Proteomic Characterisation of Serum Proteins From Atlantic Salmon (Salmo salar L.) With Cardiomyopathy Syndrome (CMS)

Janina Costa (janina.costa@moredun.ac.uk)  
Moredun Research Institute

Kim Thompson  
Moredun Research Institute

Jorge del Pozo  
University of Edinburgh

Kevin McLean  
Moredun Research Institute

Neil Inglis  
Moredun Research Institute

Philippe Sourd  
Cooke Aquaculture Scotland

Andrei Bordeianu  
Cooke Aquaculture Scotland

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Abstract

Cardiomyopathy syndrome (CMS), caused by piscine myocarditis virus (PMCV), is a serious challenge to Atlantic salmon (*Salmo salar* L.) aquaculture. Regrettably, husbandry techniques are the only tool to manage CMS outbreaks, and no prophylactic measures are available at present. Early diagnosis of CMS is therefore desirable, preferably with non-lethal diagnostic methods, such as serum biomarkers. To identify candidate biomarkers for CMS, the protein content of pools of sera (4 fish/pool) from salmon with CMS (4 pools) and from clinically healthy salmon (3 pools) were compared using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Overall, seven proteins were uniquely identified in the sera of clinically healthy salmon, while twenty seven proteins were unique to the sera of CMS salmon. Of the latter, 24 have been associated with cardiac disease in humans. These were grouped as leakage enzymes (creatine kinase, lactate dehydrogenase, glycogen phosphorylase and carbonic anhydrase); host reaction proteins (acute phase response proteins - haptoglobin, fibrinogen, α2-macroglobulin, ceruloplasmin; and complement-related proteins); and regeneration/remodelling proteins (fibronectin, lumican and retinol). Clinical evaluation of the suitability of these proteins as biomarkers of CMS, either individually or as part of a panel, is a logical next step for the development of early diagnostic tools for CMS.

Introduction

Atlantic salmon (*Salmo salar* L.) is one of the most economically important aquaculture species produced in Europe. Its production is affected by several cardiac diseases of viral origin, which significantly impact on the industry's production, namely cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV), pancreas disease (PD) caused by salmonid alphavirus (SAV), and heart and skeletal muscle inflammation (HSMI) caused by piscine orthoreovirus (PRV).

Monthly mortality reports compiled by the Scottish Salmon Producers Association (SSPO) show CMS to be one of the main health issues affecting the Scottish salmon industry. Further, in Norway over 100 farms are affected with the disease each year\(^1\). The mortalities may be particularly impactful, as they tend to occur during the later stages of the production cycle, although more recently it has also been reported in salmon shortly after they have been transferred to sea\(^2\). Overall, the economic impact of CMS is substantial, with estimated losses of a single CMS outbreak of up to 1.9 million € in Norway\(^3\). Clinically, CMS can present acutely with sudden mortalities due to cardiac failure without previous clinical signs; or with chronic clinical signs including exophthalmos, cutaneous haemorrhages and raised scales with moderate protracted mortality\(^4,5\). This variability in the presentation and outcomes of CMS render its management, mortality and costs mitigation very challenging in the field, especially in the absence of prophylactic strategies.

The disease was first described in Norway in 1985\(^6\) and subsequently termed CMS by Ferguson *et al.*\(^4\) to account for the associated pathology. In Norway, the term “acute heart failure” has also been associated with CMS. Instances of CMS have also been reported in the Faroe Islands (1984), Scotland (1997) and Ireland (2012)\(^4,7,8\). The aetiological cause of CMS was not established until 2009, when it was confirmed to be transmissible\(^9,10\), and caused by a virus\(^11\), which was subsequently named piscine myocarditis virus or PMCV\(^12\).

Piscine myocarditis virus belongs to the Totiviridae family, with small, spherical virions, approximately 50 nm in diameter, which consist of a non-enveloped protein shell and a dsRNA genome\(^12\). This genome has three open
reading frames (ORF), with ORF1 encoding the major coat protein, ORF2 encoding the RNA-dependent RNA polymerase protein (RdRp) and ORF3 encoding a third protein, the function of which is not fully understood\(^3\). The fact that PMCV is non-culturable has been a major factor in restricting research related to both CMS and PCMV.

Definitive diagnosis of viral diseases in aquaculture has traditionally been performed by histology and detection of the pathogen with molecular methods and/or viral isolation in cell culture. Diagnosis of CMS is based on observation of cardiac lesions typical of CMS and detection of viral RNA by quantitative polymerase chain reaction (qPCR) in infected heart tissue or by histopathology. Both methods require lethal sampling of fish, frequently at the final stages of their production cycle. Further, both methods are applied to a limited number of individuals within large populations, providing rather fragmented information at a population level.

Therefore, a non-lethal diagnostic method that provides an early indication of CMS (i.e. before the onset of clinical signs), and is applicable to large numbers of individuals would be desirable. Such a test would allow salmon farmers to anticipate CMS outbreaks and establish mitigation strategies to reduce or prevent its impact. Additionally, such a tool could be used as an endpoint in the genetic selection of fish for CMS resistance. Both strategies would minimise both the mortalities and economic impact of CMS.

This is a feasible proposition, as it is possible to establish early stages of disease in other animal species portraying prodromal signs of heart disease using appropriate biomarkers\(^13\). In fact, a diverse range of serum molecules are used as biomarkers in human and other animal species to indicate the presence of cardiac disease, but the availability of such biomarkers in fish is limited. More specifically, several candidates have been highlighted as potential cardiac biomarkers for fish, including creatine kinase (CK), lactate dehydrogenase (LDH), natriuretic peptides (salmon cardiac peptide cSP) and troponins. In salmon, special relevance has been placed on CK and LDH, as CK levels are elevated in pancreas disease\(^14\), and both CK and LDH are significantly increased in CMS and HSMI, although significant correlation with histopathological lesions was only seen for HSMI\(^15\).

Increased levels of CK have also been associated with muscle injury in salmon during infection by infectious salmon anaemia virus (ISAV)\(^16\).

As cardiac disease biomarker research in salmon is limited, and host responses to cardiac disease have not been fully characterised, there is scope to identify additional novel serum biomarkers of salmon cardiac disease using serum proteomics comparing healthy and diseased individuals, a method that has been used in similar settings\(^17\). Identification of a range of candidates is valuable, as the diagnosis of a disease can require more than one biomarker, and the selection of biomarkers involves appraisal of factors such as sensitivity, specificity, costs, logistics and measurement equipment\(^18\). One of the most common methods used to identify novel biomarkers is mass spectrometry (MS) of fractionated protein samples (shotgun approach)\(^19\). MS-based proteomics allows identification of specific proteins from a wide range of biological samples and is capable of identifying proteins with high sensitivity within a large dynamic range, making it ideal for biomarker identification\(^20\). In fish, this approach has been helpful for the identification of potential biomarkers for arsenic-exposure in carp (\textit{Labeo rohita})\(^21\) or for bitumen-exposure in Pacific salmon (\textit{Onchorhynchus nerka})\(^22\).

Here we examine the differential expression of proteins in the serum of field samples including fish with CMS and clinically healthy fish, analysed using liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS), in an attempt to identify putative biomarker candidates that may be further developed to allow early diagnosis of this devastating viral disease of farmed Atlantic salmon.
Results

Sample diagnostics

All 44 CMS+ sera samples were collected from moribund fish displaying macroscopic lesions consistent with CMS (e.g. exophthalmia, ventral skin haemorrhages, raised scales), which had PMCV RNA in tissues and/or serum in q-rtPCR. Conversely, all 12 CMS- fish were clinically normal, devoid of macroscopic lesions and were negative by the same diagnostic tests above (Suppl. Table 1).

SDS gel electrophoresis

When the pools of sera were resolved by SDS-PAGE electrophoresis (Figure 1), similar protein profiles were observed between the three pools of sera from CMS+ fish and between the three pools of sera from CMS- fish. Conversely, clear differences were observed in the protein profiles between CMS+ sera and CMS- sera, with CMS+ sera presenting with a visibly higher number of protein bands between 49-28kDa and 28-14kDa.

