Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences (PARAFAG)

Philosophiae Doctor (PhD)
Thesis 2021:78

# Pathogenesis studies of cardiomyopathy syndrome (CMS) in Atlantic salmon, *Salmo salar* L.

## Studier av patogenese ved kardiomyopatisyndrom (CMS) hos atlantisk laks, *Salmo salar* L.

Camilla Fritsvold

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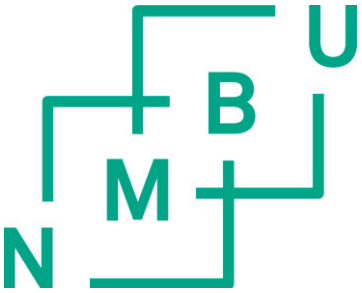
Ås, 2021

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“You never fail until you stop trying”

*Albert Einstein*

# I Table of Contents

# II Acknowledgements

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Camilla Fritsvold
*Frogner, 17. September 2021*

# III Abbreviations and definitions

bp = Basepairs
CMS = Cardiomyopathy syndrome
cDNA= Complementary DNA produced by a reverse transcriptase enzyme from mRNA and RNA
DNA = Deoxyribo-nucleic-acid
Epitope = the specific site of an antigen to which an antibody binds
HSMI = Heart and skeletal muscle inflammation
HE = Haematoxylin and eosin staining of slides for histology
ILAB = The Industrial and Aquatic Laboratory, Bergen, Norway
IHC = Immunohistochemistry
i.p. = Intraperitoneal, into the abdominal cavity
IPN = Infectious pancreatic necrosis
ISA = Infectious salmon anaemia
ISAV = Infectious salmon anaemia virus, causes ISA
ISH = *In situ* hybridisation
NMBU = Norwegian University of Life Sciences
NVI = Norwegian Veterinary Institute
PCR = Polymerase chain reaction
PD = Pancreas disease
PMCV = Piscine myocarditis virus
PRV = Piscine orthoreovirus (causing HSMI)
QTL = quantitative trait loci (a genetic marker for a trait, i.e. CMS-mortality)
RNA = Ribonucleic acid
Real-time RT-PCR = Real-time reverse-transcription polymerase chain reaction
RT-PCR = Reverse-transcription polymerase chain reaction
SAV = Salmonid alphavirus (SAV2 and SAV3 causes PD)
Site/location = aquaculture farm with cages for fish in the water
SPDV = Salmon pancreas disease virus (see SAV)

# IV Summary

Cardiomyopathy syndrome (CMS) in farmed Atlantic salmon, *Salmo salar* L., is an infectious viral disease causing severe inflammation in the cardiac spongiosum of atrium and ventricle of the salmonid heart. The disease was first described in 1985 in Norway, and is now wide-spread along the Norwegian coast. CMS usually affects large fish in their last year at sea, hence the economic impact of a moderately increased mortality or outbreaks of CMS with high mortality, is large. Together with salmon lice infestation, CMS is now considered one of the most important problems for Norwegian aquaculture, reflected both in the annual number of diagnoses and the economic losses due to mortality, and is also an increasing problem in other fish farming countries on the northern hemisphere, like Scotland, UK, Ireland and the Faroe Islands.

This study was divided into three parts. First, a challenge model for CMS in Atlantic salmon post-smolts was established, then a field outbreak of CMS in a population of young Atlantic salmon was followed until slaughtered and, finally, different methods and sampling material of CMS diagnostics were compared in another challenge trial.

Until 2010/2011, the aetiology of the disease was unknown, and several hypothesis excisted, ranging from CMS as a physiological condition, to CMS being caused by environmental factors or to CMS having an infectious, probably viral cause. In this study, we present the results of the first successful experimental trial, confirming the transmissibility of CMS. Unvaccinated post-smolts of Atlantic salmon were intraperitoneally (i.p.) injected with tissue homogenate made of cardiac and kidney tissue of large, farmed Atlantic salmon with CMS, and cardiac lesions in accordance with earlier descriptions of CMS in large, farmed Atlantic salmon developed in the experimental fish. Despite a very long observation period, the prevalence and severity of the cardiac lesions continued, and no indications of healing were observed.

In our next study, a population of young Atlantic salmon diagnosed with typical CMS only few months after transferred to sea was followed with several samplings until

slaughtered almost 10 months later. The level of PMCV specific RNA was unusually high, and both prevalence and load remained high through the observation period. Cardiac tissue was indisputable the tissue of choice when sampling for detection of PMCV specific RNA in these late, severe stages of CMS, and no difference in load was found between the three sampled compartments atrium, ventricular spongiosum and ventricular compactum. CMS was not diagnosed in any of the other five cages at the site, before 10 weeks after slaughtering of the study cage. The detected PMCV variant was similar to previous sequenced PMCV of Norwegian outbreaks.

In the last study, pre-smolt Atlantic salmon were i.p. challenged. Histopathology and real-time RT-PCR results were compared to results of real-time RT-PCR of blood and mucus, of immunohistochemistry (IHC) and of a new RNAscope *in situ* hybridisation (ISH) method. The results confirmed previous findings of the heart as the organ of choice for virus detection at later time points. Detection of PMCV specific RNA in plasma at early time points is indicating a viremic phase, and mid-kidney also showed a relatively high load at these time points. RNAscope ISH was shown to be both a more sensitive and robust method for *in situ* detection of PMCV compared to IHC, and be a valuable support to histopathology in CMS diagnostics in early screening and cases of untypical lesions or mixed infections.

# V Norsk sammendrag

Kardiomyopatisyndrom (CMS) i atlantisk oppdrettslaks, *Salmo salar* L., er en infeksiøs virussykdom som forårsaker alvorlig betennelse i laksehjertets indre lag, spongiosum, både i atrium og ventrikkel. Sykdommen ble første gang beskrevet i Norge i 1985, og er nå utbredt langs hele norskekysten. CMS rammer oftest stor laks det siste året de står i sjøen, og moderat økt dødelighet eller CMS-utbrudd gir derfor store økonomiske konsekvenser. Sammen med lakselus, anses CMS nå som en av de viktigste problemene for norsk oppdrettsnæring, noe som gjenspeiles i et høyt antall diagnoser pr. år, og summen av de økonomiske tapene p.g.a. dødelighet. CMS er et økende problem også i andre oppdrettsnasjoner på den nordlige halvkule, som Skottland, resten av Storbritannia, Irland og Færøyene.

Denne studien var tredelt. Først ble det etablert en smittemodell for CMS i post-smolt av atlantisk laks, før et feltutbrudd av CMS i ung atlantisk laks ble fulgt tett fram til utslakting av fisken. Til slutt ble prøvemateriale fra et smitteforsøk brukt i en sammenlignende studie av ulike diagnostiske metoder og prøvetyper for CMS-diagnostikk.

