prepared and run on capillary electrophoresis-mass spectrometry (CE-MS)
145 with modifications to the methods described previously [11]. Briefly, 0.1% PMSF was added
146 to each milk sample. Aliquots of 150 ul were diluted with the same volume of 2 M urea,
147 100 mM NaCl, 10 mM NH_4OH and 0.02% SDS. High Mw molecules were filtered with a
148 cut-off >20 kDa Centrisart ultrafiltration tube (Sartorius, Germany) for 1h, 3,400 rpm, 4°C.
149 To discard urea and electrolytes, a NAP-5 column (GE Healthcare, Sweden) was used,
150 equilibrated as recommended by the manufacturer. To elute the peptides from the column,
151 700 µl of the NH_4OH were used. Protein concentration was determined by a bicinchoninic
152 acid (BCA) assay, using BSA as standard. Aliquots were restored to a final concentration
153 of 2 µg/µl prior to CE-MS analysis.

154 For the CE-MS analysis a Beckman Coulter P/ACE MDQ CE system (Fullerton, USA)
155 was used. Before analysis, samples were centrifuged at 14,000 x g for 10 min at 4ºC. The
156 peptides eluting from the CE were ionised using an electro-spray ionisation (Agilent
157 Technologies, Palo Alto, CA, USA) which was grounded to achieve electric potential of 0,
158 and the electro-spray interface potential of the microTOF mass spectrometer (Bruker
159 Daltonics, Bremen, Germany) was set between -4 and -4.5 kV. The mass calibration of the
160 microTOF was performed on a weekly basis using the standard protein/peptide solution
161 (0.5 pmol/µl) for CE-MS analysis. The acquisition of data and MS were automatically
162 controlled by the CE via contact close-relays and MS spectra accumulated every 3 s, over a
163 m/z range 350-3000 for 55 min.

164 MosaiquesVisu software was used to interpret the mass spectral ion peaks representing
165 identical molecules at different charge states and thus, those signals were deconvoluted

166 into single masses[34]. The software automatically examined all mass spectra from a CE-MS
167 analysis for signals with a signal-to-noise ratio of at least 4 present in three consecutive
168 spectra. Additionally, the isotopic distribution was assessed, and charge was assigned on
169 the basis of the isotopic distribution, as well as conjugated masses, with a probabilistic
170 clustering algorithm. This operation resulted in a list wherein all signals that could be
171 interpreted are defined by mass/charge, charge, migration time, and signal intensity (ion
172 counts). Time-of-flight MS data were calibrated with Fourier transform ion cyclotron
173 resonance MS data as reference masses applying linear regression. CE migration time was
174 calibrated by local regression with 488 reference signals or ¨housekeeping polypeptides¨.
175 The obtained peak lists characterize each polypeptide by its molecular mass [Da],
176 normalized CE migration time [min] and normalized signal intensity. All detected peptides
177 were deposited, matched, and annotated in a Microsoft SQL database allowing further
178 statistical analysis. For clustering, peptides in different samples were considered identical
179 if mass deviation was <50 ppm. CE migration time was controlled to be below 0.35
180 minutes after calibration.

181 **2.6. Peptides selection and statistical analysis**

182 For the identification of potential IMI biomarkers, the normalized levels of cow milk
183 peptides were compared between time point 0h (non-infected or control group, n=6) and
184 time point 81h (infected, n=6). Only peptides that were detected with a minimal frequency
185 of 4 of 6 in at least one of the diagnostic groups were considered for statistical analysis.
186 Unadjusted P values were calculated for the comparison between the non infected and
187 infected cow groups with the Wilcoxon rank-sum test followed by adjustment for multiple
188 testing with the method described by Benjamini and Hochberg [35]. Only peptides with a
189 corrected $P < 0.05$ were considered significant.
190 The number of peptides with differential abundance was reduced to a support vector
191 machine (SVM) classifier with 77 peptides (IMI77) by a take-one-out procedure.
192 Sensitivity and specificity of the biomarker classifier in the discovery set, and 95%
193 confidence intervals (95% IC) were calculated using receiver operating characteristic
194 (ROC) plots (MedCalc versión 14.8.1, MedCalc Software, Belgium).
195

196 **2.7 Liquid chromatography and mass spectrometry for peptide biomarker**
197 **identification**

198 In order to determine the sequences of significant biomarker polypeptides, LC-MS/MS
199 peptide sequencing was carried out as previously described [11]. Briefly, the milk extracts
200 were analysed on a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly

201 UK). The samples were eluted with a gradient of solvent A: 0.1% formic acid and
202 acetonitrile (98:2) versus solvent B: 0.1% formic acid and acetonitrile (20:80) starting at
203 5% B rising to 50% B over 100 mins. The column was washed using 90% B before being
204 equilibrated prior to the next sample being loaded.

205 The eluate from the column was directed to a Proxeon nano spray ESI source (Thermo
206 Fisher Hemel UK) operating in positive ion mode then into an Orbitrap Velos FTMS
207 (Thermo Fisher Hemel UK). The ionisation voltage was 2.5 kV and the capillary
208 temperature was 250°C. The mass spectrometer was operated in MS/MS mode scanning
209 from 380 to 2000 amu.

210 Raw spectral data from LC-MS/MS analysis of the samples were uploaded to Thermo Pro-
211 teome Discoverer 1.3. Only peptides with signal to noise ratio higher than 1.5 and belong-
212 ing to precursor peptides between 380 – 6000 Da were considered. Peptide and protein
213 identification was performed with the SEQUEST algorithm. An in-house database contain-
214 ing proteins from the latest version UniProt SwissProt database was compiled to include
215 only *Bos taurus* and *S. uberis* entries. No enzyme cleavage was selected and oxidation of
216 methionine and proline were chosen as variable modifications. Precursor mass tolerance
217 was set at 5 parts per million (ppm) and 0.1 Da for MS/MS fragment ions. Resulting pep-
218 tides and protein hits were further screened by excluding peptides with an error tolerance
219 higher than 10 ppm and by accepting only those hits listed as high confidence by Proteome
220 Discoverer software. Target false discovery rate (FDR) was 0.01 (strict) or 0.05 (relaxed).

221 3 Results

222 3.1 High Abundance Proteins

223 The alteration in the high abundance proteins of milk during the experimental infection
224 with *S. uberis* is shown in Figure 1A with milk protein from a single cow (cow 6) from 0 to
225 312 h PC separated by SDS-PAGE. Similar gels for samples from all cows are given in
226 supplementary files (Figure S1). The identity of the separated milk protein bands was
227 determined by MS analysis of bands cut from a reference gel of healthy and mastitic milk
228 (Fig 1B) with the proteins identified listed in Table 1. Similar patterns of change after
229 infection of the high abundance proteins of milk were obtained in samples of milk from all
230 the infected quarters, though with some variation in the timing evident in Figure S1. For
231 instance the fall in the casein proteins at Mw 28-31 kDa was apparent in all cows but was
232 first noticeable at 30 h (cow 2 & 3), 36 h (cow 1, 4, 6) or 42 h (cow 5) in different cows.
233 Although the identity of most proteins in Fig 1A and S1 was determined by comparison to
234 the reference gel (Table 1) the identity of the proteins at Mw 28-31 kDa was less certain.

235 The protein band at 31 kDa in healthy milk is α_{s1}-casein and the protein at 28 kDa was β-
236 casein, whereas in the mastitic milk both of these bands were IgG light chain. The protein
237 bands at 28-31 kDa appearing from 72 h PC could be either caseins or IgG light chain.
238 Overall the normal pattern of milk protein was found in the initial samples with αs_1- and
239 β caseins, β-lactoglobulin and α-lactalbumin predominating. Thereafter, taking sample 6
240 as an exemplar (Fig 1A) these proteins are reduced between 30 and 81 h PC while there is
241 an increase in albumin, lactoferrin (LF) and IgG heavy chain. Of these, an observable
242 increase in albumin and IgG took place at 36 h PC with LF having a more delayed
243 response. In comparing the albumin and LF protein bands, from 36-57 h PC the albumin
244 band was more intense while from 96-192 h PC the LF band was more intense than the
245 albumin (Fig 1A). In the last sample taken (312 h PC) all of the high abundance proteins
246 were still present, although infection had been resolved in the majority of quarters[4].
247

248 **3.2 Acute phase proteins**

249 The profiles of Hp, M-SAA3 and CRP over time during the *S. uberis* mastitis challenge are
250 shown in Figure 2, 3 and 4 respectively with the median value and the individual values
251 shown for the six infected quarters from cows 1, 2, 3, 4, 5, 6 (cow numbers consistent with
252 Tassi et al [4]).

253 The earliest rise in Hp concentration was seen at 36 h PC with concentrations over 100-
254 fold the median for pre-challenge (0h PC) observed in 4 challenged quarters, and with all
255 samples reaching this level by 48 h PC. The maximum median concentration of 421 µg/ml
256 (Figure 2a) was observed at 72 h PC. At the final time of sampling (312 h), two quarters
257 still had elevated Hp concentrations relative to basal values (cow 3 and 4 in Figure 2b). In
258 control samples (n=42), the range of Hp concentration was <0.4-6.38 µg/ml, and in pre-
259 challenge samples (0 h, n=6) it was <0.4-1.26 µg/ml.

