Proteomic characterisation of serum proteins from Atlantic salmon (*Salmo salar* L.) from an outbreak with Cardiomyopathy Syndrome

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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 a CMS outbreak (3 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 fish, while twenty seven proteins were unique to the sera of CMS fish. 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, α 2-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 (Fritvold et al., 2019). 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 (Fritvold, 2016). Overall, the economic impact of CMS is substantial, with estimated losses of a single CMS outbreak cases costing up to 1.9 million € in Norway (Garseth et al., 2018). 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 (Ferguson et al., 1990, Brun et al., 2003). 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 (Amin et al., 1988) and subsequently termed CMS by Ferguson et al. (1990) to account for the associated pathology. In Norway, the term "acute heart failure" has also been associated with the disease. Instances of CMS have also been reported in the Faroe Islands (1984), Scotland (1997) and Ireland (2012) (Ferguson et al., 1990, Poppe et al., 2003, Rodger et al., 2014). The aetiological cause of CMS was not established until 2009, when it was confirmed to be transmissible (Bruno et al., 2009, Fritsvold et al., 2009), and caused by a virus (Løvoll et al., 2010), which was subsequently named piscine myocarditis virus or PMCV (Haugland et al., 2011).

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 (Haugland et al., 2011). 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 (Garseth et al., 2018). The fact that PMCV is non-culturable has been a major factor in restricting research related to both CMS and PCMV.

Diagnosis of viral diseases in aquaculture has traditionally been performed by detecting the pathogen with molecular methods or by isolating the virus in cell culture. Diagnosis of CMS is based on detection of viral RNA by quantitative polymerase chain reaction (qPCR) in infected heart tissue or by histopathology, observing pathological changes in heart tissue typical of CMS. Both methods require lethal sampling of fish, frequently at the final stages of their production cycle. This diagnostic method brings limited information when used on its own and is often used with histopathology to assess lesion severity. Further, both methods are applied to a limited number of individuals within large populations, providing rather fragmented information at a population level.

A non-lethal diagnostic method that provides an early indication of PMCV infection (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 (Mayeux, 2004). 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 to assess heart disease 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. Special relevance has been placed on CK and LDH, as CK levels are elevated in pancreas disease (Rodger et al., 1991), and both CK and LDH are significantly increased in CMS and HSMI, although significant correlation with histopathological lesions was only seen for HSMI (Yousaf et al., 2012). Increased levels of CK have also been associated with muscle injury in salmon during infection by infectious salmon anaemia virus (ISAV) (Rojas et al., 2018)

As the cardiac disease biomarker repertoire research in salmon is limited, and host responses to cardiac disease have not been fully characterised in salmon, there is scope to identify additional novel serum biomarkers of heart disease in salmon using serum proteomics comparing healthy and diseased individuals. Identifying and understanding these differences is important for establishing novel biomarkers of disease (Barbosa et al., 2012). This is valuable information, 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 (Byrnes et al., 2018). One of the most common methods used to identify novel biomarkers is mass spectrometry (MS) of fractionated protein samples (shotgun approach) (Ahrens et al., 2010). 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 (Geyer et al., 2017). In fish, this approach has been helpful for the identification of potential biomarkers for arsenic-exposure in carp (*Labeo rohita*) (Banerjee et al., 2017) or for bitumen-exposure in Pacific salmon (*Onchorhynchus nerka*) (Alderman et al., 2017).

Here we examine the differential expression of proteins in the serum of field samples from a confirmed CMS outbreak compared to that of 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.

Materials And Methods

2.1 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 from one pen on the CMS+ site and from 12 at the control site. The blood was allowed to clot at ambient temperature and serum was collected within 3h post sampling, by centrifuging the blood for 3m 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.

