Developments in Immunoassay and Omic analysis for Biomarkers of Innate Immunity in Dairy Cows

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Institute of Biodiversity, Animal Health and Comparative Medicine
University of Glasgow
• **Biomarkers of Innate Immunity and Mastitis**
  – Acute phase proteins

• **MASTITOMICS**
  – Integrated omic investigation of mastitis, starting with milk
  – Peptidome – peptides <25 kDa
  – Proteome – quantitative analysis of milk protein
  – Metabolome – quantitative analysis of milk metabolites

• **Immunooassays for biomarkers of innate immunity**
  – Immunoturbidimetric assay of bovine haptoglobin in serum
  – On-farm diagnostic immunoassay for bovine milk haptoglobin
Acute phase proteins in serum and milk from dairy cows with clinical mastitis

APP in Milk: *Staphylococcus aureus* mastitis

Somatic Cell Count

- **RH infected**
- **LH non-infected**
- **RH & LH control cows**

Innate immune genes in mastitis

Real time PCR on genes from the innate immune response in mammary gland alveolar tissue after infection with S. aureus

Whelehan et al Vet Imm Immunopath 140 (2011) 181-189
APP in mammary gland during mastitis

Secretion of APP and somatic cells into milk

Somatic cells

bacteria

IL-6

TNF

M-SAA

Hp

Alb
SAA
Hp?

to liver

mammary epithelium
Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 3. Untargeted metabolomics

Funmilola Clara Thomas,†a Manikhandan Mudaliar,†a,b Riccardo Tassi,†c Tom N. McNeilly,†c Richard Burchmore,†d Karl Burgess,†d Pawel Herzyk,†e Ruth N. Zadoks†c and P. David Eckersall*†
- Six healthy Holstein cows, one quarter infected *Strep uberis* FSLZ1-048
- Milk samples taken at 19 timepoints, 0 to 312 hours for bacteriological analysis, somatic cell count & cytokine and acute phase protein analysis
- Selected time points (*0, 36, 42, 57, 81 & 312 hours* post-challenge) analysed for:
  - Peptidomic biomarkers
  - Label-free quantitative proteomic analysis
  - Quantitative metabolomic analysis
  - Collaboration with Moredun Research Institute

Bacterial Count, Rectal Temp & SCC

Cytokine responses to the *Strep uberis* infection

One dimensional electrophoresis SDS-PAGE of milk post *Strep uberis* challenge
Acute phase proteins

Haptoglobin

Mammary associated SAA3
Milk Peptidomics by CE-MS

Sample prepared by ultrafiltration
Protein >25kDa removed
Peptide biomarker panel: *S. uberis* mastitis

Peptides <25 kDa separated by ultra filtration

Capillary electrophoresis: P/ACE MDQ CE (Beckman Coulter)

Mass spectrometry: electrospray microTOF (Agilent/Brucker)

77 peptides showed differences between pre-challenge and all post challenge samples

MosaiquesVisu software for de-convolution

Intra mammary infection 77 peptide classifier score (IMI77) versus time post challenge

Peak of peptides at 81h post challenge

Most peptides are casein degradation product

Also, glycosylation dependent adhesion molecule 1, β-lactoglobulin, serum amyloid A
Molecular Pathophysiology of Mastitis

* Decrease in caseins
- Label free relative quantitative proteomics
- Changes related to pre *Strep uberis* challenge
- Ultracentrifugation 150,000g, 60 min, 4°C to remove caseins
- Dionex UltiMate 3000 RSLCnano liquid chromatography system
- Thermo Scientific Orbitrap Elite mass spectrometer
- MaxQuant software with Andromeda search engine used for protein identification and quantification
- Hierarchical clustering, principal component analysis & Ingenuity pathway analysis performed
Quantitative proteomics (relative to pre-challenge)

The 15 proteins with the highest fold increases at different times:

- **36 h post challenge**
  - Up: Q8SP7, Peptidoglycan recognition protein 1
  - Up: PS4229, Cathelicidin-5
  - Up: PS4229, Cathelicidin-7
  - Up: PS4229, Cathelicidin-1
  - Up: Q2TB00, Haptoglobin
  - Up: F1N463, Uncharacterized protein GN=KBTBD8
  - Up: E1BC6, Uncharacterized protein GN=TCN1
  - Up: Q9TU03, Rho GDP-dissociation inhibitor 2
  - Up: P52176, Matrix metalloproteinase-9
  - Up: P30346, Cathelicidin-4
  - Up: PQ5CQ, Pentraxin-related protein PTX3
  - Up: Q58CQ, Painethasine
  - Up: Q3MXK, Uncharacterized protein (Fragment)
  - Up: Q2BO05, Complement factor H
  - Up: Q3ZV7, Hemozoin

- **81 h post challenge**
  - Up: Q2TB00, Haptoglobin
  - Up: PS4229, Cathelicidin-5
  - Up: PS4229, Cathelicidin-7
  - Up: P22226, Cathelicidin-1
  - Up: Q3M0, Uncharacterized protein GN=PTX3
  - Up: PS4229, Cathelicidin-5
  - Up: E1BC6, Uncharacterized protein GN=KBTBD8
  - Up: Q2BO05, Complement factor H
  - Up: Q3ZV7, Hemozoin

- **57 h post challenge**
  - Up: Q8SP7, Peptidoglycan recognition protein 1
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  - Up: PS4229, Cathelicidin-7
  - Up: PS4229, Cathelicidin-1
  - Up: P22226, Cathelicidin-1
  - Up: P30346, Cathelicidin-4
  - Up: Q9TU03, Rho GDP-dissociation inhibitor 2
  - Up: F1N1F8, Uncharacterized protein GN=PTX3
  - Up: F1MY5, Uncharacterized protein GN=PTX3
  - Up: Q3ZC8, Dipeptidyl peptidase 1
  - Up: P02584, Profilin-1
  - Up: P48616, Vimentin
  - Up: P19660, Cathelicidin-2
  - Up: E1BI6, Uncharacterized protein GN=PTX3
  - Up: A5PJH7, LOC78112 protein GN=LOC78112

- **312 h post challenge**
  - Up: Q2TB00, Haptoglobin
  - Up: PS4229, Cathelicidin-5
  - Up: PS4229, Cathelicidin-7
  - Up: PS4229, Cathelicidin-1
  - Up: PS4229, Cathelicidin-5
  - Up: E1BI6, Uncharacterized protein GN=PTX3
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  - Up: E1BI6, Uncharacterized protein GN=PTX3
  - Up: A5PJH7, LOC78112 protein GN=LOC78112

Log2 Fold Change and P-value for each protein at each time point.
5 Most Up-regulated

- Cathelicidins
- Peptidoglycan recognition prot.
- LPS-binding protein
- Serum amyloid A protein
- Haptoglobin

5 Most Down-regulated

- Alpha lactalbumin
- Xanthine oxidase
- Butyrophilin subfamily 1
- Beta-1,4-galactosyltransferase
- Calcium-binding protein
2553 non redundant bovine peptides identified

562 bovine proteins quantified

3 major clusters by time:

57 & 81 h - peak response
36 & 42 h - early response
0 & 312 h - pre-challenge and resolution phase

By protein:

A: present in all samples
B: present at peak response at 57 & 81 h
Principal Component Analysis

PCA based on 562 proteins generated using the Partek Genomic suite.