Fram til 2010/2011 var etiologien til sykdommen ukjent, og flere hypoteser var framsatt, og spente fra CMS som en fysiologisk tilstand, via at CMS var forårsaket av miljøfaktorer til at CMS var en smittsom sykdom, trolig forårsaket av virus. I denne studien presenterer vi resultatene fra det første vellykkete smitteforsøket som viser at CMS er en overførbar sykdom. Uvaksinerte post-smolt av Atlantisk laks ble injisert intraperitonealt (i.p.) med vevshomogenat laget av hjerte- og nyrevev fra stor oppdrettslaks med CMS, og forsøksfisken utviklet hjerteforandringer i tråd med tidligere beskrivelser av CMS i stor oppdrettslaks. Tross en svært lang observasjonsperiode, holdt både prevalens og alvorlighetsgraden av hjertelesjonene seg, og det ble ikke sett tegn på tilheling.

I vår neste studie ble en populasjon av ung atlantisk laks diagnostisert med typisk CMS bare få måneder etter sjøsetting, og denne fiskegruppen ble fulgt med flere

prøveuttak fram til utslakting nesten 10 måneder senere. Mengden PMCV spesifikt RNA påvist var uvanlig høy, og både prevalens og mengde holdt seg gjennom hele observasjonsperioden. Hjertevev var uten tvil det foretrukne vevet ved prøvetaking for påvisning av PMCV spesifikt RNA i slike sene, alvorlige stadier av CMS, og det ble ikke påvist forskjell i mengde mellom atrium, ventrikkelens spongiosum eller ventrikkelens kompaktum. CMS ble ikke diagnostisert i noen av de fem andre merdene på lokaliteten før en nabomerd fikk en CMS –diagnose 10 uker etter at den undersøkte merden var slaktet ut. PMCV-varianten liknet tidligere sekvenserte PMCV fra norske utbrudd.

I den siste studien ble pre-smolt av atlantisk laks smittet i.p.. Histopatologiske og real-time RT-PCR-undersøkelser av vevsprøver ble sammenlignet med resultater fra real-time RT-PCR-undersøkelser av blod og slim, fra immunhistokjemi (IHK) og fra en ny RNAscope *in situ* hybridiseringsmetode (ISH). Resultatene bekrefter tidligere funn av hjertevev som best egnet for prøvetaking sent i sykdomsforløpet. Påvisning av PMCV spesifikt RNA i plasma i tidlige prøveuttak indikerer en viremisk fase, og det var relativt mye i midt-nyre også på disse tidspunktene. RNAscope ISH-metoden var både mer sensitiv og robust for *in situ*-påvisning av PMCV enn IHK-metoden, og kan være et verdifullt supplement til histopatologi i CMS-diagnostikk i tidlig-fase screening og i tilfeller med utypiske hjerteforandringer eller blandete infeksjoner.

# VI List of papers

## PAPER I

Fritsvold, C., Kongtorp, R. T., Taksdal, T., Orpetveit, I., Heum, M., & Poppe, T. T. (2009).
**Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar***
Diseases of Aquatic Organisms, 87, 225–234.
DOI: 10.3354/dao02123

## PAPER II

Fritsvold, C., Mikalsen, A. B., Poppe, T., Taksdal, T. & Sindre, H.
**Characterization of an outbreak of cardiomyopathy syndrome (CMS) in young Atlantic salmon, *Salmo salar* L.**
Manuscript in press, Journal of Fish Diseases (September 2021).
DOI: 10.1111/jfd.13521.

## PAPER III

Fritsvold, C., Mikalsen, A. B., Haugland, Ø, Tartor, H. & Sindre, H.
**Characterisation of early phases of cardiomyopathy syndrome (CMS) pathogenesis in Atlantic salmon (*Salmo salar* L.) through various diagnostic methods**
Manuscript ready for submission

# 1 Introduction

## 1.1 Farming of Atlantic salmon in Norway

Since the very beginning of farming of Atlantic salmon (*Salmo salar* L.) in the late 1960s and early 1970s, Norwegian aquaculture has grown into a large and technically complex industry.

As the salmon prefers sea water temperatures between +8 and +14°C, and experience increased stress above +16°C [1], most farming are restricted geographically to cooler temperate climatic zones of both hemispheres, and the largest salmon producing countries today are Norway, Chile, United Kingdom, Faroe Islands, Canada, Australia (Tasmania), Ireland, USA, Russia and Iceland.

On annual basis, Norway has the largest production of Atlantic salmon of the world. In 2020, ~1.4 million metric tonnes of Norwegian Atlantic salmon worth almost 65 milliards (eng., $10^9$) NOK (~6,5 milliards (eng., $10^9$) €), were produced, of which 1,3 million metric tonnes were exported, mostly as fresh, whole fish. The main market for Norwegian Atlantic salmon is Europe, but also USA, Japan, South Korea and China are important markets. 162 companies is involved in production of salmonids (both Atlantic salmon and Rainbow trout) in Norway, of which there are ten major companies that in total slaughter about 2/3 of all fish [2].

In 2020, 986 sea water sites producing Atlantic salmon or Rainbow trout (*Oncorhynchus mykiss*) for consumption were registered in Norwegian aquaculture, most of these producing Atlantic salmon [2].

Compared to 2019, preliminary harvest statistics for 2020 indicate a 2.7 % increase of the production, a slightly smaller increase compared to the previous years. However, the biomass in the sea at the end of year 2020, increased by almost 100 000 tons, implying a considerably increase in total production for 2021 [2, 3].

Approximately 300 million smolts were put to sea in 2020. Of the 60.3 million Norwegian farmed Atlantic salmon registered as lost between transferred to sea and harvested (Figure 1), 52.1 million (86.4 %) were mortality-related, - almost as high as the record number of 2019 [2]. The major cause of mortality is infectious diseases, especially viral diseases related to horizontal transfer routes and lack of efficient vaccines.

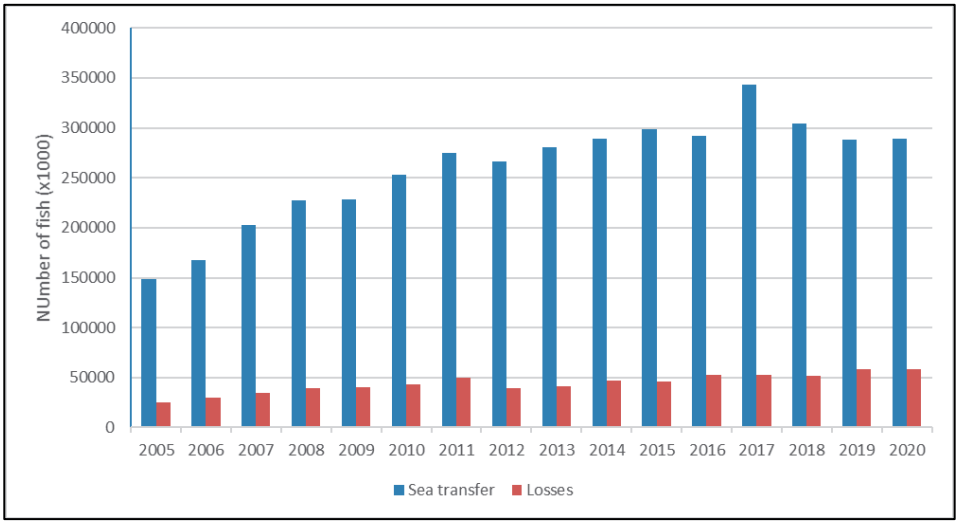


**Figure 1** Losses in seawater compared to number of Atlantic salmon transferred to sea water from 2005 to 2020 [2].

## 1.2 Health situation in Norwegian salmon farming

In the 1970s and 1980s, the dominating health problem in Norwegian salmon farming was bacterial diseases [4], and i.e. furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), vibriosis (*Vibrio anguillarum*, *Vibrio ordalii*) and cold-water vibriosis (*Vibrio salmonicida*) caused high mortalities and losses between 1987 and 1993. Concurrently a major peak in antibiotic used for treatment was seen. As efficient oil-adjuvanted vaccines against several of these bacterial diseases were introduced in the early 1990s, the occurrence of bacterial diseases rapidly decreased, and the use of antibiotics in salmonid production has since this been insignificant, i.e. less than 2 % of the fish were treated in 2020, and antibiotic resistance is almost non-existing. However, the increased resistance to the medical treatments of Salmon lice (*Lepeoptheirii salmonis*) and increasing amounts of lice has become a large challenge