The disease status of fish at each farm site was assessed by gross pathology, cardiac histopathology, and PMCV specific RT-qPCR (heart and/or serum). Gross pathology and histopathology were conducted using standard methods, and the RT-qPCR was provided by Pharmaq Analytiq (Bergen, Norway). Fish were classified as CMS+ if at least two of the disease diagnose methods (gross pathology, histology and RT -qPCR) were consistent with CMS and PCMV infection respectively, and as CMS- if this was not the case. Of the 44 CMS+ sera samples, 12 (4 fish x 3 pools) were selected for analysis by proteomics. The hearts of 8 of these 12 CMS+ fish (Table 1) were not analysed by histology or RT-qPCR since the gross pathology was so severe that these hearts were used in a parallel study that aimed to isolate PMCV by cell culture.

2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Pools of sera were used to reduce variability between individual fish. Three CMS+ sera pools (CMS+11, CMS+2 2, ICMS+ 3; n=4 CMS+ fish/pool) and three CMS- sera pools (CMS- 1, CMS- 2, CMS- 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 29.6 ($\pm$ 6.2)mg ml⁻¹ and 72.8 ($\pm$ 5.6) 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 (20µL 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.

2.3 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 (Shevchenko et al., 1996). 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 Chromeleon™ chromatography software was used to perform the liquid chromatography analysis. A micro-pump with a flow rate of $246 \mu\text{l min}^{-1}$ was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of $3 \mu\text{l min}^{-1}$ through a 5 cm x 200 mm ID monolithic reversed phase column (Thermo) maintained at 50°C. The samples (4 ml) 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 3ml 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 ml 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. (2006).

2.4 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™ V2.3 (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. (2005) 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 ^{13}C 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 a minimum of four contiguous amino acid residues.

2.5 Data analysis

Proteins identified in the three CMS+ pools were compared in order to select proteins common to all infected samples pools. 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 IntrePro 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.

Results

3.1 Sample diagnostics

Forty four fish were sampled from a pen in a farm with a known ongoing CMS outbreak. The sera 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. Conversely, all 12 CMS- fish were clinically normal, devoid of macroscopic lesions and were negative by the RT-qPCT and histopathology (Suppl. Table 1).

3.2 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 49-28k Da and 28-14 kDa.

3.3 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, with 85 proteins found in common between the three pools. Of these 85 proteins, 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. Of these, 57 protein sequences were common to all three pools, 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.

3.4 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 2 and Suppl. Table 2) and 10 unique to CMS- pools (Table 3 and Suppl. Table 3).

Of the 37 proteins unique to CMS+ pools, 8 (21.6%) were identified with over 60 % sequence coverage in the 3 CMS+ pools, where 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. $\alpha=2$, $\beta=3$), 3 as fibrinogen ($\alpha=1$, $\beta=1$, $\gamma=1$), 2 as apolipoprotein-Eb-like and 2

as retinol-binding. Therefore, 29 protein sequences unique to CMS+ pools were then 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.

Discussion

Cardiomyopathy syndrome is a viral disease of Atlantic salmon, which causes severe chronic cardiac lesions in various regions of the heart tissues. 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 of cardiac diseases such as myocardial infarction (MI) and heart failure (HF), is heavily based on individual profiling of protein biomarkers (Smith et al., 2017), which allow diagnosis of subclinical/prodromic presentations (Mayeux, 2004). As defined by the Biomarkers Definition Working Group (2001), “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 (Ahrens et al., 2010). This analysis is usually performed using liquid chromatography-mass spectrometry (LC-MS/MS), a qualitative technique capable of analysing complex samples such as serum (Zhou et al., 2020). The scope of this work was, in a preliminary way to study if it was possible to observe proteomic differences between CMS infected and non-infected fish. In this study, we used a shotgun approach to identify potential biomarkers for the disease (Ahrens et al., 2010), by comparison of CMS+ (diseased) and CMS- (clinically healthy) sera. This method is faster and more affordable than other proteomics such as Mascot, and within its limitations in sensitivity and depth proved to be useful by permitting to highlight the proteomic differences between CMS+ and CMS- fish. 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 (Barbosa et al., 2012).