260 The first rise in M-SAA3 levels was also observed at 36 h PC with 5 of 6 milk samples
261 showing at least a 20-fold increase over the median of the 0 h PC samples and with all
262 samples showing more than a 100-fold the 0 hPC median by 48 h PC. The maximum
263 median concentration of M-SAA3 was at 96 h was 9900 µg/ml (Figure 3a). At 312 h, two
264 quarters had high M-SAA3 concentration; these two quarters were the same ones which
265 had higher Hp concentration at 312 h (cow 3 and 4, Figure 3b). A range of <0.6- 18.68
266 µg/ml was found in control samples and <0.6 -19.22 µg/ml in pre-challenge samples (0 h).

267 For CRP, the first rise in concentration in milk was at 30 h PC with 3 of 6 samples at least
268 300x the 0 hPC median concentration and with all samples having over 1000x the value at

48 h PC. Peak median concentrations of CRP were achieved at 72 h at 16,687 ng/ml (Figure 4a). At 120 h PC there was a peak of CRP in cow 3 at 102,000 ng/ml while at 240 h CRP concentrations in cows 2 and 3 were noticeably higher than in the other cows (Figure 4b). The range of CRP in control samples was <1.8-41.44 ng/ml and was <1.8 ng/ml in pre-challenge samples.

3.3 IMI77 classifier based on CE-MS datasets

In order to detect IMI in cows, CE-MS datasets from 6 cows were analysed. According to specific guidelines on biomarker studies [36], samples were split into the discovery cohort formed by 12 samples, 6 samples from 0 h PC (non infected, NI) and 6 infected cows from 81 h PC (infected, I). The validation cohort consisted of 23 milk samples collected at 36, 42, 57 and 312 h PC (for all time points n=6, except for 36 h PC where n=5 as there was insufficient volume for one sample).

Comparison of the peptide profiles from the two sets of samples in the discovery cohort led to the identification of 460 peptides with adjusted BH p-value significant ($P<0.05$) that were present in at least 66% of the control or diseased groups. Those displaying an AUC=1 were further considered for the study (205 peptides). LC-MS/MS analysis, and data matching with those from Mansor et al. [11] allowed 77 sequences to be obtained from these 205 peptides (Table 2). Peptide maps (CE-MS peaks) of potential biomarkers of *S. uberis* mastitis which were up-regulated or down regulated during infection at 36, 42, 57 and 81 h PC relative to 0 h (pre-challenge) are shown in Figure 5. Out of the 77 peptides, 50 showed qualitative differences between the 0 and 81 h PC (being totally absent at one time as against the other), and 27 displayed quantitative changes with the course of infection. Fifty-five polypeptides were increased in abundance. Among them, the most abundant fragments corresponded to proteins such as alpha-S1-casein and alpha-S2-casein (36 peptides), beta-casein (22 peptides), serum amyloid and Glycosylation-dependent cell adhesion molecule 1 (GDCAM) (5 peptides each). The 77 sequence peptides were then used in a support vector machine (SVM) classifier called IMI77. After applying cross-validation of the discovery data, no peptide was left out from the final classifier. Scoring the animals from the discovery cohort with the resulting IMI77 classifier clearly separated non infected cows from the infected ones. In the next step, the classifier was applied to the 23 samples that were not used in the discovery cohort to see its performance in the progression of IMI. The distribution of IMI77 scores for the discovery and validation cohort showed a pattern where the score increased with the time of infection up to 81 h PC but with samples from 312 h PC the score was more comparable to control than infected animals (Fig. 6).

304 **3.4 Liquid chromatography and mass spectrometry**

305 Liquid chromatography-tandem mass spectrometry allowed for sequencing of the 77 pep-
306 tides in the biomarker panel which were matched with 3 multi-consensus reports and a re-
307 port of Mansor *et al.* [11]. Along with some of their characteristics, they are listed in Table 2.
308 Mass to charge ratio (m/z) range of the sequenced peptides was from 498.93 to 1008.88 Da
309 and mass range from 1016.5 to 3610.74 Da. Most of the sequenced peptides arose from
310 cleavages of alpha-S1-casein and other caseins. A few were from SAA and GDCAM pro-
311 teins. Some of the peptides derived from SAA protein were up regulated by several thou-
312 sand folds during peak of infection, for example; GADKYFHARGNYDAA, GAD-
313 KYFHARGNYDAAQRGPGGAWAA and SGKDPNHFRPAGLPDKY.

314 The greatest fold change (12,223x) occurred with the polypeptide GWRLPEY-
315 TVTQESGPAHRKEFTMTCRVERF which had sequences matching into the RISC-
316 loading complex subunit protein. This peptide was the most up regulated peptide identified
317 followed by SGKDPNHFRPAGLPDKY derived from SAA protein (10,457x). There were
318 22 peptides which were down regulated among the total 77 sequenced and these were de-
319 rived mainly from alpha-caseins and GDCAM proteins.

320 **4 Discussion**

321 In order to integrate the results on the high abundance proteins, the APP and peptides in
322 milk in relation to changes already described by Tassi et al. [4], Figure 7 shows the change in
323 selected analyte levels from the current and the previous studies based on the percentage of
324 the maximal increase for each. To further enable interpretation and integration of data
325 Figure 8 shows the mean bacterial count and rectal temperatures of the infected cows as
326 previously described [4]. Bacterial count in milk was the first parameter to increase being
327 observed at 12 h PC, reaching a peak at 36 h PC and falling to around 50% of peak bacteria
328 from 72 h PC to the end of study. It should be noted that IMI would normally be defined
329 based on the presence of bacteria in milk samples, whereby three consecutive negative
330 samples are needed to declare an animal free of IMI. The SCC first increased at 30 h PC,
331 reached a peak at 48h PC and plateaued at this level virtually to the end of the study.
332 Among the cytokines, IL1β, TNFα and IL6 reached peaks between 36-72 h PC and de-
333 clined to low levels by 120 h PC.

334 **4.1 High abundance proteins of milk**

335 The IMI with *S. uberis* caused significant change in the high abundance milk proteins and
336 increases in milk APP. While there was between animal variations in the response of high

337 abundance proteins to IMI, there were consistent changes seen along the time course of the
338 infection in the sets of milk samples from each infected udder quarter. The decrease in
339 caseins, β-lactoglobulin and α-lactabulin and increase in albumin, LF and IgG following
340 infection of the mammary are well known [18, 19, 29] but here the timing of the responses has
341 been identified. With cow 6 (Figure 1) as an example the fall in caseins of 28-38 kDa was
342 seen first at 36 h PC, occurring after bacterial count and SCC increases which were at 12 h
343 and 30 h PC respectively but at the same time as increases in cytokines such as TNFα and
344 IL1β [4]. There was a subsequent increase in the protein at 28-38 kDa from 72 h PC but in
345 mastitic milk (Fig 1B) Ig light chain has a similar mobility and with one dimensional
346 electrophoresis it is not possible to differentiate between these proteins. Two dimensional
347 electrophoresis or immunoassay would be needed to achieve this purpose. Increases in
348 albumin and IgG occurred later, at 81 h PC, while the peak of LF was further delayed to
349 120 h PC. Thus changes in the concentrations of high abundance proteins of milk
350 following IMI are not uniform across proteins. It may be that, by monitoring relative
351 concentrations of these proteins, alone or as part of a diagnostic panel, the stage of
352 infection could be identified. Although IMI had been resolved in 5 of 6 animals by 312 h
353 PC[3],the composition of high abundance proteins had not reverted to pre-challenge levels.
354 By contrast, SCC levels were still high in all cows at 312 PC[4], implying that host rather
355 than bacterial proteases are responsible for protein degradation.

356 **4.2 Acute phase proteins**

357 For the APP, the time course of increase in Hp and M-SAA3 have been described in
358 response to *S. aureus* mastitis [10] but the changes in milk CRP during any experimental
359 model of mastitis have not previously been demonstrated. In respect to the cytokine
360 response the maximum of Hp, M-SAA3 and CRP concentration were after the peak
361 cytokine responses (Figure 7).

362 There was variation between the individual cows in APP response, as there had been in
363 clinical and bacteriological response[4]. Milk Hp was first increased from basal values at 36
364 h PC in 4 cows with the median Hp across all cows peaking at 72 h PC. Notably, over
365 several hundred fold increase in milk Hp concentration was observed at the peak,
366 highlighting the strong response of milk Hp to the IMI. Elevation of milk CRP was the
367 earliest to occur with the initial increase being observed at 30h pi in 3 out of the six cows
368 while M-SAA3 was the last to be raised with only a 20x increase seen at 36 h PC. There
369 were differences between animals as well as between the APP, but the APP responses were
370 consistent in a number of aspects. At least 24 h passed between infection and any
371 elevation of the milk APP concentration. The APP showed over a thousand fold increase in

372 their concentrations with maximum median concentrations at 72-96 h PC, thereafter falling
373 though in some cows the basal level of the APP had not been reached by 312 h PC. The
374 fall in APP after 72 h PC occurred even though the SCC remained elevated for the duration
375 of the 312 h of the study and in the resolution phase more closely resembled the profile of
376 bacterial counts in the milk than the SCC. Hence, APP may be a better biomarker of IMI
377 than SCC.