for salmon farming the last 8-10 years. To limit the infection pressure towards wild salmon, prevention of high louse levels in farmed salmon is crucial, and lice levels below the maximum treatment threshold seems to have little direct impact on the welfare of farmed fish. This has led to relatively rapid development of several different non-medical delousing methods, mainly based on three different principles: Thermal, mechanical (brushing and/or water jets) and use of fresh water exposure. A common element of all methods, is the need for crowding of the fish before they are pumped into the delousing system. In addition to mechanical or thermal damage potentially caused by some of the methods, this represent a series of stressful situations for the fish. With almost 3000 registered delousing treatments in 2020, and almost 1000 active sea water sites, the average individual salmon goes through delicing about 3 times a year [3]. As the effect of delousing seldom is completely efficient or long-lasting, fish are put through these stressful events several times a year, and post-treatment mortality is not uncommon and can be high, especially in fish groups already vulnerable by other health problems and diseases.

For the last 30 years, the impact of the viral diseases has become more evident in the aquaculture industry, and in recent years, Norwegian salmonid aquaculture has been affected by several viral diseases: pancreas disease (PD)[5], infectious salmon anaemia (ISA), infectious pancreatic necrosis (IPN), heart and skeletal muscle inflammation (HSMI) in Atlantic salmon and HSMI-like disease in rainbow trout, cardiomyopathy syndrome (CMS) and gill disease caused by Salmon gill pox virus (SGPV). Vaccines based on inactivated viruses have been available for the Norwegian industry against IPN, PD and ISA [6-9], and recently a DNA vaccine against PD was included [10], but unfortunately, viral vaccines seem so far not to have as high protection efficiency against disease as the bacterial vaccines.

Intensive fish farming in Norway has a relatively short history, and appearance and discovery of new diseases is continuously ongoing, as proven by the detection of pancreas diseases (PD), heart- and skeletal muscle inflammation (HSMI) [11], cardiomyopathy syndrome (CMS)[12] and salmonid gill poxvirus disease (SGPVD)[13]. A diagnostic challenge of the most common viral diseases in farmed Atlantic salmon, is that four of them all have histopathological cardiac lesions as one

of their hallmarks: ISA, PD, HSMI and CMS. In addition, it is not unusual to find more than one disease at a fish farm site or even in a single pen, and it is not uncommon to have viral co-infections, or even triple viral infections, at the same time even in a single individual [14, 15].

NVI summarise annually the heath situation in Norwegian aquaculture in its report "The health situation in Norwegian aquaculture" [15]. In 2020, 154 sites were registered with a clinical CMS diagnosis, and piscine myocarditis virus (PMCV), the causative agent of CMS, was detected by PCR on 203 sites (not necessarily as part of a CMS diagnosis)[16]. Geographically, CMS is widespread along the Norwegian coast, but still, with most cases in the original "hot spot" of mid-and south-Norway. In the same report, CMS was ranked as the most important cause of mortality in the on-growing phase at sea in 2020, in a survey of fish health personnel and inspectors at the Norwegian Food Safety Authority [17].

## 1.3 Historical background of CMS

CMS was first observed in Norwegian farmed Atlantic salmon in 1985 [12], and a further description of what we know as CMS today was published in 1990 [18]. Since the mid-1980s, the disease has been detected in the Faroe Islands [19, 20], Scotland [21] and Ireland [22], and a similar disease has been observed in Canada [23]. Wild salmon with CMS-like lesions, but without clinical disease, have been described [19], and PMCV have been detected in a small amount (0.25 %) of Norwegian wild Atlantic salmon spawners [24, 25].

Although there is an increasing number of reports of CMS diagnosis in younger fish, the disease primarily affects large Atlantic salmon in their second year at sea, close to harvest (Figure 2). Stressful events, like transports, net-cleaning, re-stocking or lice treatments, can initiate outbreaks of CMS mortality in fish groups without prior clinical signs, or in fish groups that have experienced moderately elevated mortality for a longer period of time. This late time of onset give the disease significant

economic impact, and CMS can cause substantial economic losses, in addition to reduced animal welfare and large management-related challenges.

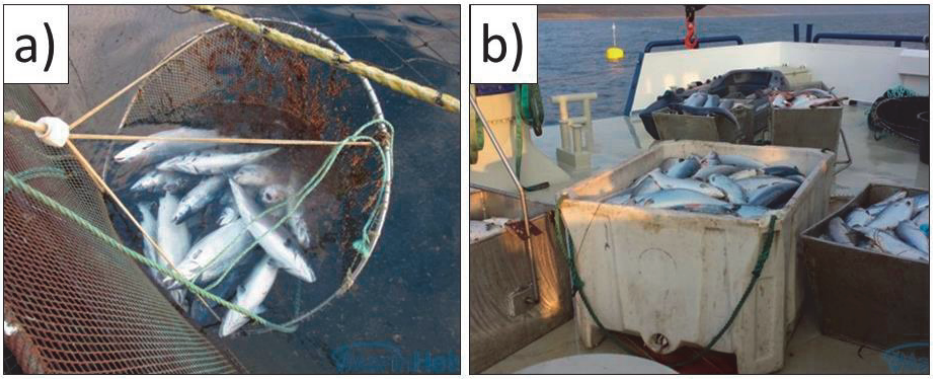


**Figure 2** CMS can cause mortality outbreaks in large Atlantic salmon in good condition.
**a)** Collection of CMS morts at a fish farm. **b)** Large amounts of CMS morts. Both photos: Per Anton Sæthre, Marin Helse AS. Used and re-printed with permission from photographer.

In the 1980s and 1990s, the cause of CMS was still unknown, and several hypotheses were proposed. Farmed Atlantic salmon have a less stressful life than their wild counterparts and are often fed ad libitum and vaccinated against some of the most severe diseases. In addition, various cardiac anomalies have been described in farmed Atlantic salmon, i.e. misshaped or reduced size of cardiac ventricle, septum transversum defects and situs inversus of the heart. A common clinical finding of these fish is low stress tolerance and decreased oxygen blood levels when handled i.e. when transported, during lice treatments or other stressful crowding events [26], as can be experienced with CMS. Even though no consistent physical cardiac anomalies were found in CMS fish, an early hypothesis was that a combination of unknown, negative effect on cardiac function (i.e. breeding related) combined with lack of exercise might have resulted in salmon hearts more vulnerable to stress, particularly when combined with low or lower oxygen levels. Environmental factors like temperature, nutritional factors and the location of sites relative to sea currents and a possible connection to low oxygen levels at sites with recurrent CMS diagnoses were also discussed as possible contributing factors [27].

