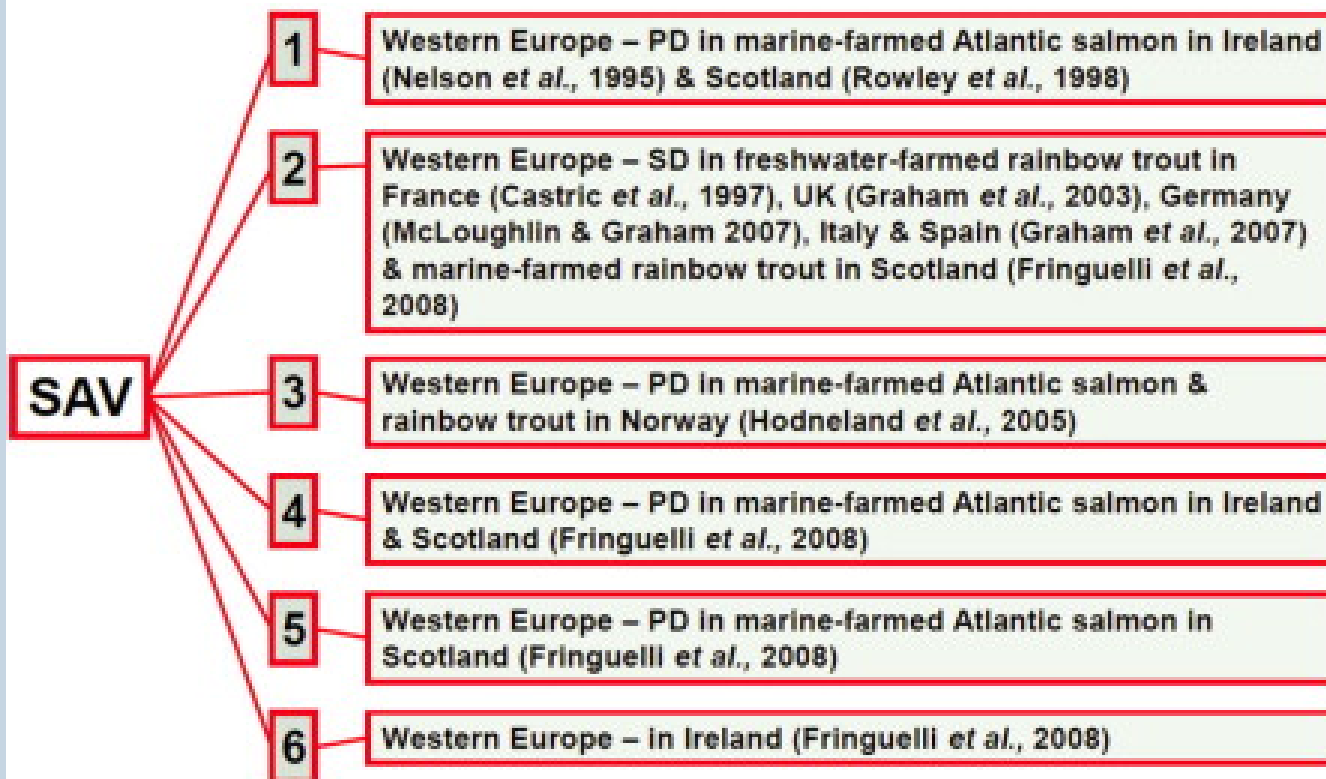# Analysis of the Serum Proteome of Atlantic salmon, *Salmo salar*, During Pancreas disease (PD)

Mark Braceland, Ralph Bickerdike, David Cockerill, Marian McLoughlin, Richard Burchmore, Phillip Cash, Mark McLaughlin, Peter David Eckersall, John Tinsley

University of Glasgow

- **Background**

- Salmonid Alphavirus (SAV)

6 subtypes of SAV & their geographic location & type of fish

SAV		
1	Western Europe – PD in marine-farmed Atlantic salmon in Ireland (Nelson et al., 1995) & Scotland (Rowley et al., 1998)	
2	Western Europe – SD in freshwater-farmed rainbow trout in France (Castric et al., 1997), UK (Graham et al., 2003), Germany (McLoughlin & Graham 2007), Italy & Spain (Graham et al., 2007) & marine-farmed rainbow trout in Scotland (Fringuelli et al., 2008)	
3	Western Europe – PD in marine-farmed Atlantic salmon & rainbow trout in Norway (Hodneland et al., 2005)	
4	Western Europe – PD in marine-farmed Atlantic salmon in Ireland & Scotland (Fringuelli et al., 2008)	
5	Western Europe – PD in marine-farmed Atlantic salmon in Scotland (Fringuelli et al., 2008)	
6	Western Europe – in Ireland (Fringuelli et al., 2008)	

(Kibenge et. al., 2012)