Each spot = one cow per time

0 hrs pre-challenge (white),
36 hrs PC (dark blue),
42 hrs PC (teal),
57 hrs PC (green),
81 hrs PC (red)
312 hrs PC (maroon).
- 57 hours post challenge
- Highly significant proteins identified using repeated measures ANOVA
- Ingenuity Pathway Analysis (IPA)
- Increases in
- Acute phase pathways
- LXR = liver receptor pathways
- RXR = retinoic acid receptor pathways
- FXR = farenoid (bile acid) receptor pathway
## Mastitomics 2: quantitative proteomics

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<sup>a</sup> Data provided by [University of Glasgow](https://www.gla.ac.uk).
Reviews of (Farm) Animal Proteomics

Journal of Proteomics Special Issue No 14 2012 Vol 75
- Acute phase in ruminants
- Pig proteomics
- Milk proteomics
- Aquaculture & proteomics
- Meat processing

Proteomics in Veterinary Medicine: Applications and Trends in Disease Pathogenesis and Diagnostics
Ceciliani F et al Vet Pathol 2014 51:351

Animal board invited review: advances in proteomics for animal and food sciences
A. M. Almeida1,2,3,4, A. Bassols5, E. Bendixen6, M. Bhide7, F. Ceciliani8, S. Cristobal9,10, P. D. Eckersall11, K. Hollung12, F. Lisacek13, G. Mazzucchelli14, M. McLaughlin15, I. Miller16, J. E. Nally17, J. Plowman18, J. Renaut19, P. Rodrigues20, P. Roncada21, J. Staric22 and R. Turk23


Molecular BioSystems Themed Issue 2016 Vol 12
Omics in Animal Sciences:
- Proteomics and pathogens
- Bovine milk microbiota
- Vaccine candidates & vector borne disease
- Mastitomics

COST Action FA1002 Farm Animal Proteomics
www.cost-faproteomics.org

COST European Cooperation in Science and Technology
• Samples from *Strep uberis* experimental infection
• Chloroform/methanol extraction
• Dionex UltiMate 3000 RSLCnano liquid chromatography system
• Thermo Scientific Exactive 80 Orbitrap mass spectrometer
• IDEOM 20 software package (version 95 18)
• Metabolites exported from IDEOM to Pathos 28 and iPath 29 for web-based metabolomics
• >3000 peaks
• ~600 metabolites identified
• Most change at 81 h post challenge
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Red for increase; Blue for decrease
Pathos
A metabolomics tool from Glasgow Polyomics

Upload File  Analyse  Feedback  Instructions

Organism: [All Organisms]  Run

Base Condition:  Mean: Time0  Experimental Condition:  Mean: Time01
Cut-offs for colour-flagging:  10.0 x  6.0 x  2.0 x  0.5 x  0.2 x  0.1 x

File: 'pathos_KEGGlist.txt'
Kegg Maps for All Organisms with compounds from input list

KEY  hide
• View metabolites found for a particular map (or all maps) — toggles list on and off.
• Link to a graph of results for a particular metabolite. Change indication scale — [increase G G G G G G G G, decrease]

Arginine and proline metabolism: 17 metabolites out of 67 (15 changed) ✓
- (S)-1-Pyrroline-5-carboxylate CS7H7N2
- 1-Pyrroline-4-hydroxy-2-carboxylate CS7H7N3
- 2,5-Dioxopentanoate CS8H6O4
- 4-Aminobutyraldehyde C4H9NO
- Creatine C4H9N3O2
- Creatinine C4H7N3O
- D-Proline CS9H9N2
- L-1-Pyrroline-3-hydroxy-5-carboxylate CS7H7N3
- L-Arginine C6H14N4O2
- L-Glutamate CS7H9N4
- L-Glutamate 5-semialdehyde CS9H9N3
- L-Osmibine CS12H12N2O2
- L-Proline CS11H12N2O2
- N-Acetylputrescine C6H14N2O
- N-Carboxyanilsorosamine C4H8N2O3
- N2-Succinyl-L-ornithine CS18H18N2O5
- Phosphocreatine C4H10N3O5P

Generate map of Arginine and proline metabolism highlighting potential metabolites.
**Biosynthesis of unsaturated fatty acids:** 12 metabolites out of 49 (10 changed)