Autoimmune heart failure, a kind of post-viral myocarditis, have been described in human medicine [28, 29]. As CMS is often found in larger fish that have survived viral

infections, CMS was early suggested to be a possible autoimmune disease occurring secondary to another viral infection or viral infections.

A viral aetiology was suggested in the first published description [12], and a few years later nodavirus like particles were observed in cardiac tissue of CMS fish[30, 31]. However, attempts to cultivate a possible viral agent from tissue homogenates of CMS diseased fish did not succeed, and have not yet been published. In 2009, both researchers at Marine Institute, Aberdeen, Scotland [32] and the NVI finally demonstrated the experimental transmissibility of CMS, using intra peritoneal (i.p.) injections of cardiac and mid-kidney tissue homogenate from CMS diseased fish (paper I) [33]. This strongly indicated a viral aetiology, and shortly after, in 2010/2011, two separate research groups presented studies demonstrating PMCV as the causative agent of CMS [34, 35].

### 1.3.1 Clinical signs, macro- and microscopic lesions

Since the early detections, CMS diseased fish has been characterized as typically large fish in good condition, either without external signs, or with some degree of exopthalmia, ventral skin haemorrhages and oedematous skin scale pockets [36] (Figure 3).

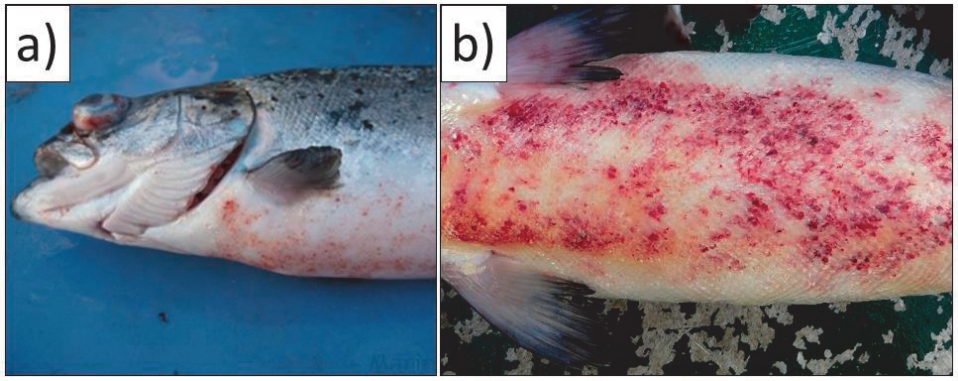


**Figure 3** Clinical findings of CMS: **a)** Exopthalmia and sparse-moderate ventral petechiae. Photo: Per Anton Sæther, Marin Helse. **b)** Severe ventral petechiae. Photo: Trygve T. Poppe, NMBU. Used and re-printed with permission from both photographers.

Autopsy findings include ascites, discoloured and/or dark liver with fibrinous casts and enlarged, sometimes ruptured atrium and/or sinus venosus and a blood or blood clot filled pericardial cavity (Figure 4) [37].

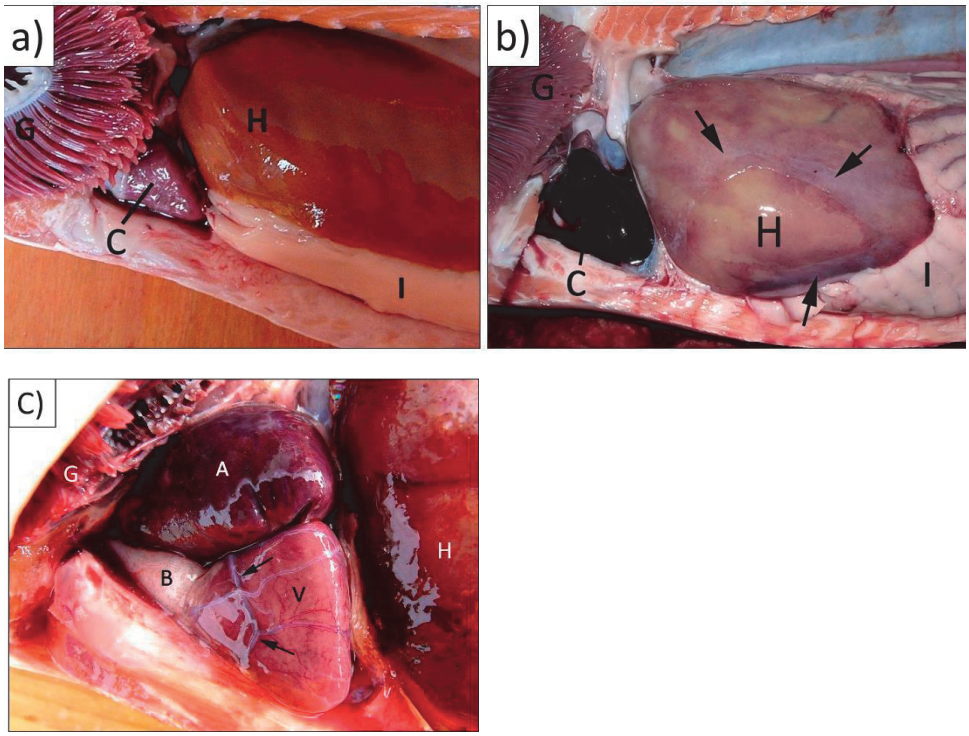


**Figure 4** In situ presentation at autopsy of heart, liver and visceral fat of a large of farmed Atlantic salmon. G = gills, C = heart, H = liver, I = intestines and fat. **a)** Normal fish. Photo: Trygve T. Poppe, NMBU. **b)** CMS diseseased fish with ruptured heart covered almost completely by a large blood clot, liver with multifocal haemorrhaging, discoloration and fibrinous pseudomembranes (F). Photo: Brit Tørud, NVI. **c)** Enlarged atrium. Photo: Trygve T. Poppe, NMBU. Used and re-printed with permission from both photographers.

Typical histopathological changes in severe cases have been described as a multifocal to diffuse subendocardial myocarditis of the atrial (Figure 5) and ventricular spongiosum (Figure 6), in most cases leaving the compactum of the ventricle free of lesions. The first inflammatory lesions are usually observed in the atrium, before also the spongiosum of the ventricle are affected. The highly cellular myocarditis is accompanied by necrosis and, to some degree, degeneration of spongious myocardium, and a hypertrophic, sometimes also hyperplastic, endocardial cell layer. The cellular inflammatory infiltration is mainly mononuclear, usually with some

lymphocytes and macrophages. Circulatory disturbances and congestion caused by increasing impairment of the heart function as the myocarditis progress, result in the observed external and internal signs, including secondary lesions in other internal organs like liver and spleen.