• **Background**

– Economic impact

Larsson et. al., 2011

- **Current Diagnostic Tools and Associated Problems**

– Virus specific RT-PCR and histopathology: Destructive

– Pathogen specific antibody detection: Slower synthesis of Igs in fish (temp. dependent), difficult to quantify disease stage
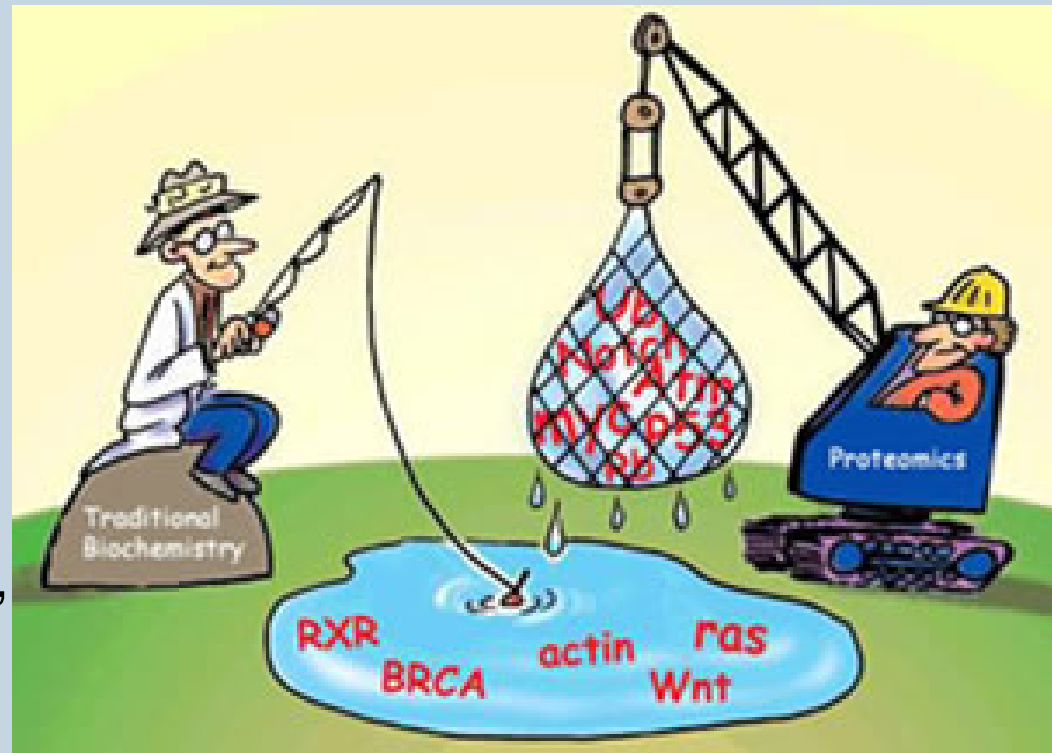
- **Aims**

–   To characterize the changing serum proteome of Atlantic salmon during PD

–   Attain a clearer understanding why these changes occur (pathology or immune response)
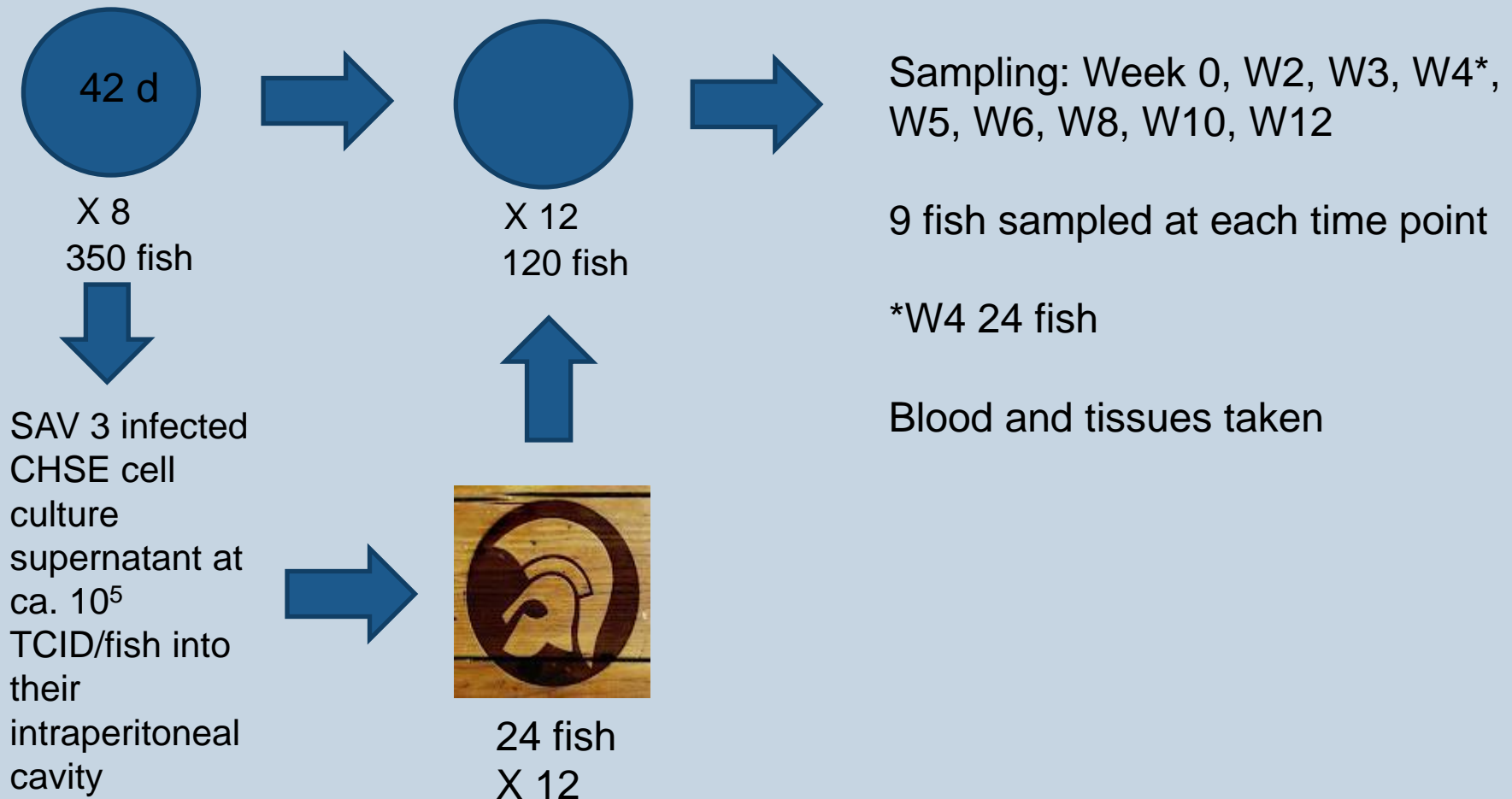
–   Use within the field