Common proteins within pool types (CMS+ and CMS-)

The data mining of the peptides obtained with LC-ESI-MS/MS analysis of the three CMS+ pools (Inf 1-3), identified 136, 136 and 158 protein sequences respectively (Suppl. Table 2), with 85 proteins found in common between the three pools. Of these 85 proteins (Suppl. Table 3), 13 were complement-related, 11 were haemoglobin-related, and there were 5 apolipoprotein, 4 alpha-2-macroglobulin, 4 fibrinogen, 3 fibronectin, 3 fibronexin, 2 hemopexin, 2 lipocalin, 2 retinol binding, 2 serum albumin and 2 serotransferrin proteins. Proteins for which only one sequence was identified included: pyruvate kinase, creatine kinase, C-reactive protein, haptoglobin-like, L-lactate dehydrogenase, angiotensinogen, myosin and keratin.

For CMS- pools (Ctr 1-3), 88, 97 and 98 protein sequences were identified, respectively (Suppl. Table 4). Of these, 57 protein sequences were common to all three pools (Suppl. Table 5), with 13 being complement-related, 7 haemoglobin-related, 4 apolipoprotein, 3 alpha-2-macroglobulin, 2 hemopexin, 2 serotransferrin, 2 serum-albumin, 2 protein LEG1 homolog-2 and 2 fibronectin. Proteins with only one sequence identified were angiotensinogen, C-reactive protein, fibrinogen, myosin and lipocalin.

Differences in protein content between pool types (CMS+ and CMS-)

When the lists of proteins common to CMS+ and CMS- pools were compared, 37 protein sequences were found to be unique to CMS+ pools (Table 1 and Suppl. Table 6) and 10 unique to CMS- pools (Table 2 and Suppl. Table 7).

Of the 37 proteins unique to CMS+ pools, 8 (21.6%) were identified with over 60 % sequence coverage, and 34 (92%) had a molecular weight below 100 kDa. After sequence analysis of proteins exclusively present in CMS+ pools, there were three instances of several sequences pertaining to the same protein: 5 were identified as haemoglobin (i.e. α=2, β=3), 3 as fibrinogen (α=1, β=1, γ=1), 2 as apolipoprotein-Eb-like and 2 as retinol-binding. Therefore, 29 protein sequences unique to CMS+ pools were considered for subsequent analysis. As for the sequence analysis of the 10 proteins exclusively present in CMS- pools, there was one instance of two sequences pertaining to the same protein: protein LEG1 homolog. Therefore, 9 protein sequences unique to CMS- pools were considered for subsequent analysis.

Functional proteomic analysis of CMS+ and CMS- pools
All unique proteins identified in CMS+ and CMS- sera pools were analysed using Blast2GO software, in order to identify the protein domains in each protein sequence, to assign them to a biological process or molecular function, and to establish any relation to a cellular component using Gene Ontology (GO). This analysis revealed that a further 3 proteins were common between CMS+ and CMS- sera pools: haemoglobin, apolipoprotein and Beta-2-glycoprotein 1 precursor (also known as apolipoprotein H). These 3 proteins were therefore excluded from further analysis (i.e. CMS+ sera unique protein n=26; CMS- sera unique protein n=7).

Of 26 proteins uniquely identified in the CMS+ pools (Table 3), 3 (11.5 %) had no assigned molecular function, while 4 (17.4 %) bind to minerals, 3 (13 %), are involved in energy metabolism, 3 (13 %), are associated with cleavage of peptide bonds, and 2 (8.7%) are involved in signalling receptor binding. The remaining proteins – i.e. with 1 molecular function each- are involved in protein, DNA or actin binding, L-lactate, and retinol activity. Regarding cellular component, this was not assigned to 15 (57.5%) of the proteins. Of the proteins with cellular component identified, 4 (36.4%) are allocated to extracellular region, 2 (18.2%) with fibrinogen complex and the remainder with keratin filament, actin cytoskeleton or collagen matrix. Eight (30.8%) proteins had no biological process assigned, and of the ones with assigned function, 6 (33.3%) were related to energy production, 3 (16.7%) involved glycolytic processes, 2 (11.1%) with platelet activation, and the remainder – i.e. with 1 process each- were related to proteolysis, protein folding, retinol transport or complement activation.

Of the 7 proteins unique to CMS- sera pools (Table 4), 2 had no molecular function assigned, and 4 (80 %) proteins are involved with lipid, protease or carbohydrate binding. The cellular component was identified in 4 proteins, with 3 (42.8%) allocated to the extracellular region. Three proteins (42.8%) were assigned to one of the following biological process: anti-bacterial defence, lipid transport or under-regulation of endopeptidase activity.

**Discussion**

Cardiomyopathy syndrome is a viral disease of Atlantic salmon, which causes severe chronic cardiac inflammation and injury of the atrium and spongy myocardium. Early diagnosis and even prognosis of CMS is desirable for its control, and this could be potentially achieved by detecting and quantifying serum protein biomarkers.

In human medicine, diagnosis and prognosis of cardiac diseases such as myocardial infarction (MI) and heart failure (HF), is heavily based on individual profiling of protein biomarkers\(^{23}\), which allow diagnosis of subclinical/prodromic presentations\(^{13}\). As defined by the Biomarkers Definition Working Group\(^{24}\), "a biological marker or biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". The use of biomarkers has been central in the development of accurate prognosis and diagnosis of human disease. In order to identify potential biomarkers, several methodologies have been applied, with particular emphasis on proteomic analysis of serum. Proteomic analysis presents the entire set of proteins expressed by an individual, cell or organ at a given time, including post-transcriptional, post-translational and protein complexes\(^{19}\). This analysis is frequently performed using liquid chromatography-mass spectrometry (LC-MS/MS), a qualitative technique capable of analysing complex samples such as serum\(^{25}\). In this study, we used a shotgun approach to identify potential biomarkers for the disease\(^{19}\), by comparison of CMS+ (diseased) and CMS- (clinically healthy) sera. An analytical approach involving digestion by a proteolytic enzyme to create a complex peptide mixture (bottom-up approach) was chosen, since it offers several advantages in terms of its reproducibility and sensitivity when
analysing complex serum samples\textsuperscript{17}. In order to eliminate the individual variability between sera, samples were analysed as pools of sera (n = 4 fish/pool) and only common proteins to each pool type (CMS+/CMS-) were taken forward for analysis.

In our study, twenty-seven proteins were uniquely identified in CMS + sera and seven proteins were unique to CMS- sera. Of the proteins unique to CMS + sera, the majority have been previously associated with cardiac disease (24 proteins), and the remainder are involved in cell structure (2 proteins) or sexual endocrine function (1 protein). Further, included in the list are previously described myocardial injury biomarkers, such as creatine kinase, L-lactate dehydrogenase, glycogen phosphorylase and carbonic anhydrase\textsuperscript{26}. These are all cardiomyocyte cytoplasmic enzymes found in healthy cardiomyocytes, which leak into the serum during cardiomyocyte damage (leakage enzymes). Other putative candidate biomarkers were detected, and all candidates are summarized in Fig. 2, which includes the aforementioned leakage proteins, but also proteins involved in host reaction to disease, regeneration/remodelling and energy metabolism.

**Leakage enzymes**

Creatine kinase (CK), L-lactate dehydrogenase (L-LDH), Glycogen phosphorylase (GP) and Carbonic anhydrase (CA) are all used as direct or indirect myocardial injury biomarkers in other species. However, their use is not restricted to myocardial injury, as they are also detected in serum during skeletal muscle and liver disease or even during extreme exercise\textsuperscript{27,28}. This limits their usefulness as specific biomarkers of cardiac disease when used on their own, but this limitation can be overcome if they are part of biomarker panels.

Creatine kinase is a dimer consisting of subunits M and/or B, which combine to form isomers CK-BB, CK-MM and CK-MB. In humans, CK-MB is found almost exclusively in the myocardium, and high levels in serum is a highly specific and sensitive indicator of myocardial cell wall injury\textsuperscript{29}. In our study a CK-M type protein was identified in CMS + sera. As CMS does not cause skeletal muscle lesions, it is very likely that the CK-M identified was of cardiac origin. The potential of using CK to diagnose piscine disease was first suggested by Rodger \textit{et al.}\textsuperscript{14} for pancreas disease (PD), a viral disease causing heart and muscle lesions in Atlantic salmon. This was supported by serum proteomics by Braceland \textit{et al.}\textsuperscript{30}, who found a correlation between serum CK levels and PD-associated lesions. In CMS, elevation of CK serum levels has been noted, but this is not correlated with CMS cardiac histopathology scores\textsuperscript{15}. This suggests that there may be a factor other than CMS lesion severity driving CK serum levels in CMS diseased fish.