378 There are differences to previous reports on APP in mastitis. Pedersen and others (2003)[23]
379 and Jacobsen et al. (2005)[37] demonstrated an earlier rise in M-SAA3 than Hp during the
380 course of an *S. uberis* intramammary challenge. The difference in comparison to our results
381 could be due to strain differences in the *S .uberis* used for challenge leading to different
382 cytokine activation pathways [16] and could also be influenced by a difference in host
383 genotype or phenotype. While assays for bovine CRP have only recently become available
384 it could be that using such an analyte with a lower detection limit and a large dynamic
385 range will accentuate the value of this APP in detecting mastitis. Previously, although CRP
386 had been identified as a milk APP [24] it has not generally been regarded as a bovine APP for
387 use as a biomarker of mastitis, but availability of the immunoassay used here for bovine
388 CRP will allow its diagnostic value to be assessed at a larger scale. Currently, of the three
389 APP, Hp is the easiest to measure with availability of specific antibody for the
390 development of varied immunoassay formats and a large response even if its peak response
391 is later than that of CRP. The stage of IMI and the species of pathogen are known to cause
392 differing mammary responses[38, 39]. While attempts to differentiate pathogen and stage of
393 IMI by APP analysis have yielded disappointing results[40] an aim of the current series of
394 studies is to determine whether differentiation is possible with inclusion not only of Hp,
395 MSAA3 and CRP but also change in the high abundance proteins, peptides and metabolites
396 possibly yielding a diagnostic algorithm similar to those being developed for protein
397 profiles being developed in clinical proteomics [41] and could yield diagnostic value for
398 mastitis detection and monitoring.

399 **4.3 Peptidomics**

400 A limitation of previous investigations of the responses of milk proteins to mastitis has
401 been that, due to the lack of suitable methods, the low Mw proteins and peptides in milk
402 are frequently ignored. Recently the use of methods specific for peptides of <25kDa have
403 suggested that there are major changes in these molecules in mastitis. CE-MS analysis of
404 bovine milk during natural mastitis [11] detected peptide differences between milk samples
405 from control and naturally infected udders (31 polypeptides) and between milk from mas-

406 titic udders caused by two separate pathogens (14 polypeptides). This method of peptide
407 analysis has been described as a powerful hyphenated technique for the study of pep-
408 tidomic profiles [42] and has been exploited for the generation of biomarker panel of peptides
409 for conditions such as renal [43] and cardiovascular [44] disorders in humans.

410 A majority of the successfully sequenced changing peptides from this challenge study
411 arose from cleavages of alpha-S1-casein (n=31) and beta-casein (22 milk proteins), in
412 agreement with the reports of Dallas et al (2014)[45], Mansor *et al*. (2013)[11] and Larsen *et*
413 *al*. (2010b)[46] and despite differences in causative agents between studies. This further ex-
414 plains the general decrease in milk caseins associated with clinical mastitis [20] and shown
415 here in Figure 1. It has been postulated that *S. uberis* is dependent on casein cleavage to
416 obtain nutrients during IMI[47], but shifts in protein and peptide distributions persist beyond
417 resolution of infection so casein cleavage is not dependent on the pathogen.

418 A few of the peptides showing change were not from casein degradation but from
419 GDCAM, (mainly down regulated), and SAA (up regulated) cleavages. These two proteins
420 have been identified as immune related proteins [29, 48, 49]. Presence of GDCAM could relate
421 to the role proposed for host glycosaminoglycans in the pathogenesis of *S. uberis*
422 mastitis[50, 51]. Proteases play a central role in the type and amounts of peptides detected in
423 milk during mastitis and endogenous peptides such as plasmin, cathepsins, elastase, and
424 amino- and carboxypeptidases have been suggested as being crucial during the IMI as they
425 are increased in milk due to release from the influx of neutrophils (PMNs) and other phag-
426 ocytic cells, measured as the SCC, that occurs during mastitis [46, 52]. These proteases were
427 also reported to have specificities towards alpha-S1 and beta caseins. Pathogen related pro-
428 teases have also been suggested to contribute to the proteolysis observed in milk during
429 mastitis [46].

430 Similar to the study of Wedholm *et al*. (2008) [53], peptides from alpha-S1, alpha-S2 and be-
431 ta-caseins were identified but in addition two kappa-caseins fragments were found and se-
432 quenced during infection but were absent in pre-challenge samples. This corresponds to
433 the effect of LPS infusion in an experimental mastitis model generating proteolytic chang-
434 es of milk over time [52].

435 Three polypeptides sequenced in this study were similarly identified in both the multi-
436 consensus and Mansor *et al*. (2013)[11] reports. Two of these peptides were fragments from
437 GlyCAM-1 protein and one was from cleavage of fibroblast growth factor-binding protein
438 (FGFBP). All of these three polypeptides were found in pre-challenge samples and absent

439 during infection, while in the study of Mansor *et al.* (2013)[11], these polypeptides only dif-
440 ferentiated between healthy and mastitic samples and not between the two different masti-
441 tis pathogen species studied (i.e. *E. coli* and *S. aureus*). The matching of these peptides
442 from the present study, the study of Mansor *et al.* (2013)[11] and with reports from previous
443 CE-MS milk analysis substantiates their probability as peptide markers of mastitis irre-
444 spective of the causal agent of mastitis.

445 As a time-point-based peptidomic study of mastitis progression, this study offers additional
446 advantage over other previous investigations in detecting and identifying peptides and in
447 showing significant difference from pre-challenge controls, as early as 36 h PC. The prob-
448 ability exists that the peptidomic profile at earlier time points (before 36 h) may signifi-
449 cantly differentiate pre-challenge samples from commencement of infection but were not
450 analysed here due to resource limitations. As an objective for future studies, it would be
451 useful to determine the earliest time point during which peptide changes are able to signifi-
452 cantly differentiate healthy from infected samples to provide an early warning of impend-
453 ing mastitis.

454 The increase in IMI77 classification score up to 81 h PC shows that peptide proteolysis
455 increases while the bacterial count declined after 30 h PC. The proteolytic activity may
456 thus be more likely to be emanating from endogenous proteases rather than those of bacte-
457 rial origin. It was of interest that at 81 h PC there were no peptides derived from albumin,
458 lactoferrin or IgG despite these being the most abundant proteins in the milk at this time
459 point. These proteins may be more resistant to degradation by the proteases present in the
460 milk than the caseins. This could be a part of an anti-bacterial function of the alteration of
461 the milk proteome in mastitis by depriving bacteria of protein as a nutrient but still provid-
462 ing protein in the milk that would be digested by the neonate's gastro-intestinal tract.

463 In respect of a peptide panel that could differentiate mastitis caused by *S. uberis* from other
464 pathogens, 72 of the polypeptides which were sequenced in this study, did not match any
465 of the polypeptides detected in Mansor *et al*'s study[11] of *S. aureus* and *E. coli* mastitis or
466 any of the multi-consensus reports. Therefore, these 72 peptides could represent a panel of
467 peptides specific to *S. uberis* mastitis. Validation of this claim would be required using
468 other *S. uberis* mastitis models such as natural infection and infections by different strains
469 of *S. uberis.*

470 The time points that were selected for peptidomic analysis were based on the clinical and
471 bacteriological course of infection, whereby the peak of infection seemed to have ended by

472 81 hrs post challenge (Fig. 8). Surprisingly, the biggest peptidomic difference between pre-
473 challenge and post-challenge samples was detected in the validation set, using samples
474 from 81 hrs post challenges. Indeed, changes in high abundance proteins, APP and pep-
475 tidomic profiles all persisted beyond the clinical and bacteriological peak of IMI, indicat-
476 ing that bacteriological, clinical and peptidomic events are partly out of synch. This is con-
477 sistent with the idea that changes in proteins and peptides are largely driven by the host
478 immune response and SCC influx rather than directly by bacteria. At the last observed time
479 point, 312 hrs PC, the IMI77 classifier scores were still significantly different from the pre-
480 challenge time point, but much closer to pre-challenge values than for any other time point
481 considered in this study. At 312 hrs PC, 5 of 6 cows had resolved the IMI and all cows and
482 quarters appeared clinically normal[3]. Thus, the change in IMI77 score reflects the natural
483 resolution of IMI. It would be interesting to explore the relationship between bacteriologi-
484 cal status and peptide profile at individual cow level for multiple time points during the
485 IMI resolution phase but samples to do so were not available from the current study.