Establish humoral Biomarkers of PD

• **Why Proteomics?**

– Functional molecules are being studied i.e. 'end product'

– Post translational modifications that cannot be predicted from linear genetics

– Strong gene expression = abundant mRNA

(does not necessarily correspond to abundant protein, active nor that this protein shall be secreted)

Experimental Model

42 d

X 8
350 fish

SAV 3 infected CHSE cell culture supernatant at ca. 10^5 TCID/fish into their intraperitoneal cavity

X 12
120 fish

24 fish
X 12

Sampling: Week 0, W2, W3, W4*, W5, W6, W8, W10, W12

9 fish sampled at each time point

*W4 24 fish

Blood and tissues taken

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- **Proteomics**

– Pools made from each sampling point using each tank (Cohabitation trial)

– Gels ran in triplicate

– 2DE carried out: pI 3-10 and SDS PAGE (BioRad)

– SameSpots power value of >0.8 and ANNOVA significance score of <0.05 to ensure reproducibility

– Protein spots identified via ion trap mass-spectrometry and comparison to the MASCOT protein database
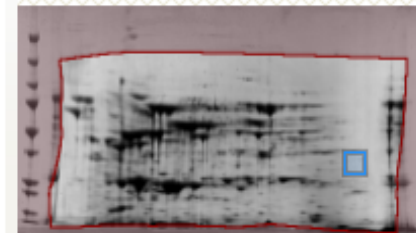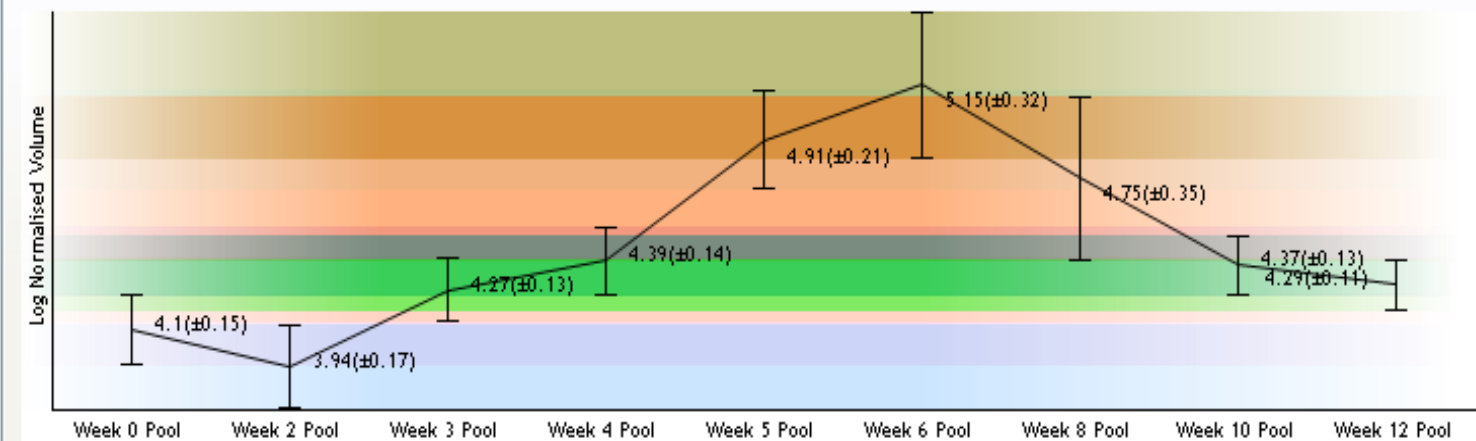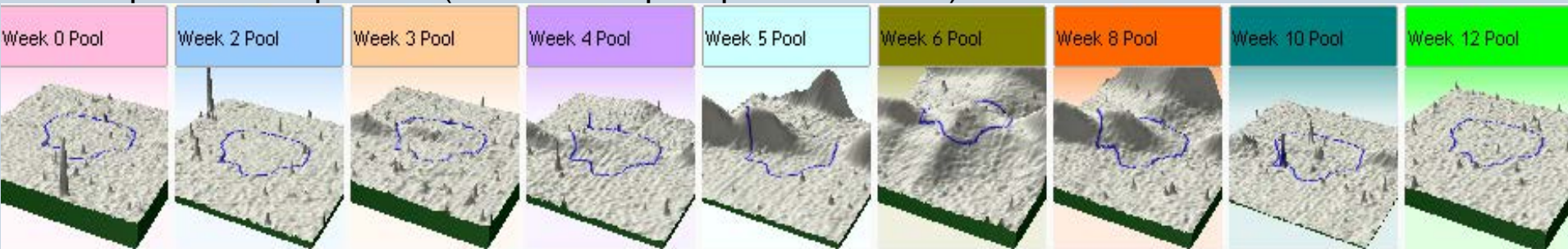
W0
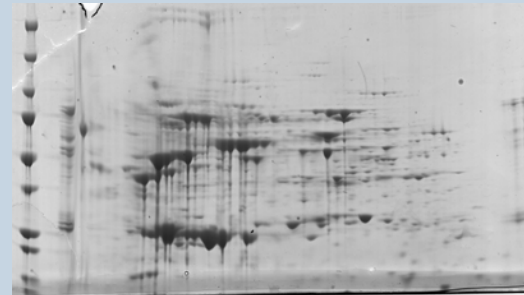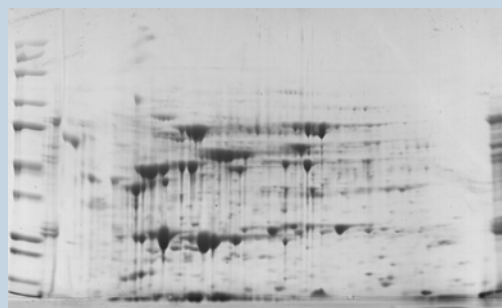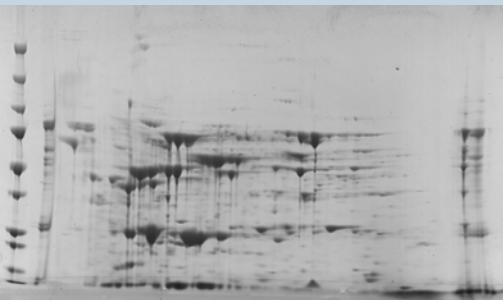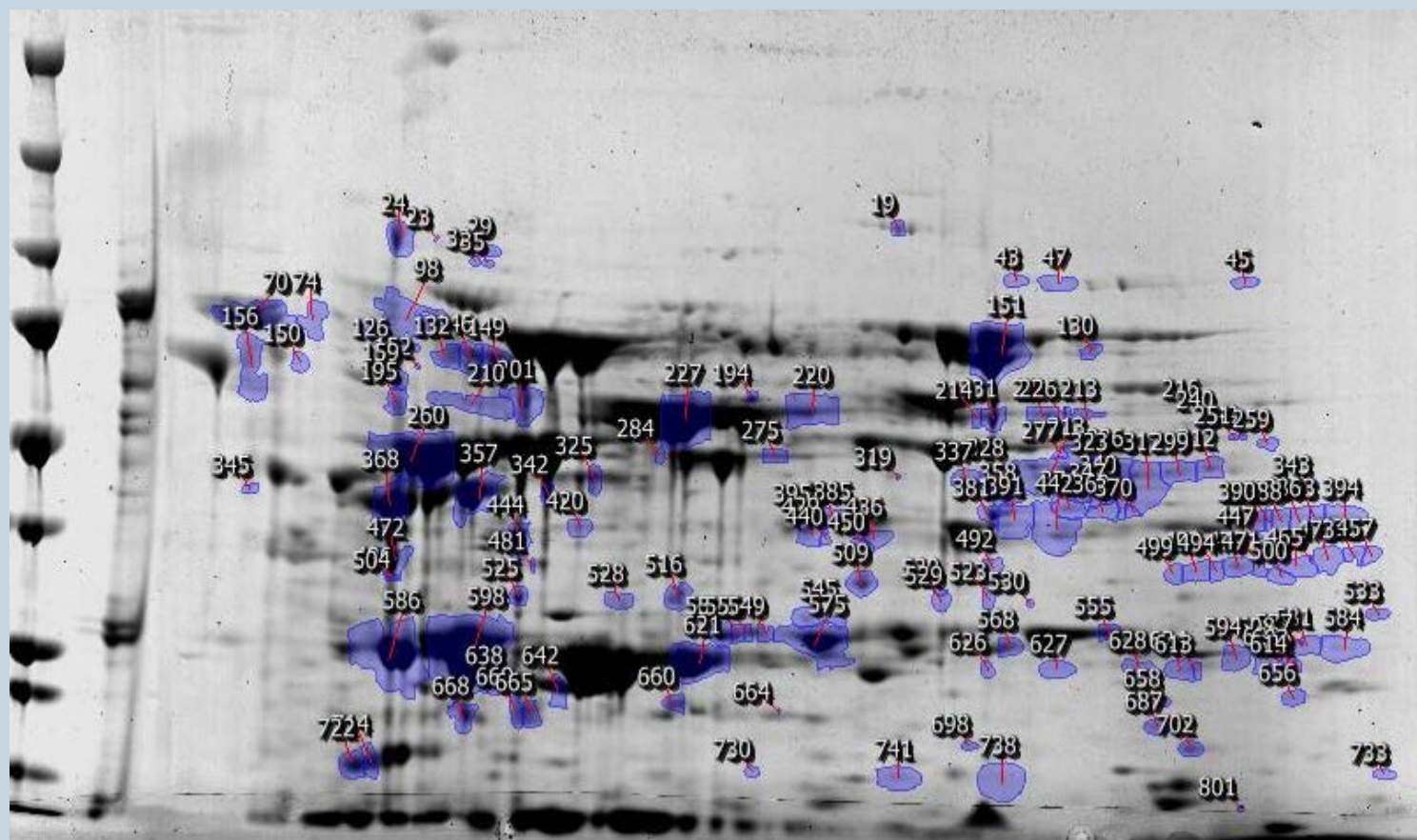
- **Progenesis Same Spots (Nonlinear dynamics, Newcastle U.K.)**

In total >800 spots were identified:
-Statistical analysis + Manually reviewed = 72 differentially expressed (Power >0.8, ANNOVA <0.05)

Example shown spot 473 (Fructose-bisphosphate aldolase):

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- **Novel proteomic analysis via ArrayStar**