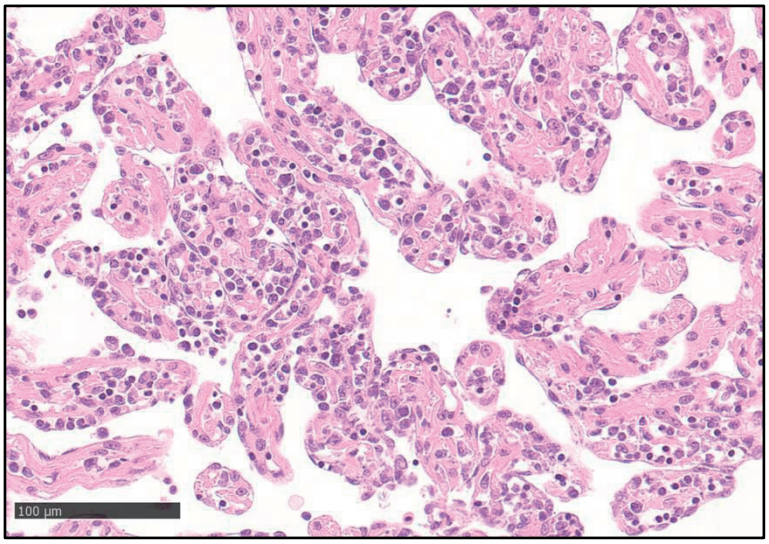


**Figure 5:** Severe Atrial CMS lesions showing infiltration of various inflammatory cells, both subendocardially and in the myocardium (200 x magnification, bar = 100 µm, HE staining).

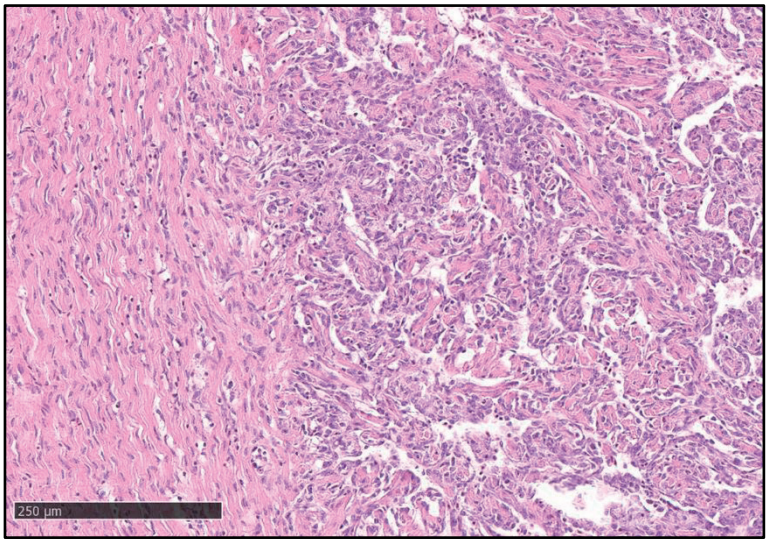


**Figure 6:** Histopathology of severe to very severe CMS, first sampling of field study described in paper II: Ventricular compactum layer to the left, with no or only very sparse infiltration of inflammatory cells. Ventricular spongiosum to the right, with intense inflammatory cell infiltration (100 x magnification, bar = 250 µm, HE staining).

## 1.4 Cardiac anatomy, physiology and pathology

### 1.4.1 The heart

The Atlantic salmon has a four-chambered, single circuit heart, where the deoxygenated blood returning from the main body passes just once. The four chambers sinus venosus, atrium, ventriculum and bulbus venous (Figure 7) are placed in this order, forming an S-shape [38]. Separated from the abdominal cavity by septum transversum, a strong fibrous connective tissue, the heart is located caudally to the gills, cranio-ventrally in the pericardial cavity, which is lined with an outer, parietal layer of the pericardium, separated from the inner, visceral pericardium by a serous fluid, which reduces friction during cardiac contractions [39].

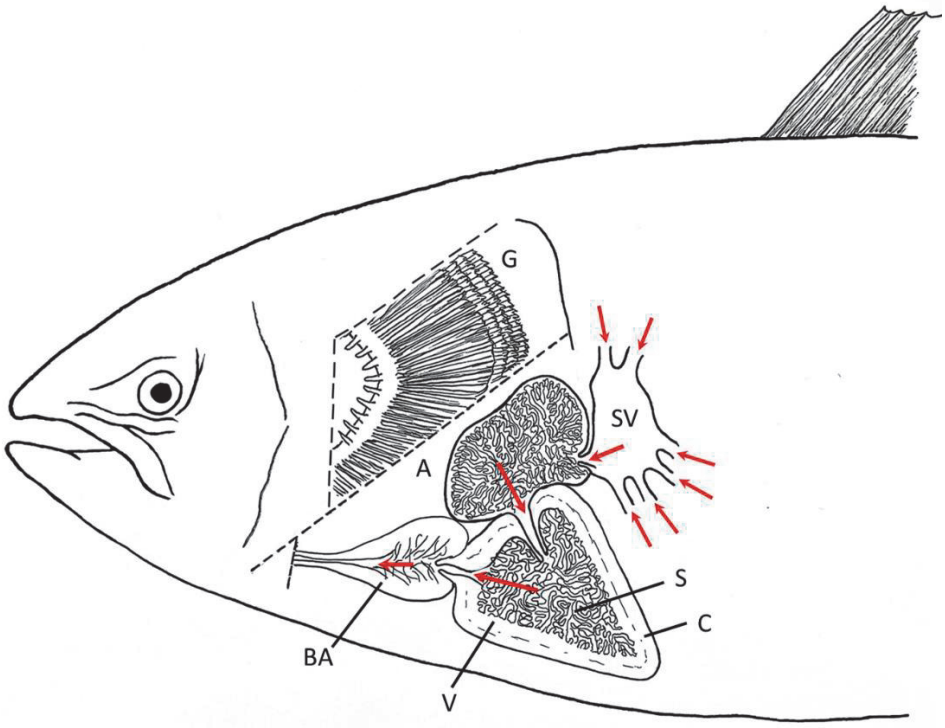


**Figure 7** The Atlantic salmon heart, direction of blood indicated by red arrows. SV = Sinus venosus, A = atrium, V = ventricle, BA = Bulbus arteriosus, C= compactum of ventricle, S = spongiosum of ventricle and G = gills (Drawing: C. Fritsvold).

Atrium and ventricle have a three-layered cardiac wall. The outer layer, the thin epicardium, is the simple squamous cell epithelium of the visceral pericardium, contains a small amount of connective tissue, and covers the myocardium, the layer of cardiac muscles. The cardiac lumen side of the myocardium is covered by endocardium, another simple squamous epithelium. Endocardium of the atrium are usually a little higher than in the ventricle [38]. The epithelium of the endocardium, like the endothelium lining the capillaries, is extremely thin, facilitating rapid diffusion, but do not offer much protection of the underlaying tissues.

Cardiomyocytes of both spongious and compact muscle are made up of cross-striated muscle cells with a diameter of about 1-12.5 μm (in contrast to 10-25 μm in mammals) [26] where the repetitive pattern of myofibrils, forming bands called sarcomeres, which can be observed as cross-striated structure by light microscopy. The individual cardiomyocyte has a central nucleus, is branched, and connected to adjacent cells by intercalated discs, with gap junctions allowing for rapid signal transmission causing cardiac contractions.