486 Early detection and differential diagnosis of the mastitis causing pathogen would be valua-
487 ble for the dairy industry, for earlier and more effective treatment and also to reduce the
488 use of ineffectual antimicrobials which would lead to a reduction in resistance to these
489 therapeutics. On large dairy farms operating under high economic pressure and on farms
490 with automated milking systems, clinical symptoms would not be noticed because regular
491 observation of individual animals does not take place. Under those circumstances, alterna-
492 tive diagnostic indicators are potentially of great value. It is clear that both APP and pep-
493 tide analysis could play a role in this scenario and when combined with quantitative prote-
494 omics [12] and metabolomics [13], that integration of protein assay and omic technologies has
495 major potential for delivering a unified and substantial means to provide a molecular in-
496 sight into a complex biological system and to stimulate biomarker development across
497 omic boundaries.

498 **5. Conclusion**

499 The high abundance protein and APP profiles of milk during an experimental *S. uberis*
500 mastitis challenge were investigated, with a shift in abundance from caseins, β-
501 lactoglobulin and α-lactalbumin to albumin, lactoferrin and IgG being observed following
502 infection. The APP profiles of Hp, M-SAA3 and CRP were closer to the bacterial count
503 than the SCC in milk from infected quarters and may have value in diagnosing and moni-
504 toring the stage of IMI. Analysis of the peptide profile in milk across selected time points
505 of the experimental challenge, showed a panel of peptides, which as early as 36 h PC,

506 could significantly differentiate infected from non-infected milk, thus suggesting potential
507 as biomarkers of bovine mastitis. Moreover, the identification of peptidomic markers that
508 were not detected in clinical mastitis due to other pathogens suggests that pathogen specif-
509 ic diagnosis is possible.

510 **Acknowledgements**

511 University of Agriculture Abeokuta, Tertiary education trust fund and Zoetis are gratefully
512 acknowledged for the funding the studentship and the research. Proteomics for gel band
513 identifications was performed in Glasgow Polyomics, which is supported by the Wellcome
514 Trust (grant no. 097821/Z/11/Z). The work was financially supported by the Moredun In-
515 novation Fund (RT) and the Scottish Government's Rural and Environment Science and
516 Analytical Services Division (TM and RZ). Suzanne McGill (Glasgow Polyomics) and
517 Chris McComb (University of Glasgow) are thanked for technical assistance.

518

519 **References**

520 1. H. Hogeveen, K. Huijps and T. J. G. M. Lam, *New Zealand Veterinary Journal*,
521 2011, **59**, 16-23.
522 2. A. Lago, S. M. Godden, R. Bey, P. L. Ruegg and K. Leslie, *Journal of Dairy*
523 *Science*, 2011, **94**, 4441-4456.
524 3. A. Almeida, A. Bassols, E. Bendixen, M. Bhide, F. Ceciliani, S. Cristobal, P.
525 Eckersall, K. Hollung, F. Lisacek, G. Mazzucchelli, M. McLaughlin, I. Miller, J.
526 Nally, J. Plowman, J. Renaut, P. Rodrigues, P. Roncada, J. Staric and R. Turk,
527 *Animal*, 2015, **9**, 1-17.
528 4. R. Tassi, T. McNeilly, J. Fitzpatrick, M. Fontaine, D. Reddick, C. Ramage, M.
529 Lutton, Y. Schukken and R. Zadoks, *Journal of Dairy Science*, 2013, **96**, 5129-5145.
530 5. R. N. Zadoks, *CAB Reviews: Perspectives in Agriculture, Veterinary Science,*
531 *Nutrition and Natural Resources*, 2007, **2**, 15 pp.-15 pp.
532 6. J. Verbeke, S. Piepers, K. Supre and S. De Vliegher, *Journal of Dairy Science*, 2014,
533 **97**, 6926-6934.
534 7. H. L. Wang, Z. X. Li, L. J. Wang, H. He, J. Yang, L. Chen, F. B. Niu, Y. Liu, J. Z.
535 Guo and X. L. Liu, *Research in Veterinary Science*, 2013, **95**, 508-514.
536 8. O. M. Keane, K. E. Budd, J. Flynn and F. McCoy, *Veterinary Record*, 2013, **173**,
537 268-+.
538 9. J. Katholm, T. W. Bennedsgaard, M. T. Koskinen and E. Rattenborg, *Journal of*
539 *Dairy Science*, 2012, **95**, 5702-5708.
540 10. P. D. Eckersall, F. J. Young, A. M. Nolan, C. H. Knight, C. McComb, M. M.
541 Waterston, C. J. Hogarth, E. M. Scott and J. L. Fitzpatrick, *Journal of Dairy*
542 *Science*, 2006, **89**, 1488-1501.
543 11. R. Mansor, W. Mullen, A. Albalat, P. Zerefos, H. Mischak, D. C. Barrett, A. Biggs
544 and P. D. Eckersall, *Journal of Proteomics*, 2013, **85**, 89-98.
545 12. M. Mudaliar, R. Tassi, F. Thomas, T. McNeilly, S. Weid, M. McLaughlin, D.
546 Wilson, R. Burchmore, P. Herzyk, P. Eckersall and R. Zadoks, *Molecular*
547 *Biosystems, Submitted*.
548 13. F. Thomas, M. Mudaliar, K. Burgess, R. Burchmore, W. Mullen, R. Tassi, T.
549 McNeilly, P. Herzyk, R. Zadoks, P. Eckersall and *Molecular Biosystems, Submitted*.

550 14. H. Moshage, *Journal of Pathology*, 1997, **181**, 257-266.
551 15. C. J. Whelehan, K. G. Meade, P. Eckersall, F. J. Young and C. O'Farrelly, *Veterinary*
552 *Immunology And Immunopathology*, 2011, **140**, 181-189.
553 16. J. G. Bode, U. Albrecht, D. Haeussinger, P. C. Heinrich and F. Schaper, *European*
554 *Journal of Cell Biology*, 2012, **91**, 496-505.
555 17. H. B. Singh, M. Thompson, A., *Milk Proteins from Expression to Food*, Academic
556 Press, Amsterdaam, 2014.
557 18. K. Kato, K. Mori and N. Katoh, *Japanese Journal of Veterinary Science*, 1989, **51**,
558 1275-1278.
559 19. L. Kaartinen, K. Veijalainen, P. L. Kuosa, S. Pyorala and M. Sandholm, *Journal of*
560 *Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health*,
561 1988, **35**, 353-360.
562 20. C. J. Hogarth, J. L. Fitzpatrick, A. Nolan, F. J. Young, A. Pitt and P. D. Eckersall,
563 *Proteomics*, 2004, **4**, 2094-2100.
564 21. K. Ostensson, *American Journal of Veterinary Research*, 1993, **54**, 231-238.
565 22. F. Ceciliani, J. J. Ceron, P. D. Eckersall and H. Sauerwein, *Journal of Proteomics*,
566 2012, **75**, 4207-4231.
567 23. L. H. Pedersen, B. Aalbaek, C. M. Rontved, K. L. Ingvartsen, N. S. Sorensen, P. M.
568 H. Heegaard and H. E. Jensen, *Journal of Comparative Pathology*, 2003, **128**, 156-
569 164.
570 24. W. Schrodl, M. Kruger, T. T. Hien, M. Fuldner and R. Kunze, *Tierarztliche Praxis*,
571 1995, **23**, 337-341.
572 25. J. Hamann, M. Kruger, C. Kretzschmar, B. Nipp and P. Gyodi, *Milchwissenschaft*,
573 1997, **52**, 546-550.
574 26. M. Kruger and A. Neumann, *Tierarztliche Praxis Ausgabe Grobtiere Nutztiere*,
575 1999, **27**, 164-167.
576 27. F. C. Thomas, M. Waterston, P. Hastie, T. Parkin, H. Haining and P. D. Eckersall,
577 *Bmc Veterinary Research*, 2015, **11**.
578 28. T. A. Reinhardt, R. E. Sacco, B. J. Nonnecke and J. D. Lippolis, *Journal of*
579 *Proteomics*, 2013, **82**, 141-154.
580 29. M. Danielsen, M. C. Codrea, K. L. Ingvartsen, N. C. Friggens, E. Bendixen and C.
581 M. Rontved, *Proteomics*, 2010, **10**, 2240-2249.
582 30. S. L. Bislev, U. Kusebauch, M. C. Codrea, R. J. Beynon, V. M. Harman, C. M.
583 Rontved, R. Aebersold, R. L. Moritz and E. Bendixen, *Journal of Proteome*
584 *Research*, 2012, **11**, 1832-1843.
585 31. J. Siwy, W. Mullen, I. Golovko, J. Franke and P. Zuerbig, *Proteomics Clinical*
586 *Applications*, 2011, **5**, 367-374.
587 32. A. Albalat, H. Husi, J. Siwi, J. Nally, P. D. Eckersall and W. Mullen, *Current*
588 *Protein and Peptide Science*, 2014, **In Press**.
589 33. M. Braceland, R. Bickerdike, J. Tinsley, D. Cockerill, M. F. Mcloughhlin, D. A.
590 Graham, R. J. Burchmore, W. Weir, C. Wallace and P. D. Eckersall, *Journal of*
591 *Proteomics*, 2013, **94**, 423-436.
592 34. N. V. Neuhoff, T. Kaiser, S. Wittke, R. Krebs, A. Pitt, A. Burchard, A. Sundmacher,
593 B. Schlegelberger, W. Kolch and H. Mischak, *Rapid Communications in Mass*
594 *Spectrometry*, 2004, **18**, 149-156.
595 35. Y. Benjamini and Y. Hochberg, *Journal of the Royal Statistical Society Series B-*
596 *Methodological*, 1995, **57**, 289-300.
597 36. H. Mischak, G. Allmaier, R. Apweiler, T. Attwood, M. Baumann, A. Benigni, S. E.
598 Bennett, R. Bischoff, E. Bongcam-Rudloff, G. Capasso, J. J. Coon, P. D'Haese, A. F.
599 Dominiczak, M. Dakna, H. Dihazi, J. H. Ehrich, P. Fernandez-Llama, D. Fliser, J.
600 Frokiaer, J. Garin, M. Girolami, W. S. Hancock, M. Haubitz, D. Hochstrasser, R. R.
601 Holman, J. P. A. Ioannidis, J. Jankowski, B. A. Julian, J. B. Klein, W. Kolch, T.
602 Luider, Z. Massy, W. B. Mattes, F. Molina, B. Monsarrat, J. Novak, K. Peter, P.