– Arraystar heat map hierarchical clustering of spot intensities

– K-means un-centred Pearson

– Shows relationship between spots and proteome as a whole throughout PD,

– No need for manually looking through each expression profile

Expression level: 9 — 21

Columns (left to right): 0, 2, 3, 4, 12, 5, 6, 8, 10

Row labels (top to bottom):
494 Glyceraldehyde 3-phosphate dehydrogenase
500 Aldolase A
463 Glyceraldehyde 3-phosphate dehydrogenase
499 Proteasome
529 Alpha-2 enolase-1
493 Glyceraldehyde 3-phosphate dehydrogenase
623 Triosephosphate isomerase + Ig light ch. precursor
440 Alpha-2 enolase-1
602 Serotransferrin II + Serum albumin 2 precursor
626 Immunoglobulin light chain precursor
613 serum albumin 2
43 Serotransferrin II
45 Complement factor H
687 Serotransferrin II
656 Alpha-2 enolase-1
669 Aldolase
450 Serotransferrin II precursor
702 Prostaglandine D synthase
395 Serotransferrin II precursor
19 Serotransferrin II + Ig heavy chain C region
556 Transferrin + Immunoglobulin light chain
150 Hemopexin-like protein
360 Alpha-2 enolase-1
628 Triosephosphate isomerase
545 Serotransferrin II
624 Triosephosphate isomerase
381 Alpha-2 enolase-1 + creatine kinase
224 Hemopexin-like protein
74 No sig.
47 Alpha-2 enolase-1 + Complement factor H
627 Triosephosphate isomerase + Ig light ch. constant region
473 Fructose-bisphosphate aldolase
386 Aldolase
477 Fructose-bisphosphate aldolase- like isotype
393 Glyceraldehyde 3-phosphate dehydrogenase
479 Glyceraldehyde-3-phosphate dehydrogenase
388 Aldolase
668 apolipoprotein A-I
509 Serotransferrin II precursor
342 Serotransferrin II
565 Hemopexin-like protein
568 Transferrin
391 Muscle-type creatine kinase CKM1
738 Putative oncoprotein nm23 + Complement factor H
328 Alpha-2 enolase-1
299 Enolase
741 Nucleoside diphosphate kinase
584 Malate dehydrogenase 1a
394 Similar to aldolase a, fructose-bisphosphate, b
642 apolipoprotein A-I
146 Complement component C9
201 Antithrombin + Complement component C9
472 serum albumin 2 + Creatine kinase
444 Complement C3-1 + Cystein proteinase inhibitor
317 Enolase 1,
442 Triosephosphate isomerase
220 Hemopexin-like protein + Beta-actin
326 Alpha-2 enolase-1
98 apolipoprotein A-I precursor
313 Pyruvate kinase
357 serum albumin 2 + Complement factor B/C2-B
368 serum albumin 2 + serum albumin 1
575 apolipoprotein A-I + Ig light ch. precursor
586 apolipoprotein A-I
151 transferrin + Ig heavy chain C region
227 Hemopexin-like protein
260 serum albumin 2 + serum albumin 1
598 apolipoprotein A-I
249 Serotransferrin II
32 C1 inhibitor

Can we differentiate between biomarkers of tissue pathology and immune response?

University of Glasgow
VIA VERITAS VITA

Histopathology

Pancreatic lesions were classified as:

Score	Description
0	Normal appearance.
1	Focal pancreatic acinar cell necrosis
2	Significant multifocal necrosis/atrophy of pancreatic acinar tissue, plus some remnants remaining.
3	Total absence of pancreatic acinar tissue
R	Recovery pancreas

Heart lesions were classified as:

Score	Description
0	Normal appearance.
1	Focal myocardial degeneration ± inflammation [<50 fibres affected]
2	Multifocal myocardial degeneration ± inflammation [50-100 fibres affected]
3	Severe diffuse myocardial degeneration ± inflammation [>100 fibres affected]
R	Repair

Red and white skeletal muscle lesions were classified as:

Score	Description
0	Normal appearance.
1	Focal myocytic degeneration ± inflammation
2	Multifocal myocytic degeneration ± inflammation
3	Severe diffuse myocytic degeneration ± inflammation
R	Repair

(McLoughlin et al., 2006, Christie et al. 2007)

Histopathology
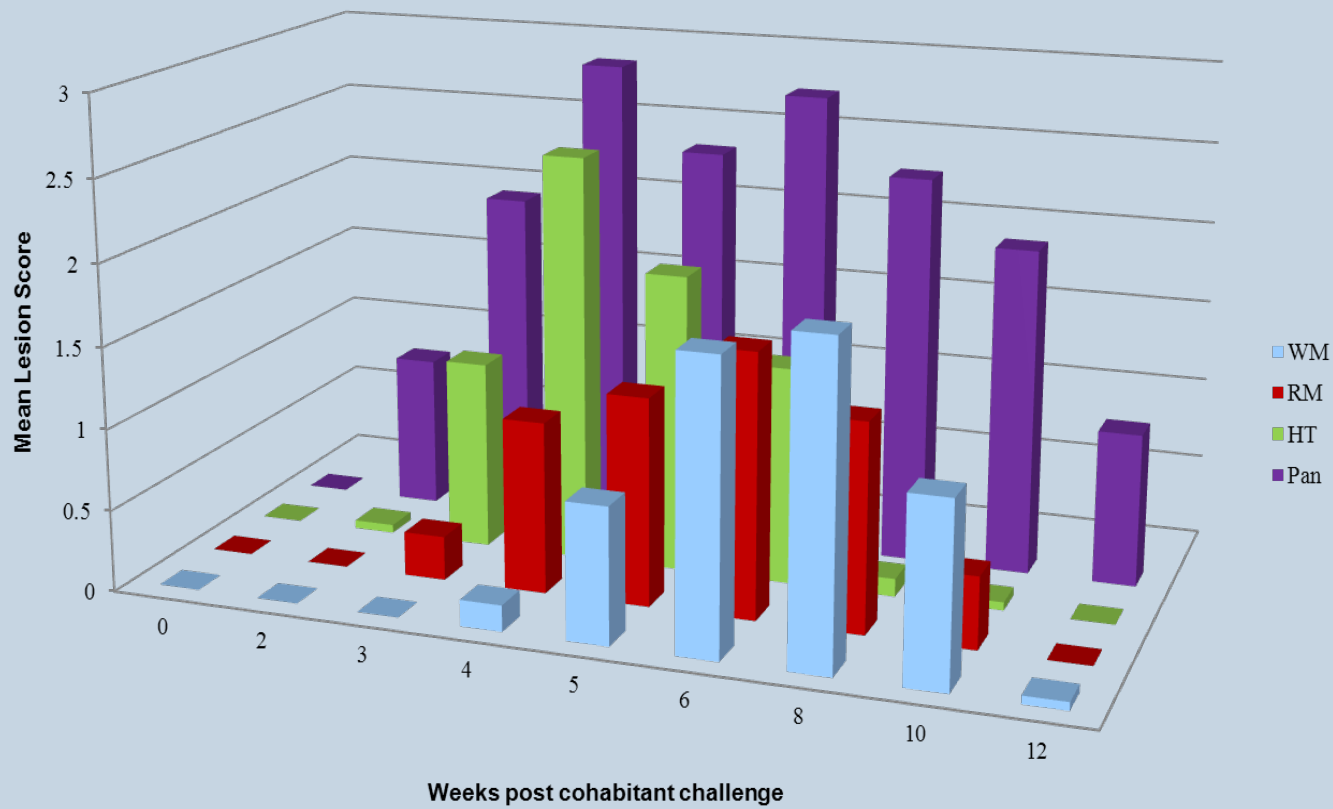
WM= White muscle, RM= Red muscle, HT= Heart. Pan= Pancreas