The proteome analysis of fish serum has been performed with pool samples ((Li et al., 2016, Alderman et al., 2017). Due to an expected large individual variability the samples were analysed as pools of sera (n=4 fish/pool). The use of serum pools makes impossible to access individual characteristics such as viral load or clinical state. However since the aim of this study was the proteomics characterisation of CMS+ and CMS- as a group and not individual characterisation or correlation with disease status, the use of serum pools is suitable. Only common proteins to each pool type (CMS+/CMS-) were taken forward for analysis. The three CMS+ pools, with 136, 136 and 158 proteins identified, just 85 were common between the 3 pools. This considerable low level of common proteins was expected when using a complex biological sample as serum, a relatively low sensitive method and the application of the strict validation regime used. 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 (Kemp et al., 2004). These are all cardiomyocyte cytoplasmic enzymes found in healthy cardiomyocytes that leak into the serum during cardiomyocyte damage (leakage enzymes). Other candidate biomarkers were seen, and all candidates are summarized in Figure 2, which includes the aforementioned leakage proteins, but also proteins involved in host reaction to disease, regeneration/remodelling and energy metabolism.

This study did not identify well known cardiac biomarkers such as troponins. This is due to a combination between serum being an extreme complex biological sample, with proteins present over a huge dynamic range, and the sensitivity of the technique employed. A more comprehensive characterisation of serum proteomes would require the use of depletion methods or extensive fraction techniques, which was not under the scope of this study.

4.1 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 (Lippi et al., 2013, Bodor, 2016). 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 (Cabaniss, 1990). 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 et al. (1991) for pancreas disease (PD), a viral disease causing heart and muscle lesions in Atlantic salmon. This was supported by serum proteomics by Braceland et al. (2013), 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 (Yousaf et al., 2012). 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 (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 (Khan et al., 2020, Klein et al., 2020). In fish, LDH has been used in toxicological, and infectious disease assessment settings in a range of species other than Atlantic salmon (Oliveira et al., 2013, Ajima et al., 2015, Elia et al., 2017, Kumar et al., 2018). Importantly, Yousaf et al. (2012) 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 (David et al., 1986). 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 (Zhu et al., 2018) and diagnosis of acute myocardial injury (Lippi et al., 2013). In fish, an increase in serum GP has been noted in starved Atlantic salmon (Sundby et al., 1991) and in *Claria bratrachus*, it has been suggested as a biomarker of cypermethrin toxicity (Begum, 2009).

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 (Lindskog, 1997). All CA isoenzymes have been used as biomarkers of neoplastic, infectious, parasitic and degenerative disease affecting a wide range of organ systems (Zamanova et al., 2019). 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 (Vuotikka et al., 2003). Interestingly, recent work has revealed membrane bound CA IV in all cardiac chambers of coho salmon (*Oncorhynchus kisutch*), which is believed to facilitate oxygen unloading from venous blood, therefore facilitating oxygenation of a heart without coronary circulation (Alderman et al., 2016). 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.

4.2 Host reaction proteins

This subgroup of proteins unique to CMS+ sera includes acute phase response proteins (haptoglobin, fibrinogen, α 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 (Latimer, 2011). 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 (Kumar et al., 2014). 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 (Andersen et al., 2012). It has been used as a diagnostic biomarker for neurological disease, diabetes, certain cancers and bovine mastitis (Nirala et al., 2020). It is also an important biomarker for humans at risk of acute MI, stroke and HF (Holme et al., 2009, Haas et al., 2011). In fish viral disease, Cordero et al. (2017) found variability in haptoglobin expression depending on the fish and viral species. In gilthead seabream (*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 (*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 *Caligus rogercresseyi* (Valenzuela-Muñoz et al., 2017). The presence of haptoglobin in CMS+ sera in our study, suggests that haptoglobin is a candidate biomarker for CMS in Atlantic salmon.