The first cardiac compartment is the sinus venosus, with no inlet valves and thin, collagenous connective tissue walls [39] containing the cardiac pacemaker, which initiate cardiac contractions. Sinus venosus works as a kind of junction, receiving blood from the rest of the fish through the hepatic veins, anterior jugular vein and the Cuverian ducts, draining it through two sino-atrial valves into the atrium (Figure 7). Atrial walls are thin and made up of cardiac muscle cells forming a trabecular three dimensional network, making up a large surface covered by endocardium. As the ventricle relaxes, atrial contractions and negative pressure moves the blood through atrio-ventricular valves into the lumen of the ventricle (Figure 7). Salmon are fast swimmers, with a pyramidal outer shape of the ventricle and a distinct, thick compact outer layer of ventricular muscle. The compact layer of the ventricle consists of two layers of concentrically arranged cardiac muscle, perpendicular to each other and separated by a thin connective tissue layer (Figure 8).

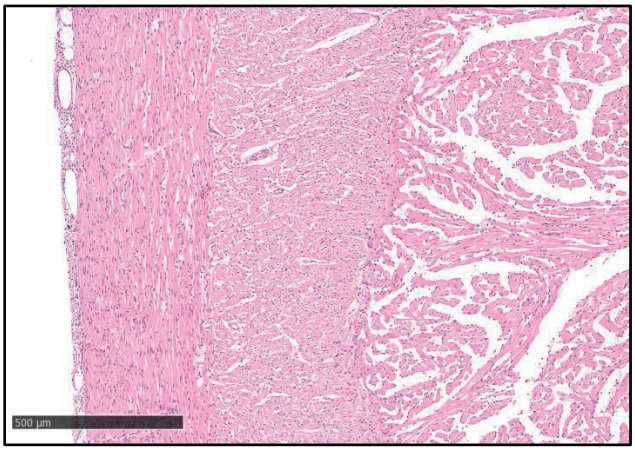


**Figure 8:** The three layers of the ventricular wall, in order from left to right: Epicardium, outer compactum and inner compactum. In addition, to the right of the compactum layers: the spongiosum in the lumen of the ventricle. (Farmed salmon, diagnostic sample NVI, HE-staining, 50 x magnification).

The ventricular lumen has a similar construction to the atrium, with almost no real lumen, but a meshwork of cardiac muscle trabechulae a little thicker than in the atrium, covered by endocardial cells. Finally, the blood is forced through a set of semilunar valves by a ventricular contraction, and into the last compartment, bulbus arteriosus (Figure 7). Bulbus arteriosus has thick, fibroelastic walls, and serves as a pressure regulator, smoothing out the pulse of the systole and keeping the blood flowing through the diastole.

### 1.4.2 Oxygenation of the heart

Coronary vessels on the surface of the ventricle ends in a capillary network surrounding the cardiomyocytes of the compact layer, supplying them with freshly oxygenated blood, before it is drained into circulation at the atrioventricular junction. The oxygen rich blood to Arteria coronaria is branching off the arteria hypobranchialis, originating from the second gill arch arteria and the dorsal aorta.

Atrial and ventricular spongious myocardial layers do not have blood vessels, and are mainly oxygenated by the venous blood passing through the cardiac lumen, dependent on diffusion through the thin endocardium. Spongious and compact layers are separated by a thin layer of connective tissue, which prevents mixing of blood between them.

The myocardial cells have a high number of mitochondriae and contains myoglobin, an oxygen binding protein resembling the haemoglobin of erythrocytes, indicating a high aerobic metabolism [38]. Spongious myocardium of atrium and ventricle contains more myoglobin than compact myocardium of the ventricle, to better maintain an aerobic metabolism at the reduced oxygen levels in the venous blood and at high activity. The differences in oxygenation are also reflected in the main type of metabolism in the two: Oxidative in the compact myocardium and glycolytic in the spongious myocardium. Teleost (bonefish, i.e. salmon) myocardial cells can use both carbohydrates and fatty acids as energy sources [26].

Cardiac output is determined by the heart rate and stroke volume, and fish primarily increase cardiac output by increasing the stroke volume.

### 1.4.3 Cardiac muscle pathology related to CMS

The tissues of the salmonid heart, has a limited range of pathological responses to damage, hence different damaging agents may cause relatively similar lesions and responses [40].

As a response to physiologically or pathologically increased workload, compensatory hypertrophy may be induced in myocardium. Hypertrophy is the increase in size of an organ or a tissue due to increased size of the cells of the tissue [40]. Hypertrophic myocardial nuclei has been described in or adjacent to cardiac necrosis and inflammation in Atlantic salmon with CMS [18], and hypertrophic endocardial cells are describes as a typical feature of CMS.

Both degeneration and necrosis can be observed in the spongious myocardium of CMS diseased hearts. In degeneration, the structure and/or the function of a cell or tissue is impaired and deteriorated. Failure to maintain homeostasis caused by a viral infection can induce degenerative changes in cells. Degeneration can be reversible, or continue further to irreversible changes like apoptosis or necrosis [41]. Apoptosis, also called programmed or targeted cell death, is a pathway by which organisms can dispose of cells damaged beyond repair in a controlled way by. External factors like a virus infection, can cause lethal injury to i.e. a myocardial cell, resulting in necrosis,

where unregulated autolysis and cell destruction induces an inflammation, followed by leukocyte invasion and phagocytosis of cellular debris [40].

In contrast to mammals, with only hypertrophic growth (increase in size) after birth, salmonids retain both hypertrophic and hyperplastic (increase in number) growth as long as they live, hence myocardial cells can regenerate. If the fish survives damage to the myocardium, the consequences may therefore not be as severe as in mammals [26]. This may be seen in both PD and HSMI, where the cardiac lesions seem to heal in surviving fish [42] [10]. Indications of healing, defined as less fish with CMS lesions and less sever lesions, was observed in a CMS challenge trial [43], in contrast to what is described as typical for CMS in field cases [18, 36].

Thrombosis, blood clot formation or presence within the cardiovascular system, is described in moderate to severe CMS [18, 20], and may be induced by exposure of subendothelial collagen when damage to endocard results in necrosis [40].

An important effect of inflammation, the response to harmful stimuli like pathogens, chemicals or damaged cells, is to ease the migration of leukocytes to the injured cells, [41] and involves a number of chemical mediators and immune cells, resulting in dilatation of blood vessels. Atlantic salmon recruit leukocytes from circulating blood cells, from cell populations in kidney and spleen and from local leukocytes in the specific issue [44]. Inflammation may also induce necrosis [40], making it difficult to determine if the necrosis observed is primary or secondary. The most common cells of the inflammatory reaction in fish are mononuclear cells, especially macrophages, lymphocytes and plasma cells [44].

## 1.5 Aetiology of CMS – piscine myocarditis virus (PMCV)

The aetiological agent of CMS, PMCV, was first described in 2011 [34, 35]. PMCV is described as a non-enveloped, spherical virus with a single, non-segmented double-stranded (ds) RNA genome of 6688 bp (Figure 9) [34].