- **Statistical Analysis Software (SAS)** regression GLM analysis of protein expression profiles (SameSpots) and their relationship with tissue pathologies

- **A number of advantages**

– When gene ontology is not clearly defined and does not take into account multiple functions

– May give insight into new isoforms of specific tissue injury (although proteomics, of this type, cannot definitively prove this but may lead to further work)

| Spot #	Identification	Probability Histo.				Fold	Estimated pI	Estimated MW
		Pancreas	Heart	Red Muscle	White Muscle			(kD)
494	Glyceraldehyde3-phosphate dehydrogenase(Q90ZF1)	0.271	0.8078	0.132	0.2332	15.3	8.2	33
360	Alpha-2enolase-1(Q9DDG6)	0.1997	0.7136	0.047	0.0002	15.3	7.77	41
317	Enolase1,(Alpha)(Q6GMI7)	0.1688	0.6918	0.0963	0.0042	7.6	7.94	45
628	Triosephosphateisomerase(Q70I40)	0.0082	0.3003	0.0001	0.0047	3.9	7.85	23
473	Fructose-bisphosphate aldolase(Q4RVI9)	0.1771	0.8644	0.0292	0.0336	16.9	8.81	34
150	Hemopexin-likeprotein(P79825)	0.872	0.3269	0.7833	0.804	9.6	4.22	68
326	Alpha-2enolase-1(Q9DDG6)	0.1489	0.6904	0.0853	0.0061	5.6	7.78	45
391	Muscle-typecreatinekinaseCKM1(Q8JH39)	0.0462	0.7088	0.004	0.0009	5.1	7.35	38
386	Aldolase(Q804Y1)	0.1753	0.911	0.0229	0.0204	13.8	8.72	38
393	Glyceraldehyde3-phosphate dehydrogenase(O42259)	0.1131	0.9435	0.0028	0.0002	15.7	8.79	38
623	Triosephosphateisomerase+ Ig light chain(Q70I40+AAG18369)	0.1508	0.5987	0.0971	0.0068	3.4	8.57	23
500	AldolaseA(Q8JH72)	0.1836	0.7625	0.039	0.0693	15.4	8.58	32
548	serumalbumin1(P21848)	0.381	0.1562	0.7586	0.2887	7	6.29	27
493	Glyceraldehyde3-phosphatedehydrogenase(O42259)	0.2358	0.7868	0.0975	0.1501	11.6	8.18	33
477	Fructose-bisphosphate aldolase-like isotype(Q8JH71)	0.1181	0.7005	0.0143	0.0255	10.1	8.7	33
687	SerotransferrinII(TRF2SALSA)	0.1577	0.0552	0.8437	0.5952	4.6	7.93	18
479	Glyceraldehyde-3-phosphatedehydrogenase(O42259)	0.0972	0.4788	0.0307	0.1032	7.1	8.43	33
440	Alpha-2enolase-1(ABONS1)	0.0534	0.8544	0.0097	0.0006	4.5	6.64	36
602	SerotransferrinII+Serumalbumin2precursor(TRF2SALSA+ABONS2)	0.0931	0.8462	0.0318	0.0005	3.6	8.63	24