603 Rossing, M. Sanchez-Carbayo, J. P. Schanstra, O. J. Semmes, G. Spasovski, D.
604 Theodorescu, V. Thongboonkerd, R. Vanholder, T. D. Veenstra, E. Weissinger, T.
605 Yamamoto and A. Vlahou, *Science Translational Medicine*, 2010, **2**.
606 37. S. Jacobsen, T. A. Niewold, E. Kornalijnslijper, M. J. M. Toussaint and E. Gruys,
607 *Veterinary Immunology And Immunopathology*, 2005, **104**, 21-31.
608 38. D. D. Bannerman, M. J. Paape, J. P. Goff, K. Kimura, J. D. Lippolis and J. C. Hope,
609 *Veterinary Research*, 2004, **35**, 681-700.
610 39. D. Bannerman, *Journal of Animal Science*, 2009, **87**, 10-25.
611 40. S. Pyorala, M. Hovinen, H. Simojoki, J. Fitzpatrick, P. Eckersall and T. Orro,
612 *Veterinary Record*, 2011, **168**, 535-U539.
613 41. X. T. Robin, N. Hainard, A. Tiberti, N. Lisacek, F. Sanchez, J-C Muller M,
614 *Translational Proteomics*, 2013, **1**, 8.
615 42. M. Herrero, E. Ibanez and A. Cifuentes, *Electrophoresis*, 2008, **29**, 2148-2160.
616 43. D. M. Good, P. Zuerbig, A. Argiles, H. W. Bauer, G. Behrens, J. J. Coon, M. Dakna,
617 S. Decramer, C. Delles, A. F. Dominiczak, J. H. Ehrich, F. Eitner, D. Fliser, M.
618 Frommberger, A. Ganser, M. A. Girolami, I. Golovko, W. Gwinner, M. Haubitz, S.
619 Herget-Rosenthal, J. Jankowski, H. Jahn, G. Jerums, B. A. Julian, M. Kellmann, V.
620 Kliem, W. Kolch, A. S. Krolewski, M. Luppi, Z. Massy, M. Melter, C. Neusuess, J.
621 Novak, K. Peter, K. Rossing, H. Rupprecht, J. P. Schanstra, E. Schiffer, J. U.
622 Stolzenburg, L. Tarnow, D. Theodorescu, V. Thongboonkerd, R. Vanholder, E. M.
623 Weissinger, H. Mischak and P. Schmitt-Kopplin, *Molecular & Cellular Proteomics*,
624 2010, **9**, 2424-2437.
625 44. C. Delles, E. Schiffer, C. von zur Muhlen, K. Peter, P. Rossing, H. H. Parving, J. A.
626 Dymott, U. Neisius, L. U. Zimmerli, J. K. Snell-Bergeon, D. M. Maahs, R. E.
627 Schmieder, H. Mischak and A. F. Dominiczak, *Journal of Hypertension*, 2010, **28**,
628 2316-2322.
629 45. D. C. Dallas, A. Guerrero, E. A. Parker, L. A. Garay, A. Bhandari, C. B. Lebrilla, D.
630 Barile and J. B. German, *Journal of Agricultural and Food Chemistry*, 2014, **62**,
631 58-65.
632 46. L. B. Larsen, K. Hinz, A. L. W. Jorgensen, H. S. Moller, O. Wellnitz, R. M.
633 Bruckmaier and A. L. Kelly, *Journal of Dairy Science*, 2010, **93**, 5613-5626.
634 47. A. J. Kitt and J. A. Leigh, in *Streptococci and the Host*, eds. T. Horaud, A. Bouvet,
635 R. Leclercq, H. deMontclos and M. Sicard1997, vol. 418, pp. 647-650.
636 48. P. Roncada, C. Piras, A. Soggiu, R. Turk, A. Urbani and L. Bonizzi, *Journal of*
637 *Proteomics*, 2012, **75**, 4259-4274.
638 49. J. Boehmer, J. Ward, R. Peters, K. Shefcheck, M. McFarland and D. Bannerman,
639 *Journal of Dairy Science*, 2010, **93**, 593-603.
640 50. R. A. Almeida, D. A. Luther, R. Nair and S. P. Oliver, *Veterinary Microbiology*,
641 2003, **94**, 131-141.
642 51. R. A. Almeida, W. H. Fang and S. P. Oliver, *Fems Microbiology Letters*, 1999, **177**,
643 313-317.
644 52. F. Moussaoui, F. Laurent, J. M. Girardet, G. Humbert, J. L. Gaillard and Y. Le Roux,
645 *Journal of Dairy Science*, 2003, **86**, 1163-1170.
646 53. A. Wedholm, H. Moller, A. Stensballe, H. Lindmark-Mansson, A. Karlsson, R.
647 Andersson, A. Andren and L. Larsen, *Journal of Dairy Science*, 2008, **91**, 3787-
648 3797.
649

Table 1 Milk proteins identified by LC-MS/MS after one dimensional SDS-PAGE separation of milk proteins

Band No	Proteins	Protein ID	Mass	pI	Mowse score	Peptides	Sequence cover %
1	IgG heavy chain	gi|7547266	36510	6.09	283	7	42
1	IgG heavy chain	gi|91982959	36562	6.49	210	6	36
1	Ceruloplasmin	gi|296491101	121901	5.68	90	9	9
2	Lactoferrin	gi|408928	80113	8.73	1896	40	61
2	Lactotransferrin precursor	gi|30794292	80002	8.69	1892	40	61
2	Serotransferrin precursor	gi|114326282	79856	6.75	403	21	33
3	Albumin	gi|1351907	71244	5.82	2449	47	68
3	Complement C3 isoform X1	gi|741932316	188675	6.41	235	14	9
4	Ig heavy chain precursor	gi|108750	51391	6.1	215	6	19
4	IgG2a heavy chain constant region, partial	gi|1699167	36402	7.7	167	38	24
5	Ig heavy chain precursor	gi|108750	51391	6.1	251	7	23
5	Ig lambda light chain	gi|15088675	25032	5.84	132	4	20
5	Alpha-S1-casein isoform X2	gi|982928492	23558	5.12	111	4	21
6	Ig lambda-like polypeptide 1	gi|741957421	25010	8.19	457	10	44
6	Ig light chain, lambda gene cluster	gi|92096965	24863	7.53	449	9	37
7	Alpha-S1-casein	gi|225632	24477	4.85	665	8	40
7	Beta-lactoglobulin	gi|2194088	18583	4.83	115	6	32
8	Beta-casein isoform X1	gi|741930202	29150	5.89	305	35	44
8	Component PP3	gi|741536	15295	5.98	144	4	26
9	Beta-lactoglobulin	gi|229460	18641	4.76	163	6	48
9	Alpha-S1-casein isoform X13	gi|528953246	20227	5.32	140	4	28
10	Beta-lactoglobulin	gi|6980895	18641	4.76	2325	18	82
11	Alpha-lactabumin	gi|68	14603	4.8	392	4	39
11	Beta-lactoglobulin	gi|2194088	18583	4.83	308	8	48

Table 2. Peptides used in IMI77 classifier. Frequency and intensity indicate the number of samples in which each peptide was detected / number of quarter milk samples per group (n=6) and the average ion counts, respectively for samples collected pre-challenge (0 h PC, non infected, NI) and at 81 h PC (infected, I).