L-lactate dehydrogenase has two subunits and five isoforms. It is an enzyme involved in energy production (\textit{i.e.} converts lactate to pyruvate and back, and NAD to NADH and back), and for this reason is found in almost all cell types. Consequently, this enzyme has been used as a human and veterinary biomarker for a wide range of diseases causing tissue damage, including myocardial, hepatic, skeletal muscle and renal disease, as well as haematological and neoplastic disorders\textsuperscript{31,32}. In fish, LDH has been used in toxicological, and infectious disease assessment settings in a range of species other than Atlantic salmon\textsuperscript{33–36}. Importantly, Yousaf \textit{et al.}\textsuperscript{15} also recorded LDH serum elevation in CMS in Atlantic salmon, which as with CK, was not correlated with CMS cardiac lesion scores.

Glycogen phosphorylase (GP) is an enzyme that breaks glycogen into glucose subunits. Its isoenzymes are found in muscle, liver and brain\textsuperscript{37}. For this reason, it has been extensively used as a biomarker in injury to those organs.
Two settings for its use in human medicine are as a biomarker of cardiac injury for cardiotoxicity evaluation\textsuperscript{38} and diagnosis of acute myocardial injury\textsuperscript{27}. In fish, an increase in serum GP has been noted in starved Atlantic salmon\textsuperscript{39} and in \textit{Claria bratrachus}, GP has been suggested as a biomarker of cypermethrin toxicity\textsuperscript{40}.

Carbonic anhydrases (CA) are a family of cytoplasmic metalloenzymes, with fourteen isoenzymes that catalyze the reversible conversion between carbon dioxide and water to bicarbonate and hydrogen ions. They are therefore ubiquitous in the body and are involved in acid-base homeostasis and fluid balance\textsuperscript{41}. All CA isoenzymes have been used as biomarkers of neoplastic, infectious, parasitic and degenerative disease affecting a wide range of organ systems\textsuperscript{42}. In the human cardiac disease context, the ratio of CA III and serum myoglobin has been used to identify post-surgical cardiac injury, as myoglobin is present in both heart and skeletal muscle and CA III is only present in skeletal muscle\textsuperscript{43}. Interestingly, recent work has revealed membrane bound CA IV in all cardiac chambers of coho salmon (\textit{Oncorhynchus kisutch}), which is believed to facilitate oxygen unloading from venous blood, therefore facilitating oxygenation of a heart without coronary circulation\textsuperscript{44}. Regrettably, in our study, the serum CA isotype detected was not recognized by the analysis, and further characterization of circulating CA in CMS fish would be required to evaluate its biomarker potential.

**Host reaction proteins**

This subgroup of proteins unique to CMS + sera includes acute phase response proteins (haptoglobin, fibrinogen, \textalpha{}2-macroglobulin, ceruloplasmin) and complement-related proteins (factor B and serine protease-like). Acute phase proteins are proteins that increase (positive) or decrease (negative) in response to inflammation\textsuperscript{45}. The complement system is part of the innate immune system, and its effector functions include recruitment and activation of inflammatory cells and opsonization and lysis of microbes\textsuperscript{46}. These host response proteins are interpreted as the result of a systemic response to disease, and could have potential as candidate biomarkers of disease in general and in CMS as part of biomarker panels.

Haptoglobin rapidly binds to haemoglobin after haemolysis and tissue damage events, protecting the tissues and cells from oxidative damage\textsuperscript{47}. It has been used as a diagnostic biomarker for neurological disease, diabetes, certain cancers and bovine mastitis\textsuperscript{48}. It is also an important biomarker for humans at risk of acute MI, stroke and HF\textsuperscript{49,50}. In fish viral disease, Cordero \textit{et al.}\textsuperscript{51} found variability in haptoglobin expression depending on the fish and viral species. In gilthead seabream (\textit{Sparus aurata}) haptoglobin was up-regulated in lymphocystis disease virus infection (LCDV), but down-regulated in nervous necrosis virus infection (NNV) – to which they are resistant. Conversely, in European seabass (\textit{Dicentrarchus labrax}) - which is susceptible to NNV- haptoglobin was up-regulated during NNV infection. Notably, levels of haptoglobin were also seen to be up-regulated in Atlantic salmon infected with sea lice \textit{Caligus rogercresseyi}\textsuperscript{52}. The presence of haptoglobin in CMS + sera in our study, suggests that haptoglobin is a candidate biomarker for CMS in Atlantic salmon.

Fibrinoten, a protein used to predict stroke and MI in human patients, fibrinogen, was also identified in CMS + sera. Fibrinogen is an acute phase protein\textsuperscript{53} and its GO biological function is assigned as “platelet activation, blood coagulation and fibrin clot formation”. Its presence in CMS + sera is not surprising, as CMS lesions feature intraluminal cardiac thrombosis and cutaneous haemorrhage\textsuperscript{3}, and all of these may indicate a coagulopathy. This idea is also supported by the presence of kininogen and a serine protease-like protein in CMS + sera. Kininogens are the precursor of kinins, which act as inflammatory mediators, but also are involved in the contact phase of the clotting cascade\textsuperscript{54}. In fact, increased levels of kininogen in plasma and tissues are linked with injury,
inflammation, myocardial infarction, and diabetes. Our study also identified a serine protease-like protein. The superfamily of serine proteases is involved in a range of processes, including such as blood coagulation, platelet activation, fibrinolysis and thrombosis, inflammation, coagulation and haemorrhaging. In our study, gene oncology assigned the molecular function of the serine protease-like protein as "serine-type endopeptidase activity", which is synonymous to "blood coagulation factor activity". The overall picture of coagulopathy in CMS + fish is also complemented by the presence of Alpha-2 macroglobin (α2-M) in CMS + serum, as this is an inhibitor of thrombin and plasmin. The cardiac isoform of this protein causes cardiac hypertrophy, and is used as an early diagnostic marker of cardiac diseases in patients with HIV and diabetes. The presence of higher levels of cardiac isoform α2-M is beneficial during the early onset of myocardial damage associated with diabetes, due to its protease inhibitor function and antioxidant effect. When Atlantic salmon were pre-treated with α2-M, then injected with a serine protease from Aeromonas salmonicida, α2-M was found to inhibit the serine protease by preventing the pro-coagulant effect of the enzyme.

Ceruloplasmin carries copper and oxidizes iron, facilitating its inclusion in transferrin. It has been detected in high levels in patients with myocardial infarction, heart failure, ischemic and non-ischemic cardiomyopathy, coronary heart disease, artheriosclerosis or angina. Ceruloplasmin correlate with the severity of cardiac failure and non-ischemic cardiomyopathy. In fish, this protein has been investigated as a possible biomarker of bacterial resistance in fish. This is possibly due to its role in reducing iron availability to the bacteria.

Protein complement factor B, usually know as factor B, is a serine protease, part of the alternative complement system pathway. Its presence in CMS + serum is not surprising, as a systemic inflammatory response is to be expected during PCMV infection, where the complement cascade is likely to promote inflammatory cell recruitment and activation, and destruction of infected cardiomyocytes.

# Regeneration/remodelling

Cardiomyopathy syndrome in Atlantic salmon frequently presents as a chronic cardiac lesion that leads to acute cardiac failure. This chronicity is aligned with the findings of our study, where several proteins associated with regeneration and remodelling were unique to CMS + serum.