Proteomic results split into 2 categories
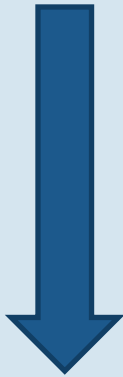
Those which alter in serum abundance due to pathology
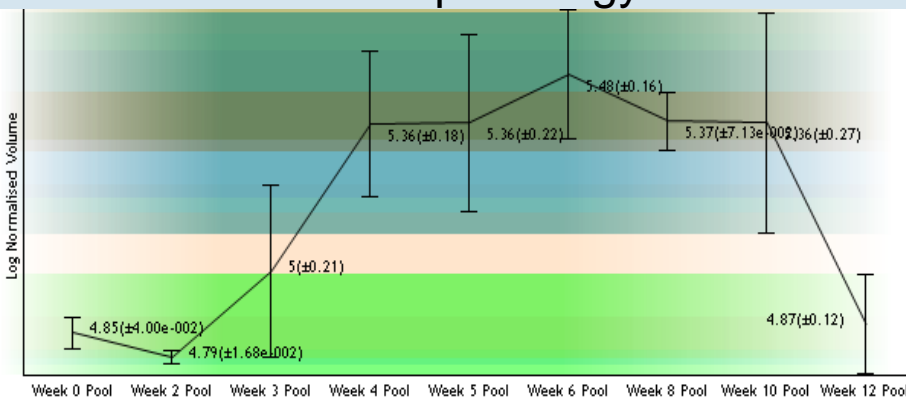
Creatine kinase (Spot 391)
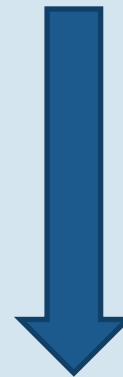
Those which alter as part of humoral immune response
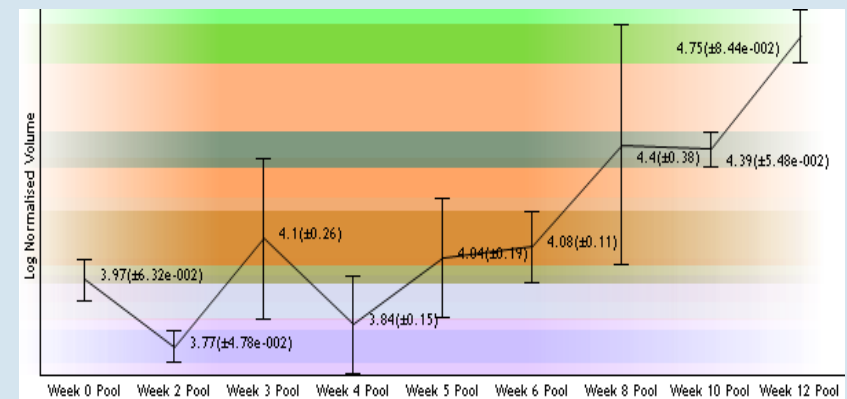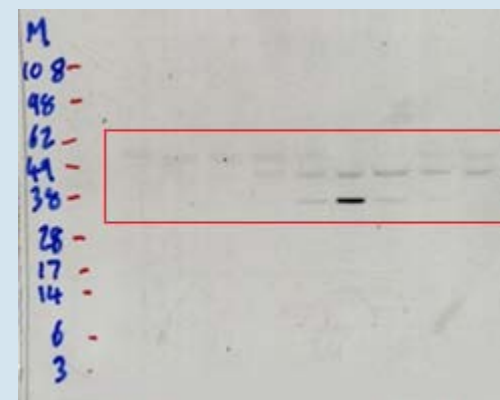
Hemopexin-like protein (Spot 150)

Validation

- **Western Blots**

– 1D: separation of protein by mass SDS-PAGE, transfer to Nitro-cellulose (NC)

– 2d: Separation of protein by pI by isoelectric focusing then mass SDS-PAGE, transfer to NC

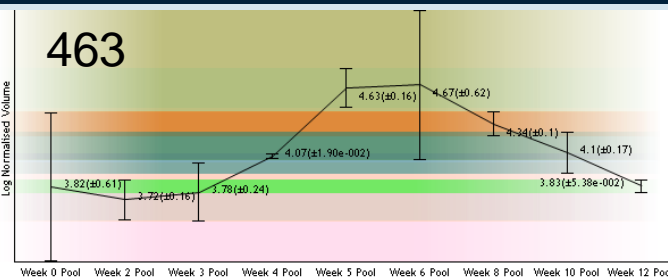– Anti-GADPH: Primary antibody = (Ms) @ 1:500 dil. Secondary Ab = Dnk to Ms HRP @ 1: 5000