Peptide _ID	Protein symbol	Protein Name	Sequence	Frequency NI	Intensity NI	Frequency I	Intensity I	Fold change I/NI	Direction I/NI
5003	GLCM1	Glycosylation-dependent cell adhesion molecule 1	SHAFEVVKT	2/6	1.7	6/6	599.2	344.3	up
5320	CASA1	Alpha-S1-casein	QQKEPMIGV	1/6	0.4	6/6	692.4	1731.0	up
7162	CASA2	Alpha-S2-casein	QKFALPQYL	1/6	3/6	6/6	968.6	1793.8	up
8859	CASB	Beta-casein	SEESITRINK	0	0	6/6	2194.7	2194.7	up
8906	CASA1	Alpha-S1-casein	NELSKDIGSES	6/6	154.2	0	0	0	down
9741	CASB	Beta-casein	YPQRDMPIQA	1/6	6/6	6/6	297.0	309.4	up
9931	LACB	Beta-lactoglobulin	EELKPTPEGDL	1/6	1/6	6/6	445.2	2473.3	up
10197	CASA1	Alpha-S1-casein	HAQQKEPMIGV	0	0	6/6	528.1	528.1	up
10508	CASA2	Alpha-S2-casein	TKVIPYVRYL	6/6	1852.2	0	0	0	down
12245	CASB	Beta-casein	LSSSEESITRIN	0	0	6/6	433.0	433.0	up
13263	CASA1	Alpha-S1-casein	HPIKHQGLPQEV	2/6	31.1	6/6	2295.1	73.7	up
13326	CASA1	Alpha-S1-casein	IPNPIGSENSEKT	6/6	671.8	2/6	17.5	0	down
14354	CASA1	Alpha-S1-casein	VAPFPEVFGKEKV	1/6	22.3	6/6	1952.1	87.5	up
14551	CASA1	Alpha-S1-casein	YKVPQLEIVPNSA	1/6	4.9	6/6	412.3	85.0	up
15151	CASB	Beta-casein	AVPYPQRDMPIQA	0	0	6/6	1440.3	1440.3	up
15287	CASA1	Alpha-S1-casein	FVAPFPEVFGKEK	6/6	343.6	0	0	0	down
15326	CASB	Beta-casein	EMPFPKYPVEPF	0	0	6/6	2261.4	2261.4	up
15395	CASA1	Alpha-S1-casein	DIPNPIGSENSEKT	0	0	6/6	416.3	416.3	up
15403	CASA1	Alpha-S1-casein	HIQKEDVPSERY	1/6	44.4	6/6	1591.8	35.8	up
15580	CASA1	Alpha-S1-casein	KHPIKHQGLPQEV	0	0	6/6	2616.1	2616.1	up
15923	LACB	Beta-lactoglobulin	SLLDAQSAPLRVYV	1/6	0.6	6/6	5381.2	9277.9	up
16011	CASA1	Alpha-S1-casein	EGIHAQQKEPMIGV	0	0	6/6	3192.3	3192.3	up
16211	CASA1	Alpha-S1-casein	EGIHAQQKEPmIGV	0	0	6/6	667.2	667.2	up
16353	SAA	Serum amyloid A protein	GNYDAAQRGPGGAWAA	1/6	61.7	6/6	2005.7	32.5	up
16692	CASA1	Alpha-S1-casein	SDIPNPIGSENSEKT	0	0	6/6	4668.6	4668.6	up
16863	CO3	Complement C3	SEETKENERFTVK	3/6	9.0	6/6	1244.5	138.3	up
17132	CASA1	Alpha-S1-casein	HIQKEDVPSERYL	1/6	11.9	6/6	9426.7	795.5	up
17453	CASB	Beta-casein	AVPYPQRDMPIQAF	0	0	6/6	754.6	754.6	up
17789	SAA	Serum amyloid A protein	GADKYFHARGNYDAA	0	0	6/6	1326.7	1326.7	up

17818	OSTK	Osteopontin-K	IRISHELDSASSEVN	0	0	6/6	2132.0	2132.0	up
18670	CASA1	Alpha-S1-casein	NELSKDIGSESTEDQA	0	0	6/6	924.8	924.8	up
18956	CASB	Beta-casein	QKAVPYPQRDMPIQA	0	0	6/6	1016.0	1016.0	up
19009	CASB	Beta-casein	HKEMPFPKYPVEPF	0	0	6/6	1767.1	1767.1	up
19028	CASB	Beta-casein	HKEMPFPKYPVEPF	6/6	2655.3	4/6	153.2	0.1	down
19318	CASB	Beta-casein	FPKYPVEPFTESQSL	1/6	8.4	6/6	2113.3	251.3	up
19331	CASA2	Alpha-S2-casein	LYQGPIVLNPWDQVK	6/6	376.6	0	0	0	down
20271	SAA	Serum amyloid A protein	RGNYDAAQRGPGGAWAAK	0	0	6/6	632.3	632.3	up
20714	CASA1	Alpha-S1-casein	SMKEGIHAQQKEPMIGV	0	0	6/6	1119.7	1119.7	up
20789	CASB	Beta-casein	QKAVPYPQRDMPIQAF	0	0	6/6	403.3	403.3	up
20919	SAA	Serum amyloid A protein	SGKDPNHFRPAGLPDKY	0	0	6/6	10457.3	10457.3	up
21739	CASK	Kappa-casein	SRYPSYGLNYYQQKPV	0	0	6/6	270.8	270.8	up
22168	CASA1	Alpha-S1-casein	EQKHIQKEDVPSERYL	0	0	6/6	2672.9	2672.9	up
22421	CASB	Beta-casein	QKAVPYpQRDMPIQAFL	0	0	6/6	935.6	935.6	up
23769	CASA1	Alpha-S1-casein	GIHAQQKEPMIGVNQELAY	2/6	34.7	6/6	10893.4	313.7	up
24045	GLCM1	Glycosylation-dependent cell adhesion molecule 1	SSRQPQSQNPKLPLSILKE	6/6	817.9	0	0	0	down
24098	FGFP1	Fibroblast growth factor-binding protein 1	RGSKASADESLALGKPGKEPR	6/6	661.2	0	0	0	down
24482	CASA2	Alpha-S2-casein	TMEHVSSSEESIISQETYK	0	0	6/6	1541.8	1541.8	up
24847	CASA1	Alpha-S1-casein	SDIPNPIGSENSEKTTMPLW	6/6	703.8	6/6	20422.2	29.0	up
25003	CASA1	Alpha-S1-casein	SDIPNPIGSENSEKTTmPLW	1/6	3.5	6/6	5216.3	1481.9	up
25030	CASA1	Alpha-S1-casein	RPKHPIKHQGLPQEVLNEN	6/6	2951/6	2/6	111.4	0	down
25054	CASA1	Alpha-S1-casein	HPIKHQGLPQEVLNENLLR	6/6	918.0	1/6	9.8	0	down
25195	CASB	Beta-casein	VLPVPQKAVPYPQRDMPIQA	0	0	6/6	1612.7	1612.7	up
25582	GLCM1	Glycosylation-dependent cell adhesion molecule 1	SSRQPQSQNPKLPLSILKEK	6/6	6164.4	0	0	0	down
25911	CASA2	Alpha-S2-casein	KNTMEHVSSSEESIISQETY	6/6	1199.6	0	0	0	down
26545	CASA1	Alpha-S1-casein	RPKHPIKHQGLPQEVLNENL	6/6	3084.5	4/6	293.0	0.1	down
26799	CASB	Beta-casein	WMHQPHQPLPPTVmFPPQSV	0	0	6/6	576.5	576.5	up
27098	CASB	Beta-casein	VLPVPQKAVPYPQRDMPIQAF	0	0	6/6	1251.4	1251.4	up
27560	CASA1	Alpha-S1-casein	HIQKEDVPSERYLGYLEQLL	6/6	2623.0	0	0	0	down
27692	CASB	Beta-casein	SWMHQPHQPLPPTVMFPPQSV	0	0	6/6	2676.3	2676.3	up
27904	GLCM1	Glycosylation-dependent cell adhesion molecule 1	ILNKPEDETHLEAQPTDASAQF	6/6	695.2	0	0	0	down
27994	CASA1	Alpha-S1-casein	RPKHPIKHQGLPQEVLNENLL	6/6	52176.1	5/6	8867.9	0.2	down