Fibronectin was uniquely identified in CMS + serum. One form of fibronectin (FN1) is a major glycoprotein in the extracellular matrix and is critical in cell adhesion, differentiation, migration, and growth. This is a protein known to be actively involved in epicardium regeneration in zebrafish after cardiac injury. Importantly, there is evidence that cardiac regeneration occurs in CMS + Atlantic salmon. The second form of fibronectin (FN2) is presented on the cell surface and was first identified in zebrafish, and then later in other species of fish, mice and humans. Bearzoti et al. suggested that fibronectin mediates in the entry of fish rhabdoviruses into cells. It was subsequently shown that FN2 can mediate the attachment and entry of infectious hematopoietic necrosis virus (a piscine rhabdovirus) into cells. It is unclear which fibronectin form, FN1 or FN2, was identified in the present study, since its identity is based on a predicted protein in a computer-annotated Atlantic salmon genome.
Lumican is an extracellular matrix proteoglycan that is required for organisation of the collagenous matrix\textsuperscript{79}, and is also present in CMS + serum. Engebretsen \textit{et al.}\textsuperscript{80} noted that cardiac lumican levels are higher in human patients with heart failure; and its role in cardiac remodelling post pressure overload has been recorded in a mouse model\textsuperscript{81}. In fish, lumican has been identified in the skin mucus of carp (\textit{Cyprinus carpio}) infected with \textit{Ichthyophthirius multifiliis}\textsuperscript{82}. We hypothesize that the presence of this protein in CMS + serum is associated with cardiac remodelling and may be an indicator of chronicity in this setting.

A retinol-binding protein was also present in CMS + serum (retinol is also known as vitamin A). In zebrafish, cardiomyocytes induce the expression of a retinoic acid-synthesizing enzyme in response to injury, promoting cardiomyocyte proliferation, and subsequent heart regeneration\textsuperscript{83}. Further, lipocalin was also identified, which among other roles is involved in transmembrane transport of retinol into cells through the plasma retinol-binding protein (RBP)\textsuperscript{84}. In the study of Yndestad \textit{et al.}\textsuperscript{85}, high levels of neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2), were observed in the serum of patients with acute MI/HF and chronic HF, which led to their suggestion as HF candidate biomarkers. The identification of these two proteins in CMS + serum suggests the possibility of cardiac regeneration in CMS diseased fish, a topic for further investigation.

**Closing remarks**

In conclusion, this proteomic study has provided an insight into the proteins present in Atlantic salmon serum with CMS. The proteins unique to CMS + serum include well-documented cardiac disease biomarkers, inflammatory biomarkers (acute phase proteins/complement), and candidate biomarkers for tissue remodelling/regeneration. The panel of proteins obtained confirms shotgun LC-MS/MS is a powerful technique for identifying candidate cardiac disease biomarkers of viral disease of farmed Atlantic salmon. Fuller assessment of the proteins identified here is need to establish their usefulness as biomarkers to assess the disease status of CMS-affected Atlantic salmon.

**Materials And Methods**

**Biological samples**

Blood was collected from Atlantic salmon at two marine production sites in Scotland. One site had an on-going CMS outbreak (CMS + site) and the other site was classified as a CMS-free farm (control site), with no previous history of the disease. Blood was collected from 44 Atlantic salmon at the CMS + site and from 12 at the CMS-site. The blood was allowed to clot at ambient temperature and serum was collected within 3 h post sampling, by centrifuging the blood for 3 m at 2680 x g (SciSpin mini-centrifuge). The serum was immediately aliquoted and stored at -20 °C until transferred to the lab and storage at -80 °C. Also collected were heart samples, which were fixed in 10% neutral buffered formalin.

The disease status of fish at each farm site was assessed by gross pathology, cardiac histopathology, and PMCV specific RT-qPCR. The former were conducted using standard methods, and the latter was provided by Pharmaq Analytiq (Bergen, Norway). Fish were classified as CMS + if histology and RT -qPCR were consistent with CMS and PCMV infection respectively, and as CMS- if this was not the case.
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (sds-page)

Pools of sera were used to reduce variability between individual fish. Three CMS + sera pools (Inf 1, Inf 2, Inf 3; n = 4 CMS + fish/pool) and three CMS- sera pools (Ctr 1, Ctr2, Ctr 3; n = 4 CMS- fish/pool) were prepared for analysis. The proteins were separated by electrophoresis, for which sera were denatured with lithium dodecyl sample buffer (NuPAGE LDS sample buffer, Invitrogen, ThermoFisher Scientific, Renfrew, UK), 50 mM dithiothreitol (DTT) (NuPAGE reducing agent, Invitrogen) and heating the sample to 70 °C for 10 min. The average protein concentration of the CMS + sera was 10.2 mg ml\(^{-1}\) and 40.6 mg ml\(^{-1}\) for the non-infected sera. The former was diluted 1:1 and the latter 1:15 to have similar protein profiles resolved on the gel. The samples were loaded (20 µL per lane) onto a pre-casted 12% NuPAGE Bis-Tris Mini Gels, using SeeBlue Plus2 pre-stained standards (Invitrogen, ThermoFisher Scientific, Renfrew, UK) as a molecular weight reference. The gels were run on an XCell SureLock Mini-Cell electrophoresis system (ThermoFisher Scientific, Renfrew, UK) for 45 min at 200 V using NuPAGE MES SDS running buffer supplemented with NuPAGE antioxidant. The resolved proteins were stained with SimplyBlue Safe Stain (Invitrogen) for 1 h and de-stained with deionised water for a further 1 h.

MS/MS

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was used to analyse the gels containing the protein profiles. Each stained gel lane (3 CMS + and 3 CMS- sera) was removed from the gel, and a series of equal gel slices of 2.5 mm deep, from top to bottom were made. The slices underwent in-gel de-staining, reduction, alkylation and trypsinolysis as described\(^{86}\). An Ultimate 3000 nano-HPLC system (Dionex) with a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromelon™ chromatography software was used to performed the liquid chromatography analysis. A micro-pump with a flow rate of 246 µl min\(^{-1}\) was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 µl min\(^{-1}\) through a 5 cm x 200 mm ID monolithic reversed phase column (Thermo) maintained at 50 °C. The samples (4 µl) were injected directly onto the column. Elution of peptides was achieved by the application of a 15 min linear gradient from 8–45% solvent B (80% acetonitrile, 0.1% (w/v) formic acid) and directed through a 3µ I UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (amaZon-ETD, Bruker Daltonics) via a low-volume (50 µl min\(^{-1}\) maximum) stainless steel nebuliser (Agilent) and ESI. The parameters for tandem MS analysis were based on those described previously by Batycka et al.\(^{87}\).

Data Mining

After importing the deconvoluted MS/MS data in .mgf (Mascot Generic Format) into ProteinScape™ V3.1 (Bruker Daltonics), proteomics data analysis software, Mascot™ V2.5.1(Matrix Science) search algorithm was used for downstream database mining of the annotated Atlantic salmon genome sequence. Protein Search” feature of ProteinScape™ was used to establish the protein content of each individual gel slice, whereas the “Protein Extractor” feature was used to compile the protein content of all gel slices into a single result file. The guidelines established by Taylor et al.\(^{88}\) were used to set Mascot search parameters, and to this end, fixed (carbamidomethyl “C”) and variable (oxidation “M” and deamidation “N,Q”) modifications were selected along with peptide (MS) and
secondary fragmentation (MS/MS) tolerance values of 0.5 Da whilst allowing for a single 13C isotope. Molecular weight search (MOWSE) scores attained for individual protein identifications were inspected manually and a list of significant protein were prepared. The proteins were considered significant only if a) two peptides were matched for each protein, and b) each matched peptide contained an unbroken “b” or “y” ion series represented by of a minimum of four contiguous amino acid residues.

Data analysis

Proteins identified in the three CMS + pools were compared in order to select proteins common to all infected samples. The same procedure was applied to select proteins common to all CMS- pools. The two sets of selected CMS + and CMS- proteins were then compared, and a list of unique proteins produced for each of them. The functional annotation and mapping of unique protein sequences were performed using Blast2Go (Götz et al 2008). Briefly, the protein sequences were blasted with NCBI Blast Service (QBLAST), then the proteins were run through InterProScan to classify them by family and to identify main domains; the final functional annotation was performed by mapping homologue sequences and annotating them with Gene Ontology databases.

Declarations

Competing interests
The author(s) declare no competing interests.

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Author contributions statement

J.Z.C., K.D.T. and N.F.I. conceived the experiment, P.S. and A.B. collect the samples, K.M. and N.F.I. conducted the proteomics experiment, J.Z.C. and J.d.P analysed the results; J.Z.C, K.D.T and J.d.P wrote the article. All authors reviewed the manuscript.