An acute phase protein used to predict stroke and MI in human patients, fibrinogen, was also identified in CMS+ sera. Fibrinogen is an acute phase protein (Jain et al., 2011) 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 (Garseth et al., 2018), 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 (Weisel et al., 1994). In fact, increased levels of kininogen in plasma and tissues are linked with injury, inflammation, myocardial infarction, and diabetes (Wong, 2016). 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 (Wu et al., 2005, Patel, 2017). In our study, gene ontology assigned the molecular function of the serine protease-like protein as "serine-type endopeptidase activity", which is synonymous to "blood coagulation factor activity" (<http://geneontology.org/>, accessed 01/06/2020).

The overall picture of coagulopathy in CMS+ fish is also complemented by the presence of Alpha-2 macroglobin (α_2 -M) in CMS+ serum, as this is an inhibitor of thrombin and plasmin (de Boer et al., 1993). 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 (Ramasamy et al., 2006, Yoshino et al., 2019). The presence of higher levels of cardiac isoform α_2 -M is beneficial during the early onset of myocardial damage associated with diabetes, due to its protease inhibitor function and antioxidant effect (Soman et al., 2011). When Atlantic salmon were pre-treated with α_2 -M, then injected with a serine protease from *Aeromonas salmonicida*, α_2 -M was found to inhibit the serine protease by preventing the pro-coagulant effect of the enzyme (Salte et al., 1993).

Ceruloplasmin carries copper and oxidizes iron, facilitating its inclusion in transferrin (Hellman et al., 2002). It has been detected in high levels in patients with myocardial infarction, heart failure, ischemic and non-ischemic cardiomyopathy, coronary heart disease, arteriosclerosis or angina (Reunanen et al., 1992, Mänttari et al., 1994, Ziakas et al., 2009, Dadu et al., 2013, Xu et al., 2013). In fact, high levels of ceruloplasmin correlate with the severity of cardiac failure and non-ischemic cardiomyopathy (Xu et al., 2013, Andreasova et al., 2018). In fish, this protein has been investigated as a possible biomarker of bacterial resistance in fish (Sahoo et al., 2013). This is possibly due to its role in reducing iron availability to the bacteria.

Protein complement factor B, usually known as factor B, is a serine protease (Hourcade et al., 1998), part of the alternative complement system pathway (Kulkarni et al., 2008, Ricklin et al., 2010). 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.

4.3 Regeneration/remodelling

Cardiomyopathy syndrome in Atlantic salmon frequently presents as a chronic cardiac lesion that leads to acute cardiac failure (Garseth et al., 2018). 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 (Pankov et al., 2002). This is a protein known to be actively involved in epicardium regeneration in zebrafish after cardiac injury (Wang et al., 2013). Importantly, there is evidence that cardiac regeneration occurs in CMS+ Atlantic salmon (Garseth et al., 2018). 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 (Liu et al., 2002, Liu et al., 2003). Bearzoti et al. (1999) suggested that fibronectin mediates 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 (Liu et al., 2002). 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 (Chakravarti et al., 1998), and is also present in CMS+ serum. Engebretsen et al. (2013) 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 (Mohammadzadeh et al., 2019). In fish, lumican has been identified in the skin mucus of carp (*Cyprinus carpio*) infected with *Ichthyophthirius multifiliis* (Saleh et al., 2018). 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 (Kikuchi et al., 2011). 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) (Flower, 1996). In the study of Yndestad et al. (2009), 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.

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 suggests shotgun LC-MS/MS has a genuine potential as a rapid, low cost technique for preliminary identification of candidate cardiac disease biomarkers of viral disease of farmed Atlantic salmon. Fuller assessment of the proteins identified here is needed to establish their usefulness as biomarkers to assess the disease status of CMS-affected Atlantic salmon.