- (13Z)-Docosenoic acid C22H42O2
- (15Z)-Tetracosenoic acid C24H46O2
- (9Z,11Z,14Z)-Icosatetraenoic acid C20H32O2
- (9Z)-Octadecenoic acid C18H34O2
- Docosanoic acid C22H44O2
- Hexadecanoic acid C16H32O2
- Icosadienoic acid C22H36O2
- Icosanoic acid C20H34O2
- Linoleate C18:2
- Octadecanoic acid C18H36O2
- Tetracosanoic acid C24H48O2

**Generate map of Glycerophospholipid metabolism** highlighting potential metabolites.

**Glycerophospholipid metabolism:** 6 metabolites out of 18 (6 changed)

- Choline C5H14NO
- Choline phosphate C5H15NO4P
- Ethanolamine phosphate C2H8NO4P
- sn-Glycerol 3-phosphate C3H9O6P
- sn-glycero-3-Phosphocholine C8H21NO6P
- sn-glycero-3-Phosphoethanolamine C5H14NO6P

**Generate map of Glycerophospholipid metabolism** highlighting potential metabolites.

- C27H48O4
- 3alpha,7alpha,12alpha-Trihydroxy-5beta-cholanate C24H40O5
- Chenodeoxycholate C24H40O4
- Glycocholate C26H43NO6
- Taurine C2H7NO3S
- Taurocholate C26H45NO6S
- Taurochenodeoxycholate C26H45NO6S

**Generate map of Primary bile acid biosynthesis** highlighting potential metabolites.
Metabolomics of mastitic milk

Increased bile acid metabolites in mastitis
• **Decreases in**
  – Carbohydrate metabolites,
  – Nucleic acid metabolites
  – Lactose
  – Hippurate

• **Increases in**
  – Amino acid, di, tri-peptides
  – Lipid metabolites
  – Bile acid metabolites
  – Lactate
### Molecular Pathophysiology of Mastitis

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**Colour code**

- nd = not determined
- 25% = light grey
- 50% = medium grey
- 75% = dark grey
- 100% = black

*Note: The table represents the expression levels of various biomarkers over different hours and days post infection.*
• Bacterial count & temperature reach peak at 36/42 h before most analytes
• SCC remain elevated while APP, peptides & proteins decline with bacteria
• APP peak at 57 and 81 h
• Peptide biomarkers peak at 81 h
• Early responding anti microbial cathelicidins peak at 57 h
• Haptoglobin highest fold increase; at 81 h
• Increased lipid and nitrogen metabolism
• Decreased carbohydrate & nucleic acid metabolism
• Metabolomics & proteomics indicate a bile acid response to mastitis
Ab-Ag complexes form and precipitate out of solution. Absorbance increases.

In spectrophotometer:
Absorbance increases at 340nm.
AN IMMUNOTURBIDIMETRIC ASSAY FOR CANINE C-REACTIVE PROTEIN

P.D. ECKERSALL, J.G. CONNER* AND J. HARVIE

- Affinity chromatography of canine CRP
- Raise antiserum in sheep
- Absorb out non-specific antibody
- Antibody + antigen → precipitate
- Quantify on biochemical analyser
• Sheep antibody raised to bovine Hp (requires large volume for high throughput analysis)

• IT assay developed & validated for bovine Hp

1 = pure bHp
2-5 = serum with Hp >0.1g/L
6 = serum with Hp <0.01 g/L

Standard curve

Correlation to established method (haemoglobin binding)
Acknowledgements

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• Mani Mudaliar
• Ruth Zadoks
• Tom McNeilly
• Richard Burchmore
• Bill Mullen
• Emily O’Reilly
• Nicola Brady

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• EU COST Action in Farm Animal Proteomics
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• Wellcome Trust
• Moredun Research Institute
• BioMar Ltd & Marine Harvest (Scotland)
• Aviagen

[Logos of BBSRC, Wellcome Trust, EU COST, and Glasgow Polyomics]