Animal statement

The animals sample for this study were sample directly from a fish farm and were sacrifice following UK regulation and under Moredun Research Institute ethic committee approval.

Methods statement

Moredun Research Institute has approved all the methodology used during this study.

References

1 Fritvold, C. & Jensen, B. B. Cardiomyopathy syndrome (CMS). 52-55 (Norwegian Veterinary Institute, Norway, 2019).

2 Fritvold, C. Cardiomyopathy syndrome (CMS). 30-31 (The Norwegian Veterinary Institute, Norway, 2016).
3 Garseth, A. H., Fritsvold, C., Svendsen, J. C., Bang Jensen, B. & Mikalsen, A. B. Cardiomyopathy syndrome in Atlantic salmon *Salmo salar* L.: A review of the current state of knowledge. *J Fish Dis* **41**, 11-26, doi:10.1111/jfd.12735 (2018).

4 Ferguson, H. W., Poppe, T. & Speare, D. J. Cardiomyopathy in farmed Norwegian salmon. *Dis Aquat Organ* **8**, 225-231 (1990).

5 Brun, E., Poppe, T., A., S. & Jarp, J. Cardiomyopathy syndrome in farmed Atlantic salmon *Salmo salar*: occurrence and direct financial losses for Norwegian aquaculture *Dis Aquat Organ* **56**, 214-247 (2003).

6 Amin, A. B. & Trasti, J. Endomyocarditis in atlantic salmon in Norwegian seafarms. A case report. *Bull. Eur. Assoc. Fish Pathol.* **8**, 70-71 (1988).

7 Poppe, T. T. & Seierstad, S. L. First description of cardiomyopathy syndrome (CMS)-related lesions in wild Atlantic salmon *Salmo salar* in Norway. *Dis Aquat Organ* **56**, 87-88 (2003).

8 Rodger, H. D., McCleary, S. J. & Ruane, N. M. Clinical cardiomyopathy syndrome in Atlantic salmon, *Salmo salar* L. *J Fish Dis* **37**, 935-939, doi:10.1111/jfd.12186 (2014).

9 Bruno, D. W. & Noguera, P. A. Comparative experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*. *Dis Aquat Organ* **87**, 235-242, doi:10.3354/dao02129 (2009).

10 Fritsvold, C. *et al.* Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*. *Dis Aquat Organ* **87**, 225-234, doi:10.3354/dao02123 (2009).

11 Løvoll, M. *et al.* A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS). *Virol J* **7**, 309, doi:10.1186/1743-422X-7-309 (2010).

12 Haugland, O. *et al.* Cardiomyopathy syndrome of atlantic salmon (*Salmo salar* L.) is caused by a double-stranded RNA virus of the Totiviridae family. *J Virol* **85**, 5275-5286, doi:10.1128/JVI.02154-10 (2011).

13 Mayeux, R. Biomarkers potential uses and limitations. *NeuroRx* **1**, 182-188, doi:10.1602/neurorx.1.2.182 (2004).

14 Rodger, H. D., Murphy, T. M., Drinan, E. M. & Rice, D. A. Acute skeletal myopathy in farmed Atlantic salmon *Salmo salar*. *Dis Aquat Organ* **12**, 17-23 (1991).

15 Yousaf, M. N. & Powell, M. D. The effects of heart and skeletal muscle inflammation and cardiomyopathy syndrome on creatine kinase and lactate dehydrogenase levels in Atlantic salmon (*Salmo salar* L.). *Scientific World Journal* **2012**, 741302, doi:10.1100/2012/741302 (2012).

16 Rojas, V. *et al.* Detection of muscle-specific creatine kinase expression as physiological indicator for Atlantic salmon (*Salmo salar* L.) skeletal muscle damage. *Aquaculture* **496**, 66-72, doi:10.1016/j.aquaculture.2018.07.006 (2018).

17 Barbosa, E. B. *et al.* Proteomics: methodologies and applications to the study of human diseases. *Rev Assoc Med Bras* **58**, 366-375, doi:10.1016/s2255-4823(12)70209-6 (2012).
18 Byrnes, S. A. & Weigl, B. H. Selecting analytical biomarkers for diagnostic applications: a first principles approach. *Expert Rev Mol Diagn* **18**, 19-26, doi:10.1080/14737159.2018.1412258 (2018).

19 Ahrens, C. H., Brunner, E., Qeli, E., Basler, K. & Aebersold, R. Generating and navigating proteome maps using mass spectrometry. *Nat Rev Mol Cell Biol* **11**, 789-801, doi:10.1038/nrm2973 (2010).

20 Geyer, P. E., Holdt, L. M., Teupser, D. & Mann, M. Revisiting biomarker discovery by plasma proteomics. *Mol Syst Biol* **13**, 942, doi:10.15252/msb.20156297 (2017).

21 Banerjee, S. *et al.* Identification of potential biomarkers of hepatotoxicity by plasma proteome analysis of arsenic-exposed carp *Labeo rohita*. *J Hazard Mater* **336**, 71-80, doi:10.1016/j.jhazmat.2017.04.054 (2017).

22 Alderman, S. L., Dindia, L. A., Kennedy, C. J., Farrell, A. P. & Gillis, T. E. Proteomic analysis of sockeye salmon serum as a tool for biomarker discovery and new insight into the sublethal toxicity of diluted bitumen. *Comp Biochem Physiol Part D Genomics Proteomics* **22**, 157-166, doi:10.1016/j.cbd.2017.04.003 (2017).

23 Smith, J. G. & Gerszten, R. E. Emerging Affinity-Based Proteomic Technologies for Large-Scale Plasma Profiling in Cardiovascular Disease. *Circulation* **135**, 1651-1664, doi:10.1161/CIRCULATIONAHA.116.025446 (2017).

24 Group, B. D. W. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* **69**, 89-95, doi:10.1067/mcp.2001.113989 (2001).

25 Zhou, B. *et al.* Plasma proteomics-based identification of novel biomarkers in early gastric cancer. *Clin Biochem* **76**, 5-10, doi:10.1016/j.clinbiochem.2019.11.001 (2020).

26 Kemp, M., Donovan, J., Higham, H. & Hooper, J. Biochemical markers of myocardial injury. *Br J Anaesth* **93**, 63-73, doi:10.1093/bja/aeh148 (2004).

27 Lippi, G., Mattiuzzi, C., Comelli, I. & Cervellin, G. Glycogen phosphorylase isoenzyme BB in the diagnosis of acute myocardial infarction: a meta-analysis. *Biochem Med (Zagreb)* **23**, 78-82, doi:10.11613/bm.2013.010 (2013).

28 Bodor, G. S. Biochemical markers of myocardial damage. *EJIFCC* **27**, 95-112 (2016).

29 Cabaniss, C. D. in *Clinical methods: The history, physical, and laboratory examinations* (eds H.K. Walker, W.D. Hall, & J.W. Hurst) Ch. 32, (Butterworths, 1990).

30 Braceland, M. *et al.* The serum proteome of Atlantic salmon, *Salmo salar*, during pancreas disease (PD) following infection with salmonid alphavirus subtype 3 (SAV3). *J Proteomics* **94**, 423-436, doi:10.1016/j.jprot.2013.10.016 (2013).

31 Khan, A. A., Allemailem, K. S., Alhumaydhi, F. A., Gowder, S. J. T. & Rahmani, A. H. The biochemical and clinical perspectives of lactate dehydrogenase: an enzyme of active metabolism. *Endocr Metab Immune Disord Drug Targets* **20**, 855-868, doi:10.2174/1871530320666191230141110 (2020).

32 Klein, R., Nagy, O., Tóthová, C. & Chovanová, F. Clinical and Diagnostic Significance of Lactate Dehydrogenase and Its Isoenzymes in Animals. *Vet. Med. Int.* **2020**, 5346483, doi:10.1155/2020/5346483 (2020).
33 Oliveira, R. et al. Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (Danio rerio). *Environ. Toxicol. Pharmacol.* **36**, 903-912, doi:https://doi.org/10.1016/j.etap.2013.07.019 (2013).

34 Ajima, M. N. O., Ogo, O. A., Audu, B. S. & Ugwoegbu, K. C. Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, Clarias gariepinus. *Drug Chem. Toxicol.* **38**, 383-390, doi:10.3109/01480545.2014.974108 (2015).