Declarations

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Data availability

The raw proteomic data can be shared if requested. All the analysed data are compiled and presented in the tables in the manuscript and in the supplemented data file.

Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1 – Fish disease status assessed by gross pathology, histology and RT-qPCR (heart and serum) of each individual sample pooled to be analysed by proteomics.

Proteomic samples	Pathology	RT-qPCR (Ct values)		
		Gross signs	Histology	Heart Serum
CMS- 1	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
CMS- 2	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
CMS- 3	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
	Neg	Neg	§	§
CMS+ 1	Pos	NP	NP	28.5
	Pos	NP	NP	30.3
	Pos	NP	NP	30.8
	Pos	NP	NP	30.1
CMS+ 2	Pos	NP	NP	33.2
	Pos	NP	NP	34.0
	Pos	NP	NP	29.9
	Pos	NP	NP	31.6
CMS+ 3	Pos	Pos	15.6	31.3
	Pos	Pos	17.7	30.2
	Pos	Pos	18.3	§
	Pos	Pos	15.7	30.8

NP - not performed

§ negative since the RT-qPCR result is "not determined"

Table 2 – 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 [%]
XP_014069247.1	Alpha-2-macroglobulin like	105.2	17	26.5	16	29.2	16	25.8
XP_014057055.1	Apolipoprotein Eb-like	35.6	6	29.7	8	34.5	8	30.1
XP_014011065.1	Apolipoprotein Eb-like	31.1	4	22.9	11	48.4	9	40.0
NP_001158744.1	Beta-2-glycoprotein 1 precursor	129.2	4	18.6	2	7.1	7	27.4
NP_001133769.1	Carbonic anhydrase-like	28.6	5	28.5	7	43.5	7	33.1
XP_013979099.1	Ceruloplasmin	38.8	18	18.6	26	27.4	15	17.3
NP_001133188.1	Creatine kinase M-type	42.9	9	30.7	13	41.7	7	31.2
XP_013984632.1	Cofilin-2-like	18.7	3	25.1	5	44.3	4	37.1
XP_014051546.1	Complement factor B-like	86.8	7	14.0	11	19.9	7	14.8
NP_001135172.1	Enolase	47.2	33	68.7	38	64.1	28	51.8
XP_014012275.1	Fibrinogen beta chain-like	54.9	18	40.9	19	40.5	29	48.1
XP_014050741.1	Fibrinogen gamma chain like	48.6	17	38.9	16	37.3	23	47.1
XP_014061843.1	Fibrinogen alpha chain-like	79.9	11	18.4	6	8.5	9	12.6
XP_014021181.1	Fibronectin-like	246.9	5	3.4	4	2.6	4	2.6
NP_001133181.1	Fructose-bisphosphate aldolase A	39.5	26	63.1	28	65.0	23	61.2
NP_001133122.1	Glycogen phosphorylase, muscle form	97.4	15	21.2	13	23.2	5	8.5
XP_014019196.1	Haptoglobin – like	34.8	31	68.0	29	68.7	28	66.5
NP_001265947.1	Haemoglobin subunit beta 1	16.0	18	89.8	18	89.8	17	89.8
XP_014048454.1	Haemoglobin subunit beta 1 – like	16.0	17	89.8	17	89.8	16	89.8