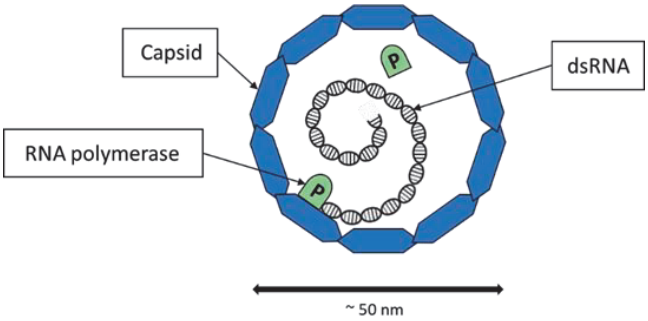


**Figure 9** Simplified figure of a PMCV virion based on comparison to viruses of *Totiviridae*. (C. Fritsvold).

On the positive sense (+) strand of the genome, three open reading frames (ORF1, ORF2 and ORF 3, are identified (Figure 10) [34]. ORF2 and encoded protein show homology to viruses of *Totiviridae* and based on further similarities of genomic characteristics and organization to totiviruses it is expected that ORF1 encodes the capsid protein and ORF 2 a RNA-dependent RNA polymerase (RdRp). Further, these two ORFs may be translated to form a capsid-RdRp fusion protein due to a -1 ribosomal frameshift (Figure 10) [34, 45] The function of the protein encoded by ORF3 has not been described yet, but is focus of extensive research (pers.comm. A. Mikalsen, NMBU). The main hypothesis is a non-structural protein involved in the viral entry/infection of cells and/or release, but the protein may also influence or modulate the immune response of the host [45].

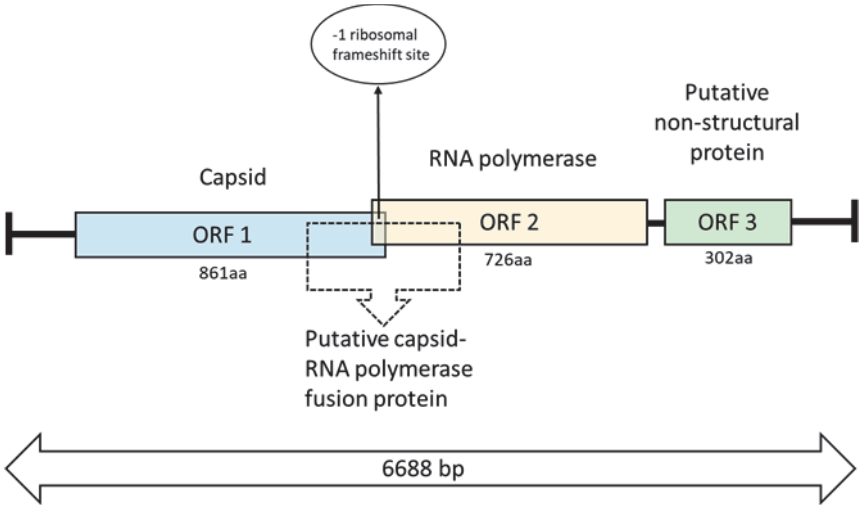


**Figure 10** Overview of the proposed organisation of the PMCV genome (Adapted from A.B. Mikalsen [46], C. Fritsvold).

As mentioned, PMCV shows similarities with viruses of *Totiviridae*, but has not been officially assigned and is at present only described as a piscine toti-like virus together with five other recently discovered toti-like viruses of fish (golden shiner toti-like virus 1 and 2 (GSTLV-1 and -2), common carp tori-like virus 1 (CCTLV-1), blue gill toti-like virus 1 (BGTLV-1) and Cyclopterus lumpus toti-like virus (CLuTLV), respectively). Virus of the Totiviridae usually infect uni-cellular organisms like protozoan parasites (i.e. Giardia lamblia, *Trichomonas sp*., *Leishmania sp*.) and fungi, in which they cause latent, persistent infections of their hosts, and are transmitted to new host cells during cell division, sporogenesis or cell fusion [45]. In contrast, several toti-like viruses infecting more advanced host arthropods (shrimp, crab, mosquito, fruit fly and ants) [47] [48] [48] [49-52], planarians (flatworms) [53] and some fish species, has recently been discovered. These toti-like viruses are shown or expected to have an extracellular transmission way, and carries extra genomic material suggested to improve cell entry and infection of more advanced hosts than the traditional Totiviridae. As PMCV and GSTLV-1, CCTLV-1 and CLuTLV are the only ones having an extra protein-coding sequences as a third ORF, and are phylogenetically close related as a distal phylogenetic branch of the Totiviridae, it has been suggested that they should be defined as a separate genus named Pistolvirus (for Piscine Toti-like virus) [45].

Norwegian PMCV strains constitute a genetically homogenous group, with some geographical clusters. However, in some farms, different isolates samples at the same site showed a relatively high variability [54]. Similar findings were found in a smaller studies of Irish isolates and between Norwegian and Irish strains [22, 55], and also strains of wild Norwegian Atlantic salmon were closely related to the Norwegian strains from farmed Atlantic salmon [24] [56]. ORF3 is shown to have a higher amino acid diversity compared to ORF1 (capsid) in both Irish and Norwegian isolates [22, 54].

## 1.6 Diagnostic methods

CMS is not a notifiable disease by OIE, neither a listed fish disease in the EU nor by the Norwegian Food Safety Authority, hence there is no official diagnostic criteria available.

A CMS diagnosis at NVI in 2021 is primary based on a histopathological evaluation of the cardiac atrium and ventricle. Secondary, a similar histopathological examination of a selection of organs, usually including gill, liver, pyloric cecae with pancreas tissue, mid-kidney, spleen and skin/muscle, are performed to eliminate other or concurrent causes of positive findings of cardiac lesions in accordance with CMS. The additional use of real-time PCR to detect PMCV specific RNA in samples of heart and kidney tissue, is increasing in routine diagnostics at NVI, and the PCR method is in common use to screen for PMCV specific RNA in fish groups without clinical signs of CMS.

### 1.6.1 Differential diagnoses and co-infections

Typical CMS is histopathological easily distinguishable from its most important differential diagnosis PD, HSMI and ISA. However, occurrence of co-infections are not uncommon, both at site and individual level, and early, sparse or untypical lesions of a disease, or different phases of two or more diseases in one individual, can make it challenging to differentiate between the cardiac diseases based on histopathology only [15]. In these cases, results of real-time PCR investigations or immunohistochemistry (IHC) can be supportive e.g. for ISA or PD. Still limitations may occur, and although the IHC for PD has strong and specific positive signals, this is only in the limited period when necrosis of the exocrine pancreas can be found.

Another example are PRV (causative agent of HSMI) that are shown to be almost ubiquitous in Norwegian salmonid aquaculture using PCR-based methods, but still virus presence do not necessary cause disease [14, 57].