35 Elia, A. C. et al. Oxidative stress and related biomarkers in cupric and cuprous chloride-treated rainbow trout. *Environ Sci Pollut Res Int* **24**, 10205-10219, doi:10.1007/s11356-017-8651-z (2017).

36 Kumar, S. et al. Effects on haematological and serum biochemical parameters of Pangasianodon hypophthalmus to an experimental infection of *Thaparocleidus* sp. (Monogenea: dactylogyridae). *Experimental Parasitology* **188**, 1-7, doi:https://doi.org/10.1016/j.exppara.2018.02.007 (2018).

37 David, E. S. & Crerar, M. M. Quantitation of muscle glycogen phosphorylase mRNA and enzyme amounts in adult rat tissues. *Biochim Biophys Acta Gen Subj* **880**, 78-90, doi:https://doi.org/10.1016/0304-4165(86)90122-4 (1986).

38 Zhu, Y. & Gius, D. Glycogen Phosphorylase: A Novel Biomarker in Doxorubicin-Induced Cardiac Injury. *Clin Cancer Res* **24**, 1516-1517, doi:10.1158/1078-0432.CCR-17-3276 (2018).

39 Sundby, A., Hemre, G.-I., Borrebaek, B., Christoffersen, B. & Blom, A. K. Insulin and glucagon family peptides in relation to activities of hepatic hexokinase and other enzymes in fed and starved Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*). *Comp Biochem Physiol Part B: Comp Biochem* **100**, 467-470, doi:https://doi.org/10.1016/0305-0491(91)90205-R (1991).

40 Begum, G. Enzymes as biomarkers of cypermethrin toxicity: response of *Clarias batrachus* tissues ATPase and glycogen phosphorylase as a function of exposure and recovery at sublethal level. *Toxicol. Mech. Methods* **19**, 29-39, doi:10.1080/15376510802205650 (2009).

41 Lindskog, S. Structure and mechanism of carbonic anhydrase. *Pharmacol. Ther.* **74**, 1-20, doi:https://doi.org/10.1016/S0163-7258(96)00198-2 (1997).

42 Zamanova, S., Shabana, A. M., Mondal, U. K. & Ilies, M. A. Carbonic anhydrases as disease markers. *Expert Opin. Ther. Pat.* **29**, 509-533, doi:10.1080/13543776.2019.1629419 (2019).

43 Vuotikka, P. et al. Serum Myoglobin/Carbonic Anhydrase III Ratio in the Diagnosis of Perioperative Myocardial Infarction During Coronary Bypass Surgery. *Scand. Cardiovasc. J.* **37**, 23-29, doi:10.1080/14017430310006992 (2003).

44 Alderman, S. L. et al. Evidence for a plasma-accessible carbonic anhydrase in the lumen of salmon heart that may enhance oxygen delivery to the myocardium. *J Exp Bio* **219**, 719-724, doi:10.1242/jeb.130443 (2016).

45 Latimer, K. S. *Duncan and Prasse's veterinary laboratory medicine: clinical pathology*. 5 edn, 178-179 (Wiley-Blackwell 2011).

46 Kumar, V., Abbas, A. K. & Aster, J. C. *Robbins & Cotran pathologic basis of disease*. 88-89 (Elsevier 2014).
47 Andersen, C. B. et al. Structure of the haptoglobin-haemoglobin complex. *Nature* **489**, 456-459, doi:10.1038/nature11369 (2012).

48 Nirala, N. R., Harel, Y., Lellouche, J.-P. & Shtenberg, G. Ultrasensitive haptoglobin biomarker detection based on amplified chemiluminescence of magnetite nanoparticles. *J. Nanobiotechnology* **18**, 6, doi:10.1186/s12951-019-0569-9 (2020).

49 Holme, I., Aastveit, A. H., Hammar, N., Jungner, I. & Walldius, G. Haptoglobin and risk of myocardial infarction, stroke, and congestive heart failure in 342,125 men and women in the Apolipoprotein MOrtality RISk study (AMORIS). *Ann. Med.* **41**, 522-532, doi:10.1080/07853890903089453 (2009).

50 Haas, B. et al. Proteomic analysis of plasma samples from patients with acute myocardial infarction identifies haptoglobin as a potential prognostic biomarker. *J. Proteom.* **75**, 229-236, doi:https://doi.org/10.1016/j.jprot.2011.06.028 (2011).

51 Cordero, H., Li, C. H., Chaves-Pozo, E., Esteban, M. Á. & Cuesta, A. Molecular identification and characterization of haptoglobin in teleosts revealed an important role on fish viral infections. *Dev Comp Immunol* **76**, 189-199, doi:https://doi.org/10.1016/j.dci.2017.06.006 (2017).

52 Valenzuela-Muñoz, V., Boltaña, S. & Gallardo-Escárate, C. Uncovering iron regulation with species-specific transcriptome patterns in Atlantic and coho salmon during a Caligus rogercresseyi infestation. *J. Fish Dis.* **40**, 1169-1184, doi:10.1111/jfd.12592 (2017).

53 Jain, S., Gautam, V. & Naseem, S. Acute-phase proteins: As diagnostic tool. *J. Pharm. Bioallied Sci.* **3**, 118-127, doi:10.4103/0975-7406.76489 (2011).

54 Weisel, J. W. et al. The shape of high molecular weight kininogen. Organization into structural domains, changes with activation, and interactions with prekallikrein, as determined by electron microscopy. *J Biol Chem* **269**, 10100-10106 (1994).

55 Wong, M. K. S. in *Handbook of Hormones* (eds Yoshio Takei, Hironori Ando, & Kazuyoshi Tsutsui) 268-e230A-263 (Academic Press, 2016).

56 Wu, Q., Kuo, H. C. & Deng, G. G. Serine proteases and cardiac function. *Biochim Biophys Acta* **1751**, 82-94, doi:10.1016/j.bbapap.2004.09.005 (2005).

57 Patel, S. A critical review on serine protease: Key immune manipulator and pathology mediator. *Allergol Immunopathol (Madrid)* **45**, 579-591, doi:10.1016/j.aller.2016.10.011 (2017).

58 de Boer, J. P. et al. Alpha-2-macroglobulin functions as an inhibitor of fibrinolytic, clotting, and neutrophilic proteinases in sepsis: studies using a baboon model. *Infect Immun* **61**, 5035-5043, doi:10.1128/IAI.61.12.5035-5043.1993 (1993).

59 Ramasamy, S. et al. Cardiac isoform of alpha 2 macroglobulin, an early diagnostic marker for cardiac manifestations in AIDS patients. *AIDS* **20** (2006).

60 Yoshino, S. et al. Molecular form and concentration of serum α2-macroglobulin in diabetes. *Sci. Rep.* **9**, 12927, doi:10.1038/s41598-019-49144-7 (2019).
61 Soman, S., Manju, C. S., Rauf, A. A., Indira, M. & Rajamanickam, C. Role of cardiac isoform of alpha-2 macroglobulin in diabetic myocardium. *Mol Cell Biochem* **350**, 229-235, doi:10.1007/s11010-010-0702-4 (2011).

62 Salte, R., Norberg, K., Ødegaard, O. R., Arnesen, J. A. & Olli, J. J. Exotoxin-induced consumptive coagulopathy in Atlantic salmon, *Salmo salar* L.: inhibitory effects of exogenous antithrombin and α2-macroglobulin on *Aeromonas salmonicida* serine protease. *J. Fish Dis.* **16**, 425-435, doi:10.1111/j.1365-2761.1993.tb00876.x (1993).

63 Hellman, N. E. & Gitlin, J. D. Ceruloplasmin metabolism and function. *Annu Rev Nutr* **22**, 439-458, doi:10.1146/annurev.nutr.22.012502.114457 (2002).

64 Reunanen, A., Knekt, P. & Aaran, R. K. Serum ceruloplasmin level and the risk of myocardial infarction and stroke. *Am J Epidemiol* **136**, 1082-1090, doi:10.1093/oxfordjournals.aje.a116573 (1992).

65 Mänttäri, M. *et al.* Serum ferritin and ceruloplasmin as coronary risk factors. *Eur Heart J* **15**, 1599-1603, doi:10.1093/eurheartj/a060440 (1994).