28202	CASB	Beta-casein	FQSEEQQQTEDELQDKIHPF	0	0	6/6	1862.6	1862.6	up
28876	GLCM1	Glycosylation-dependent cell adhesion molecule 1	SSRQPQSQNPKLPLSILKEKHL	6/6	22515.4	0	0.0	0.0	down
29718	CASB	Beta-casein	QSKVLPVPQKAVPYPQRDMPIQA	0	0	6/6	1521.7	1521.7	up
29972	SAA	Serum amyloid A protein	GADKYFHARGNYDAAQRGPGGAWAA	0	0	6/6	3021.4	3021.4	up
30120	CASA1	Alpha-S1-casein	RPKHPIKHQGLPQEVLNENLLR	6/6	15286.8	5/6	1592.0	0.1	down
31513	CASA1	Alpha-S1-casein	LKKYKVPQLEIVPNSAEERLHSM	6/6	389.1	0	0.0	0.0	down
32317	LACB	Beta-lactoglobulin	RTPEVDDEALEKFDKALKALPMHI	6/6	1491.6	0	0.0	0.0	down
32654	CASA1	Alpha-S1-casein	EERLHSMKEGIHAQQKEPMIGVNQ	6/6	3355.6	0	0.0	0.0	down
33130	CASB	Beta-casein	MAPKHKEMPFPKYPVEPFTESQSL	0	0	6/6	2502.4	2502.4	up
33228	CASB	Beta-casein	SQSKVLPVPQKAVPYPQRDMPIQAF	0	0	6/6	376.7	376.7	up
33323	CASA1	Alpha-S1-casein	HIQKEDVPSERYLGYLEQLLRLK	6/6	6037.7	1/6	115.9	0.0	down
35775	CASB	Beta-casein	LSLSQSKVLPVPQKAVPYPQRDMPIQA	1/6	5.9	6/6	1353.1	230.1	up
36371	CASB	Beta-casein	SLSQSKVLPVPQKAVPYPQRDMPIQAF	0	0	6/6	2161.4	2161.4	up
44033	CASK	Kappa-casein	TMARHPHPHLSFMAIPPKKNQDKTEIPTINT	0	0	6/6	1822.2	1822.2	up
44930	TRBP2	RISC-loading complex subunit TARBP2	GWRLPEYTVTQESGPAHRKEFTMTCRVERF	0	0	6/6	12223.3	12223.3	up
1217454	CASA1	Alpha-S1-casein	FPEVFGKEKV	1/6	4.0	6/6	3105.0	770.5	up

Legends to Figures

Figure 1: (A) One dimensional gel showing high abundance proteins from a mammary quarter challenged with *Streptococcus uberis* (panel A) (from left to right: size marker with band size in kDa, 0, 6, 12, 18, 24, 30, 36, 42, 48, 57, 72, 81, 96, 120, 144, 68, 192, 240 and 312 hours post challenge). Proteins were identified through comparison with results from reference samples shown in Figure 1B, with the main proteins shown here: LF = lactoferrin; Alb = albumin, Ig = Immunoglobulin; CN = casein; LG = lactoglobulin; LA = lactalbumin

(B) One dimensional gel showing (left to right) high abundance proteins from a healthy mammary quarter (a), high abundance proteins from a quarter with clinical mastitis of unknown etiology (b), and size marker with band sizes in kDa (c). Based on LC-MS/MS analysis (Table 1), bands were identified as 1. IgG heavy chain and ceruloplasmin; 2 lactoferrin, lactotransferrin precursor and serotransferrin precursor; 3. albumin and complement C3; 4. Ig heavy chain precursor and IgG heavy chain constant region; 5. Ig heavy chain precursor and light chain, alpha-S1-casein; 6. immunoglobulin lambda like polypeptide and light chain; 7. alpha-S1-casein and beta-lactoglobulin; 8. beta casein and component PP3; 9. beta-lactoglobulin and alpha-S1-casein; 10. beta-lactoglobulin; ;11. alpha- and beta-lactoglobulin.

Figure 2: Haptoglobin concentration in bovine mammary quarters challenged with *Streptococcus uberis* (infected, n=6) or mock challenged with phosphate buffered saline (controls, n=6). Results show median (A) and individual (B) concentrations.

Figure 3: Mammary associated SAA3 concentration in bovine mammary quarters challenged with *Streptococcus uberis* (infected, n=6) or mock challenged with phosphate buffered saline (controls, n=6). Results show median (A) and individual (B) concentrations.

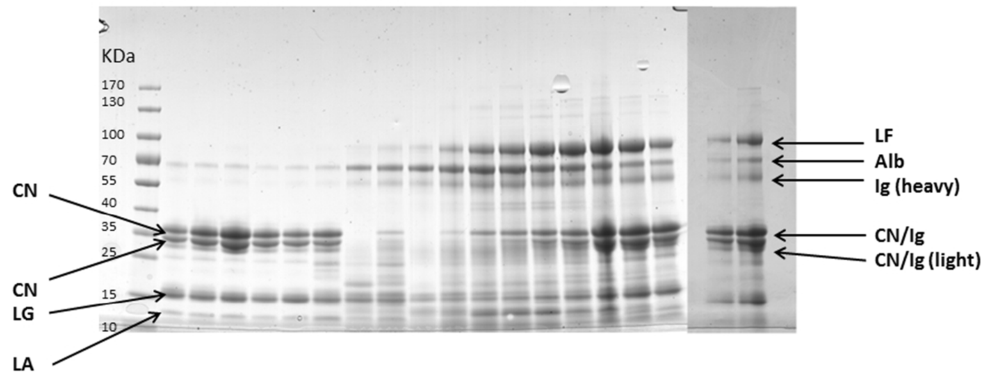
Figure 4: C-Reactive protein concentration in bovine mammary quarters challenged with *Streptococcus uberis* (infected, n=6) or mock challenged with phosphate buffered saline (controls, n=6). Results show median (A) and individual (B) concentrations.

Figure 5: Peptides detected in milk fluid and differences between non-infected cows (0 h PC) and infected (36, 42, 57 and 81 h PC). Representation of the up-regulated (left panel) and down-regulated (right panel) peptides analysed by CE-MS. Each peptide was identified by a unique identifier based on the migration time (min) and specific mass (kDa), with a peak height representing the relative abundance.

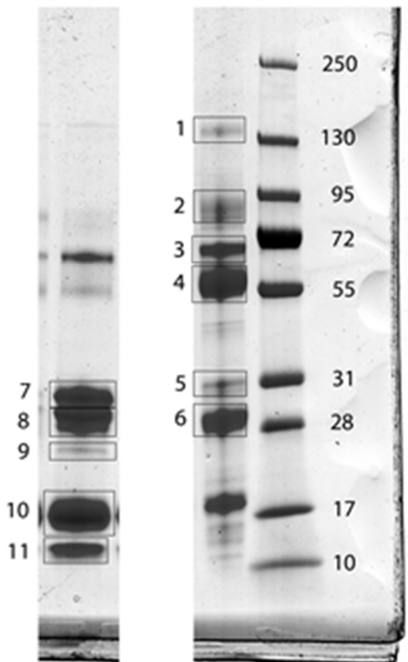
Figure 6: Performance of the classifier in the discovery cohort (0 and 81 hours) and progression of infection (36 h, 42 h, 57 h, 312 h PC). Box whisker plot according to the IMI77 score showing median, 10th, 25th, 75th and 90th percentiles.

Figure 7 The relative responses of analytes following experimental infections with *S.uberis* combining results from this investigation and those described by Tassi et al [4]. The shading represents increasing responses in relation to the peak response and represents 25%, 50%, 75% and 100% of peak response on days PC. Responses were increased from the day 0 levels except where indicated by * which were decreases with respect to the day 0 level.

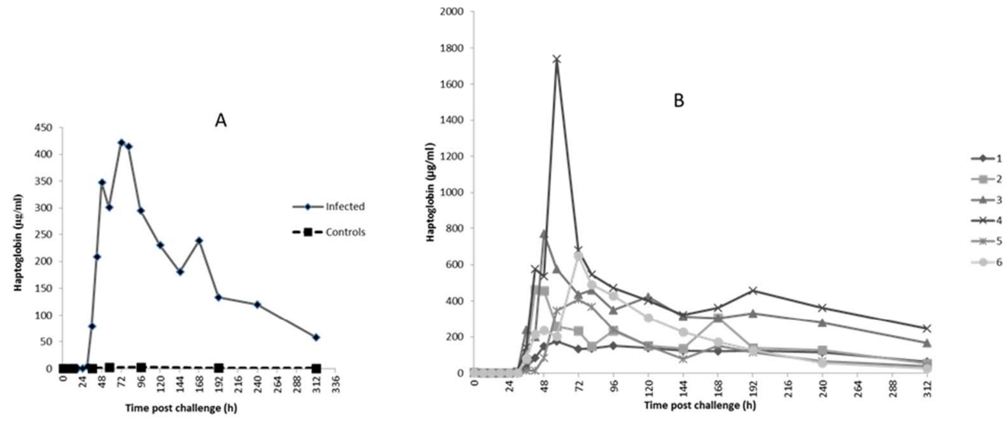
Figure 8: Course of infection in challenged cows (n=6) as indicated by average body temperature and average bacterial count in milk. Number of culture positive quarters ranged from six at 18 to 72, 105 and 129 hrs post challenge to one at 312 hrs post challenge[4]. Vertical lines indicate time points for which peptidomic analysis was conducted. normally define IMI based on the presence of bacteria in milk samples, whereby three consecutive negative samples are needed to declare an animal free of IMI