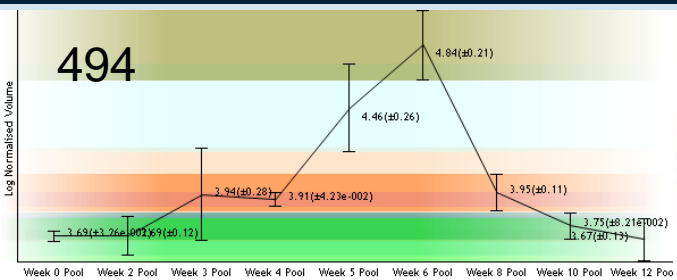# GADPH 1D Western Blot
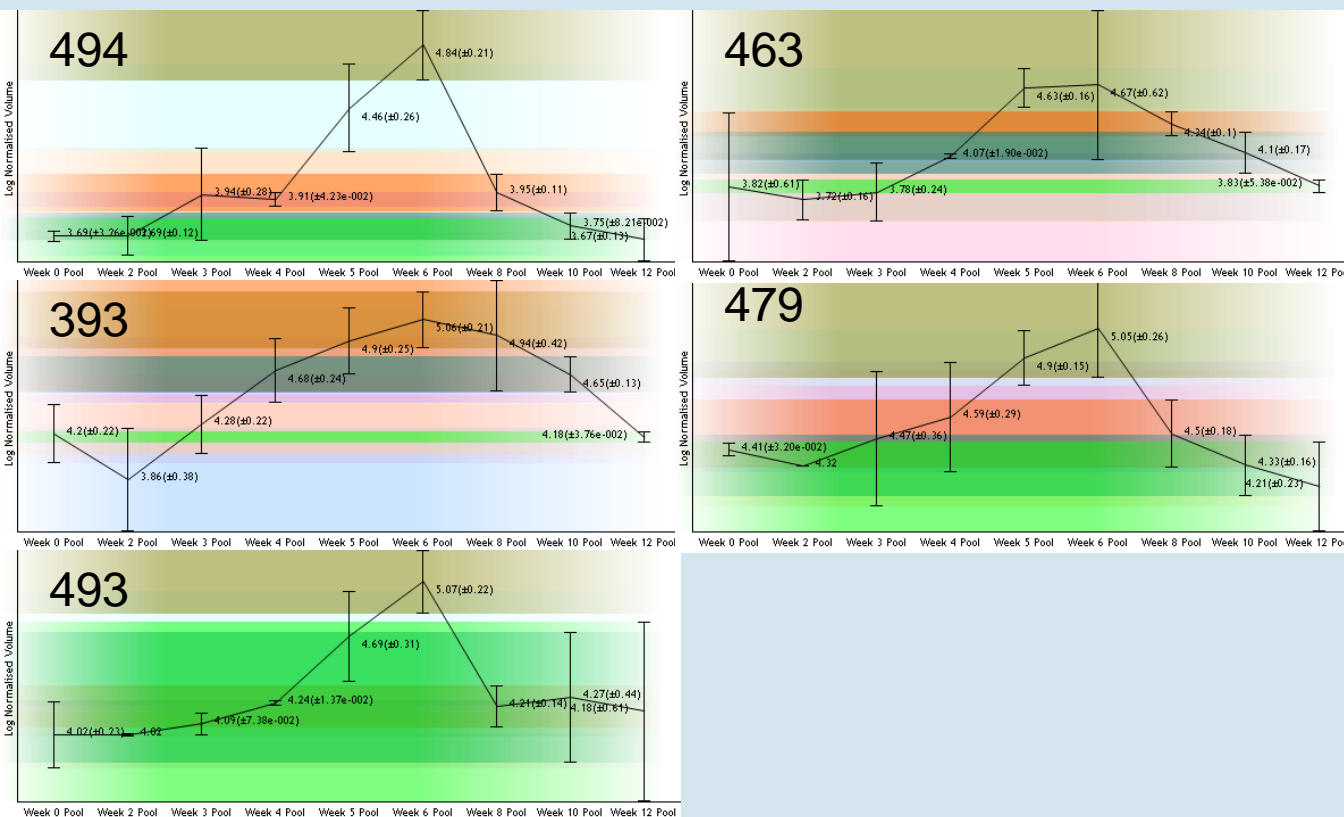
1 = W0, 2 = W2 pc, 3 = W3 pc, 4 = W4 pc, 5 = W5 pc, 6 = W6 pc, 7 = W8 pc, 8 = W10 pc, 9 = W12 pc
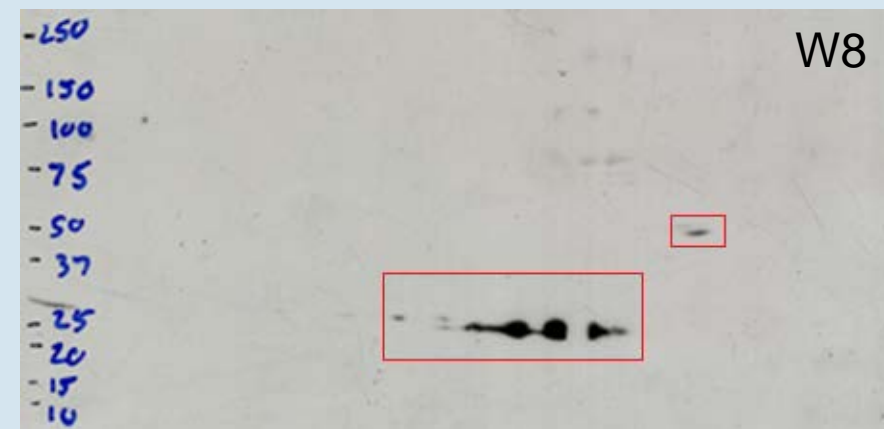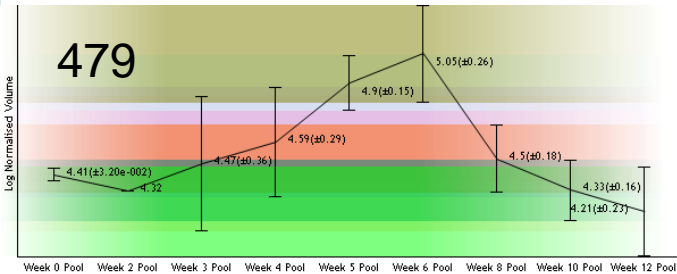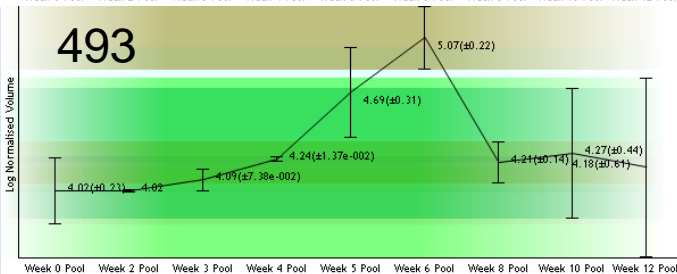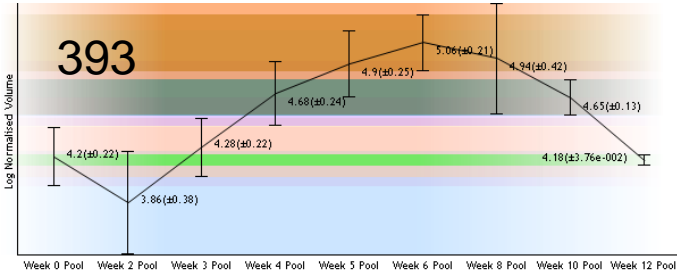
- **Conclusion**

–   Histopathology results from this cohabitation trial followed the sequential pattern of tissue damage characteristic of PD

–   Whilst proteomics may not be a front line diagnostic tool it is extremely useful in identifying possible biomarkers which after assay development may be used in the field

–   This dual approach has given new insight into the effects of pathology on the serum proteome

- **Acknowledgements**

– All my supervisors (academic and industrial)

– Marian McLoughlin for carrying out all the histopathology

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– Phillip Cash, University of Aberdeen

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