66 Ziakas, A. *et al.* Ceruloplasmin is a better predictor of the long-term prognosis compared with fibrinogen, CRP, and IL-6 in patients with severe unstable angina. *Angiology* **60**, 50-59, doi:10.1177/0003319708314249 (2009).

67 Dadu, R. T. *et al.* Ceruloplasmin and heart failure in the Atherosclerosis Risk in Communities study. *Circ Heart Fail* **6**, 936-943, doi:10.1161/CIRCHEARTFAILURE.113.000270 (2013).

68 Xu, Y., Lin, H., Zhou, Y., Cheng, G. & Xu, G. Ceruloplasmin and the extent of heart failure in ischemic and nonischemic cardiomyopathy patients. *MEDIATORS INFLAMM* **2013**, 348145, doi:10.1155/2013/348145 (2013).

69 Andreasova, T. *et al.* Evaluation of ceruloplasmin - a potential biomarker in chronic heart failure. *J. Clin. Exp. Cardiol.* **9**, doi:10.4172/2155-9880.1000601 (2018).

70 Sahoo, P. K. *et al.* Characterization of the ceruloplasmin gene and its potential role as an indirect marker for selection to Aeromonas hydrophila resistance in rohu, Labeo rohita. *Fish Shellfish Immunol* **34**, 1325-1334, doi:10.1016/j.fsi.2013.02.020 (2013).

71 Hourcade, D. E., Mitchell, L. M. & Oglesby, T. J. A conserved element in the serine protease domain of complement factor B. *J Biol Chem* **273**, 25996-26000, doi:10.1074/jbc.273.40.25996 (1998).

72 Kulkarni, P. A. & Afshar-Kharghan, V. Anticomplement therapy. *Biologics* **2**, 671-685, doi:10.2147/btt.s2753 (2008).

73 Ricklin, D., Hajishengallis, G., Yang, K. & Lambris, J. D. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* **11**, 785-797, doi:10.1038/ni.1923 (2010).

74 Pankov, R. & Yamada, K. M. Fibronectin at a glance. *J. Cell Sci.* **115**, 3861-3863, doi:10.1242/jcs.00059 (2002).

75 Wang, J., Karra, R., Dickson, A. L. & Poss, K. D. Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev. Biol.* **382**, 427-435, doi:https://doi.org/10.1016/j.ydbio.2013.08.012 (2013).
76 Liu, X. & Collodi, P. Novel form of fibronectin from zebrafish mediates infectious hematopoietic necrosis virus infection. *J. Virol.* **76**, 492-498, doi:10.1128/jvi.76.2.492-498.2002 (2002).

77 Liu, X., Zhao, Q. & Collodi, P. A truncated form of fibronectin is expressed in fish and mammals. *Matrix Biol* **22**, 393-396, doi:10.1016/s0945-053x(03)00071-4 (2003).

78 Bearzotti, M. *et al.* Fish Rhabdovirus Cell Entry Is Mediated by Fibronectin. *J. Virol.* **73**, 7703-7709, doi:10.1128/jvi.73.9.7703-7709.1999 (1999).

79 Chakravarti, S. *et al.* Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. *J Cell Biol* **141**, 1277-1286, doi:10.1083/jcb.141.5.1277 (1998).

80 Engebretsen, K. V. T. *et al.* Lumican is increased in experimental and clinical heart failure, and its production by cardiac fibroblasts is induced by mechanical and proinflammatory stimuli. *The FEBS Journal* **280**, 2382-2398, doi:10.1111/febs.12235 (2013).

81 Mohammadzadeh, N. *et al.* The extracellular matrix proteoglycan lumican improves survival and counteracts cardiac dilatation and failure in mice subjected to pressure overload. *Sci. Rep.* **9**, 9206, doi:10.1038/s41598-019-45651-9 (2019).

82 Saleh, M. *et al.* Quantitative shotgun proteomics distinguishes wound-healing biomarker signatures in common carp skin mucus in response to Ichthyophthirius multifilis. *Vet Res* **49**, 37, doi:10.1186/s13567-018-0535-9 (2018).

83 Kikuchi, K. *et al.* Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev Cell* **20**, 397-404, doi:10.1016/j.devcel.2011.01.010 (2011).

84 Flower, D. R. The lipocalin protein family: structure and function. *Biochem J* **318** (Pt 1), 1-14, doi:10.1042/bj3180001 (1996).

85 Yndestad, A. *et al.* Increased systemic and myocardial expression of neutrophil gelatinase-associated lipocalin in clinical and experimental heart failure. *Eur. Heart J.* **30**, 1229-1236, doi:10.1093/eurheartj/ehp088 (2009).

86 Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass Spectrometric Sequencing of Proteins from Silver-Stained Polyacrylamide Gels. *Anal. Chem.* **68**, 850-858, doi:10.1021/ac950914h (1996).

87 Batycka, M. *et al.* Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis. *Rapid Commun. Mass Spectrom.* **20**, 2074-2080, doi:10.1002/rcm.2563 (2006).

88 Taylor, G. K. & Goodlett, D. R. Rules governing protein identification by mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 3420-3420, doi:10.1002/rcm.2225 (2005).

**Tables**

Table 1 – Thirty seven protein sequences unique to cardiomyopathy syndrome-infected Atlantic salmon sera (CMS+), corresponding number of peptides and percentage of sequence cover (SC %) for three CMS+ sample pools (n=4 fish per pool).
| Accession       | MW [kDa] | Infected 1 | Infected 2 | Infected 3 |
|-----------------|----------|------------|------------|------------|
|                 | #Peptides| SC [%]     | #Peptides  | SC [%]     | #Peptides | SC [%] |
| NP_001265947.1  | 16.0     | 89.8       | 18         | 89.8       | 17        | 89.8   |
| XP_014048454.1  | 16.0     | 89.8       | 17         | 89.8       | 16        | 89.8   |
| XP_014022930.1  | 26.5     | 81.8       | 15         | 63.6       | 16        | 71.7   |
| NP_001135172.1  | 47.2     | 68.7       | 38         | 64.1       | 28        | 51.8   |
| XP_014019196.1  | 34.8     | 68.0       | 29         | 68.7       | 28        | 66.5   |
| XP_014049624.1  | 11.9     | 66.1       | 5          | 48.6       | 5         | 48.6   |
| XP_014047100.1  | 10.1     | 63.4       | 7          | 63.4       | 7         | 63.4   |
| NP_001133181.1  | 39.5     | 63.1       | 28         | 65.0       | 23        | 61.2   |
| XP_014048453.1  | 15.9     | 62.2       | 11         | 57.3       | 7         | 57.3   |
| XP_014048452.1  | 15.2     | 62.2       | 10         | 62.2       | 11        | 57.3   |
| NP_001266041.1  | 23.7     | 55.3       | 8          | 62.6       | 8         | 52.5   |
| XP_014013823.1  | 21.9     | 49.5       | 9          | 49.5       | 12        | 69.8   |
| XP_014034361.1  | 21.9     | 43.8       | 7          | 43.8       | 11        | 43.8   |
| XP_014011142.1  | 43.7     | 43.6       | 6          | 22.2       | 4         | 12.6   |
| XP_014012275.1  | 54.9     | 40.9       | 19         | 40.5       | 29        | 48.1   |
| XP_014046901.1  | 11.4     | 40.8       | 5          | 40.8       | 6         | 50.5   |
| XP_014050741.1  | 48.6     | 38.9       | 16         | 37.3       | 23        | 47.1   |
| NP_001135175.1  | 58.3     | 31.5       | 17         | 38.7       | 9         | 20.4   |
| NP_001133188.1  | 42.9     | 30.7       | 13         | 41.7       | 7         | 31.2   |
| Accession      | Description                                      | Peptides | SC % | SC % | SC % | SC % |
|---------------|--------------------------------------------------|----------|------|------|------|------|
| XP_014037121.1| Lipocalin-like                                    | 20.1     | 4    | 30.4 | 6    | 34.3 |
| XP_014057055.1| Apolipoprotein Eb-like                            | 35.6     | 6    | 29.7 | 8    | 34.5 |
| NP_001133769.1| Carbonic anhydrase-like                           | 28.6     | 5    | 28.5 | 7    | 43.5 |
| XP_014009124.1| Lumican-like                                      | 38.1     | 9    | 27.2 | 9    | 28.1 |
| XP_014069247.1| Alpha-2-macroglobulin like                        | 105.2    | 17   | 26.5 | 16   | 29.2 |
| XP_013984632.1| Coflin-2-like                                     | 18.7     | 3    | 25.1 | 5    | 44.3 |
| XP_014011065.1| Apolipoprotein Eb-like                            | 31.1     | 4    | 22.9 | 11   | 48.4 |
| NP_001133122.1| Glycogen phosphorylase, muscle form               | 97.4     | 15   | 21.2 | 13   | 23.2 |
| NP_001135161.1| 2-peptidylprolyl isomerase                        | 17.5     | 3    | 20.7 | 6    | 29.3 |
| XP_014024862.1| Kininogen-1-like                                  | 41.5     | 5    | 20.0 | 4    | 20.0 |
| XP_013979099.1| Ceruloplasmin                                     | 38.8     | 18   | 18.6 | 26   | 27.4 |
| NP_001158744.1| Beta-2-glycoprotein 1 precursor                  | 129.2    | 4    | 18.6 | 2    | 7.1  |
| XP_014061843.1| Fibrinogen alpha chain-like                      | 79.9     | 11   | 18.4 | 6    | 8.5  |
| XP_014049817.1| Mannose-binding protein C-like                   | 22.8     | 3    | 16.7 | 5    | 20.5 |
| XP_014051546.1| Complement factor B-like                         | 86.8     | 7    | 14.0 | 11   | 19.9 |
| XP_014063047.1| L-lactate dehydrogenase B chain                  | 36.3     | 2    | 9.0  | 2    | 9.9  |
| XP_013991158.1| Keratin type II cytoskeletal cochleal-like      | 58.2     | 2    | 4.1  | 3    | 4.1  |
| XP_014021181.1| Fibronectin-like                                 | 246.9    | 5    | 3.4  | 4    | 2.6  |