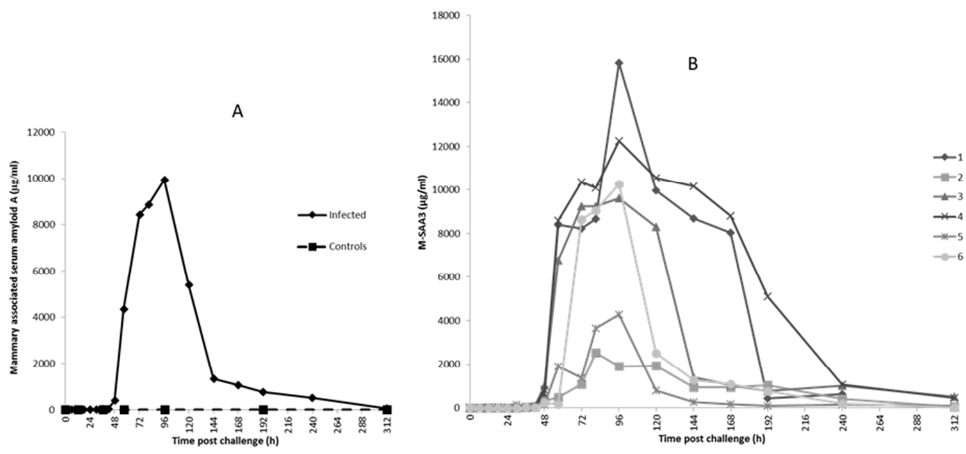
238x106mm (96 x 96 DPI)



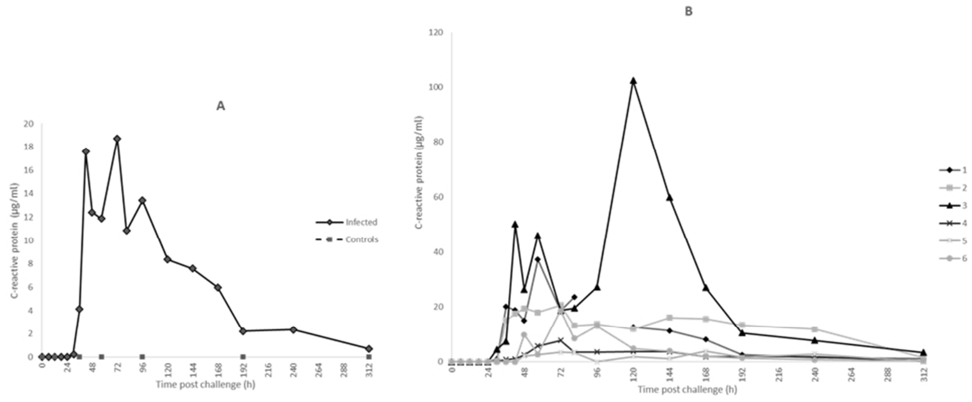
62x114mm (96 x 96 DPI)



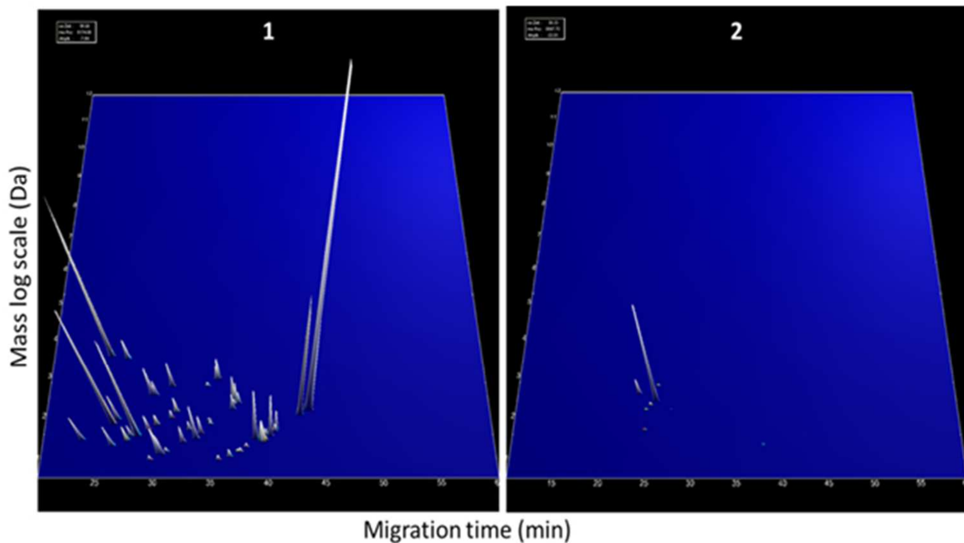
254x106mm (96 x 96 DPI)



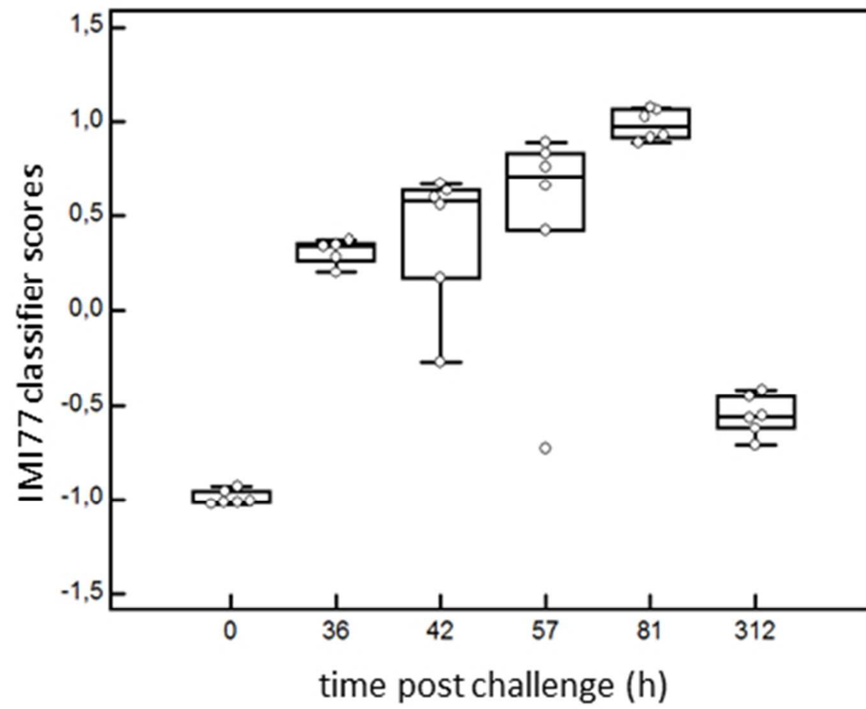
254x117mm (96 x 96 DPI)



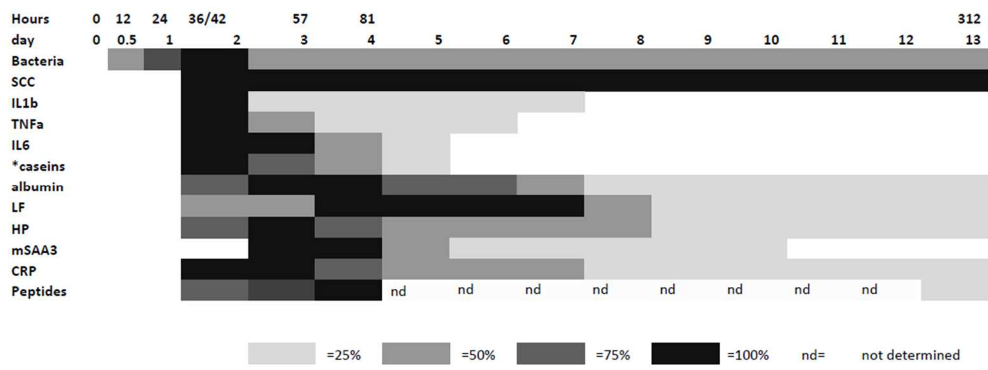
263x116mm (96 x 96 DPI)



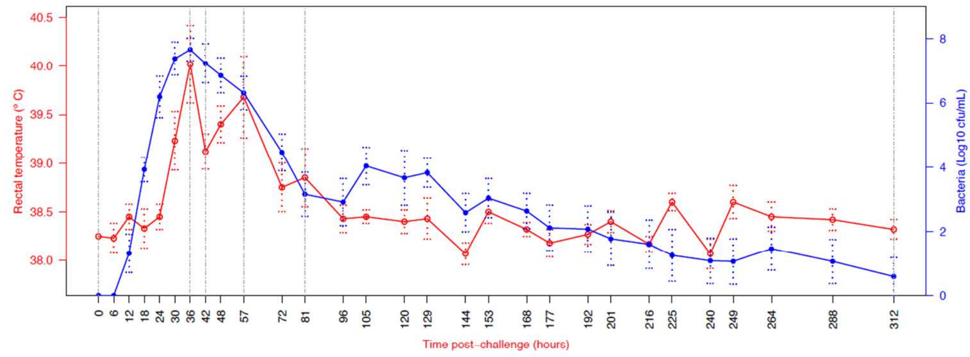
171x96mm (96 x 96 DPI)



126x99mm (96 x 96 DPI)



279x105mm (96 x 96 DPI)



338x190mm (96 x 96 DPI)