XP_014048453.1	Haemoglobin subunit alpha	15.9	11	62.2	11	57.3	7	57.3
XP_014048452.1	Haemoglobin subunit alpha-4	15.2	9	62.2	10	62.2	11	57.3
XP_014047100.1	Haemoglobin subunit beta	10.1	6	63.4	7	63.4	7	63.4
XP_014046901.1	Histone H4	11.4	5	40.8	5	40.8	6	50.5
XP_014063047.1	L-lactate dehydrogenase B chain	36.3	2	9.0	2	9.9	9	35.0
XP_014037121.1	Lipocalin-like	20.1	4	30.4	6	34.3	5	30.4
XP_014009124.1	Lumican-like	38.1	9	27.2	9	28.1	7	21.3
XP_013991158.1	Keratin type II cytoskeletal cochlear-like	58.2	2	4.1	3	4.1	3	4.1
XP_014024862.1	Kininogen-1-like	41.5	5	20.0	4	20.0	5	25.6
XP_014049817.1	Mannose-binding protein C-like	22.8	3	16.7	5	20.5	3	16.7
XP_014049624.1	Parvalbumin beta 1	11.9	7	66.1	5	48.6	5	48.6
NP_001135161.1	2-peptidylprolyl isomerase	17.5	3	20.7	6	29.3	7	42.1
NP_001135175.1	Pyruvate kinase PKM-like	58.3	13	31.5	17	38.7	9	20.4
NP_001266041.1	Serine protease-like protein	23.7	8	55.3	8	62.6	8	52.5
XP_014011142.1	Sex hormone-binding globulin	43.7	13	43.6	6	22.2	4	12.6
XP_014013823.1	Retinol-binding protein 4-B	21.9	10	49.5	9	49.5	12	69.8
XP_014034361.1	Retinol-binding protein 4 isoform X1	21.9	7	43.8	7	43.8	11	43.8
XP_014022930.1	Triosephosphate isomerase B	26.5	18	81.8	15	63.6	16	71.7

Table 3 – 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).

		Non-infected 1		Non-infected 2		Non-infected 3	
Accession no.		#Peptides	SC [%]	#Peptides	SC [%]	#Peptides	SC [%]
XP_013999412.1	Apolipoprotein B-100 isoform X4	69	26.4	68	24.8	72	31.5
XP_013979020.1	Complement factor H-like	12	34.5	12	37.1	16	53.3
XP_014018408.1	Fucolectin-6-like	8	42.0	6	21.8	9	39.6
XP_014059764.1	Haemoglobin subunit beta-1-like	13	72.8	12	72.8	15	72.8
XP_013979096.1	Histidine-rich glycoprotein-like	21	40.6	22	40.4	27	41.2
XP_014005720.1	Ladderlectin-like	5	33.9	9	53.2	5	39.8
NP_001117052.1	Lectin C type receptor B	5	18.8	6	22.9	9	38.3
XP_013999800.1	LEG1 homolog	5	17.0	6	19.0	10	33.5
XP_014060522.1	Protein LEG1 homolog	5	12.1	8	17.4	9	24.0
XP_014019752.1	Type-4 ice-structuring protein LS-12-like	5	38.6	6	44.8	6	54.5

Figures

Leakage enzymes	Host reaction	Regeneration/ Remodelling
Creatine kinase Lactate dehydrogenase Glycogen phosphorylase Carbonic anhydrase	Haptoglobin [§] Fibrinogen [§] Kininogen [§] α2-macroglobulin [§] Ceruloplasmin [§] Complement factor B ⁺ Serine protease-like Kininogen	Fibronectin Lumican Retinol-binding Lipocalin

Figure 1

Protein profiles of sera sampled from Atlantic salmon (*Salmo salar* L.): consistent with cardiomyopathy syndrome (CMS+) and negative for cardiomyopathy syndrome (CMS-), run on a 12% NuPAGE Bis-Tris SDS-PAGE gel and

stained with Coomassie blue. CMS+ pools diluted two fold (n=4 fish per pool) are in Lane 2 - CMS+1; Lane 3 - CMS+2; Lane 4 - CMS+3. CMS- pools diluted 1:16 (n=4 fish per pool) are in Lane 5 - CMS-1; Lane 6 CMS-2 and Lane 7 - CMS-3. Lane 1 is molecular weight (Mwt).

Figure 2

Summary of candidate biomarkers from a list of proteins unique to CMS+ sera pools (4 fish/pool), listed by broad type. *acute phase response proteins; +complement proteins.

Supplementary Files

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