Table 2 – Ten protein sequences unique to cardiomyopathy syndrome negative Atlantic salmon sera (CMS-), corresponding number of peptides and percentage of sequence cover (SC %) for three CMS- sample pools (n=4 fish per pool).
Table 3 - Biological processes, molecular function and cellular components assigned to proteins unique to cardiomyopathy syndrome-infected Atlantic salmon sera (CMS+), identified with InterPro-Go. The proteins annotated with GO database are shown in italic.
| Description                      | Biological process                                                                 | Cellular component        | Molecular function                                                                 |
|----------------------------------|-------------------------------------------------------------------------------------|---------------------------|-----------------------------------------------------------------------------------|
| Alpha-2-macroglobulin like        | -                                                                                   | extracellular region      | endopeptidase inhibitor activity                                                  |
| Carbonic anhydrase-like           | -                                                                                   | -                         | carbonate dehydratase activity; zinc ion binding                                  |
| Ceruloplasmin                    | iron ion transport; cellular iron ion homeostasis                                   | extracellular space       | copper ion binding; ferroxidase activity                                            |
| complement factor B-like         | complement activation                                                              | extracellular region      | serine-type endopeptidase activity                                                 |
| Cofilin-2-like                   | actin filament depolymerisation                                                      | actin cytoskeleton        | actin binding                                                                     |
| Creatine kinase M-type           | phosphocreatine biosynthetic process, phosphorylation                               | -                         | creatine kinase activity; catalytic activity; kinase activity; transferase activity; transferring phosphorus-containing groups; ATP binding |
| Enolase                          | glycolytic process                                                                  | phosphopyruvate hydratase activity | magnesium ion binding; phosphopyruvate hydratase activity                          |
| Fibronectin-like                 | -                                                                                   | -                         | protein binding                                                                   |
| Fibrinogen alpha & gamma chain like | protein polymerization, platelet activation                                        | fibrinogen complex, blood coagulation | signalling receptor binding                                                       |
| Fibrinogen beta chain-like       | protein polymerization; platelet activation; blood coagulation; fibrin clot formation | fibrinogen complex        | signalling receptor binding                                                       |
| Fructose-bisphosphate aldolase A | glycolytic process                                                                  | -                         | catalytic activity; fructose-bisphosphate aldolase activity                       |
| Glycogen phosphorylase, muscle form | carbohydrate metabolic process                                                    | -                         | glycogen phosphorylase activity; pyridoxal phosphate binding; 1,4-alpha-oligoglucan phosphorylase activity |
| Haptoglobin-like                 | proteolysis                                                                         | -                         | serine-type endopeptidase activity                                                 |
| Histone H4                       | nucleosome assembly; DNA-template transcription, initiation                          | nuclear chromosome        | DNA binding; histone binding; protein heterodimerization activity                 |
| Keratin type II cytoskeletal cochleal-like | -                                                            | keratin filament          | -                                                                                 |
| Protein Name                          | Biological Process                         | Cellular Component | Molecular Function                                                                 |
|--------------------------------------|--------------------------------------------|--------------------|------------------------------------------------------------------------------------|
| Kininogen-1-like                     | -                                          | -                  | cysteine-type endopeptidase inhibitor activity                                      |
| L-lactate dehydrogenase B chain      | oxidation-reduction process                | Cytoplasm          | L-lactate dehydrogenase activity                                                   |
| Lipocalin-like                       | -                                          | -                  | small molecule binding                                                             |
| Lumican-like                         | collagen fibril organization, visual perception | collagen-containing extracellular matrix | protein binding                                                                  |
| Mannose-binding protein C-like       | -                                          | -                  | -                                                                                 |
| Parvalbumin beta 1                   | -                                          | -                  | calcium ion binding                                                               |
| Pyruvate kinase PKM-like             | glycolytic process                        | -                  | potassium ion binding; pyruvate kinase activity; magnesium ion binding; catalytic activity; kinase activity |
| Retinol-binding protein 4-B          | retinol transport                          | -                  | retinoid binding, retinol transmembrane transporter activity                        |
| Serine protease-like protein         | Notch signalling pathway, complement activation | extracellular space | serine-type endopeptidase activity                                                 |
| Sex hormone-binding globulin         | -                                          | -                  | -                                                                                 |
| Triosephosphate isomerase B          | gluconeogenesis                            | -                  | triose-phosphate isomerase activity                                                |
| 2-peptidylprolyl isomerase           | protein folding, protein peptidyl-prolyl isomerization | -                  | peptidyl-prolyl cis-trans isomerase activity                                         |

Table 4 – Biological processes, molecular function and cellular components assigned to proteins unique to healthy Atlantic salmon sera, identified with InterPro-Go. The proteins annotated with GO database are shown in italic. Healthy serum unique proteins assigned to biological process, molecular function and cellular component with GO database.
| Description                                      | Biological process                        | Cellular component               | Molecular function                      |
|-------------------------------------------------|-------------------------------------------|----------------------------------|----------------------------------------|
| C-type lectin domain family 4 member E-like     | defence response to Gram-negative bacterium | integral component of membrane   | carbohydrate binding                   |
| fucolectin-6-like isoform X2                    | -                                         | -                                | -                                      |
| type-4 ice-structuring protein LS-12-like       | lipid transport; lipoprotein metabolic process | extracellular region            | lipid binding                          |
| complement factor H-like                        | -                                         | -                                | -                                      |
| histidine-rich glycoprotein-like                | negative regulation of endopeptidase activity | Cell; sperm flagellum            | protease binding                       |
| protein LEG1 homolog                            | -                                         | extracellular region             | cysteine-type endopeptidase inhibitor activity |
| ladderlectin-like                               | -                                         | -                                | carbohydrate binding                   |

**Figures**

**Leakage enzymes**
- Creatine kinase
- Lactate dehydrogenase
- Glycogen phosphorylase
- Carbonic anhydrase

**Host reaction**
- Haptoglobin*
- Fibrinogen*
- Kininogen*
- α2-macroglobulin*
- Ceruloplasmin*
- Complement factor B
- Serine protease-like
- Kininogen

**Regeneration/Remodelling**
- Fibronectin
- Lumican
- Retinol-binding
- Lipocalin

**Figure 2**

Summary of candidate biomarkers from a list of proteins unique to CMS+ sera, listed by broad type. *acute phase response proteins; +complement proteins.