PD, caused by salmonid alpha virus (SAV), is a notifiable disease by OIE, subject to mandatory restrictions if detected, and one of the economically important diseases in Norwegian salmon farming. A majority of the cases occur in larger fish, but can also be found the first year in seawater [15]. Typical histopathology findings in the acute phase are necrosis of the exocrine pancreatic tissue, followed by a primary necrosis of myocardial cells, and a secondary inflammation of ventricular compactum and spongious myocardium of both ventricle and atrium. In addition, an inflammation of red skeletal muscle, and sometimes also white skeletal muscle, are observed [58, 59]. The myocardial necrosis of PD is very characteristic, and can be used to distinguish PD from the other cardiac diseases if present. The myositis in red skeletal muscle, in combination with cardiac inflammation, makes PD the most important differential diagnosis of HSMI.

The first lesions of HSMI are usually seen as a mild epicarditis and a focal myocarditis in the ventricular spongiosum [60], in contrast to CMS, where initial cardiac lesions are found in the atrium. In clinical HSMI, when mortality is registered, typical cardiac lesions are moderate to severe myocarditis and necrosis in both compactum and spongiosum of the ventricle, and to some degree in the atrium, moderate to severe epicarditis and endocarditis, often together with myositis and necrosis of red skeletal muscle, in addition to multifocal liver necrosis, some times haemorrhagic, usually in a non-zonal pattern [11, 60].

Haemorrhagic liver necrosis and hepatocellular degeneration, thought to be a result of circulatory failure, have also been observed in CMS, although it is in general a more consistent findings of ISA [21, 61]. ISA is caused by infectious salmon anemia virus, and typical autopsy findings of ISA indicate anemia, circulatory disturbances and blood vessel damage, like pale gills, bleeding in the eye, dark liver, dark and swollen spleen, congestion and various haemorrhaging of inner organs [62, 63]. In the heart, multifocal lesions in myocardium can be observed, a common sign in generalised viral diseases [26]. ISA can cause high mortality, and infection with ISAV (HPR deleted) is

a notifiable disease by OIE, a listed fish diseases, category C non-exotic, in the EU, and subsequently, in Norway, through the EEA agreement.

## 1.7 Epidemiology of CMS

CMS was first detected in mid-Norway in the late 1980s, before the disease spread to all Norwegian salmon farming areas. Still a major proportion of annual CMS diagnoses in Norway are from this original geographical area (Figure 11).

Usually, CMS is diagnosed in larger fish in good condition, close to harvest, and CMS have only been diagnosed in farmed Atlantic salmon in sea-water phase. Experimental trials has shown that PMCV is transmitted horizontally from injected Atlantic salmon to cohabitants, who developed lesions in accordance with CMS [34]. As CMS is a contagious disease, the principal preventive measure is to block introduction to aquaculture facilities, by keeping the number of introductions, sources of origin and contact points with other farms as low as possible, to keep the overall infection pressure low.

As CMS has been found in young Atlantic salmon a possible vertical transmission route has been studied and discussed in several studies [64, 65]. Still, although PMCV specific RNA has been detected by PCR-based methods in progeny from PMCV-positive parents, the prevalence seems to be decreasing with development stage, and also, there is no proof that PMCV specific RNA detected in such progeny represent viable, infectious virions.

In an epidemiology study, following cohorts of 12 sites from sea transfer to slaughter, the initial PMCV infection was detected between 1 and 7 months post sea-water transfer, and median time from this first detection to an outbreak of CMS (in 6 of 12 sites), was 6.5 months [66]. Another study, including all salmon cohorts from 2004 to 2012, observed a median time from sea transfer to CMS diagnosis of 16 months (interquartile range 13-19 months), in fish with an average weight of 3.6 kg at the time of CMS diagnosis [27].

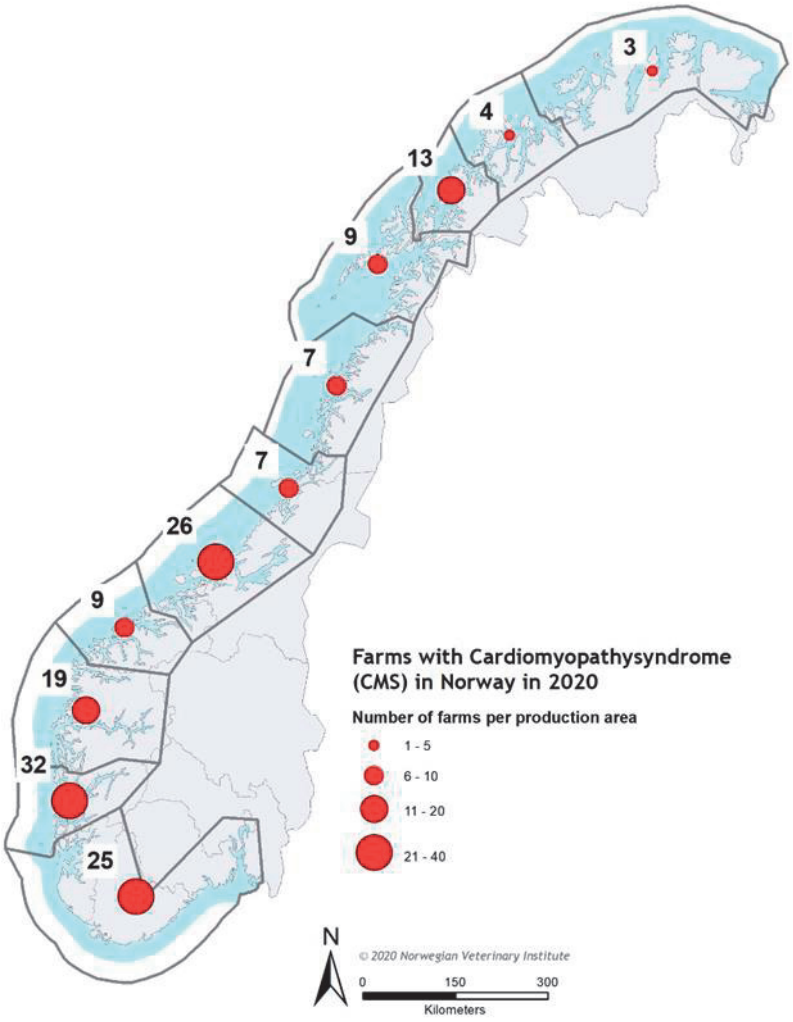


**Figure 11** Map of farms with CMS in Norway 2020. Since 2017, the Norwegian coast were divided into 13 geographically defined "production areas" (In Norwegian: Produksjonsområder, POs) (B. Bang Jensen from [15]).

Potential marine reservoirs of PMCV has been discussed, and to some extent studied [67], but based on published studies, the Atlantic salmon is the only confirmed source of PMCV infection. Prevalence studies showing low prevalence of PMCV in wild Atlantic salmon [56], suggests that wild salmon is of minor importance as a reservoir for farmed Atlantic salmon. PMCV has been detected in two species of cleaner fish in

an Irish Atlantic salmon farm, corkwing wrasse *Symphodus melops* L. and ballan wrasse *Labrus bergylta* (Ascanius), with unspecific cardiac pathology [68].

Similar to other infectious diseases, length of time in the sea, increasing cohort size and infection pressure increases the probability of developing CMS [27]. Stressful events, like transports, stocking, net-cleaning and delousing are also considered potential triggers of CMS outbreaks [15]. CMS in previous cohorts, and HSMI in the same cohort, were also identified as risk factors [27]. Reuse of cleaner fish may represent another potentially risk factor [68].

### 1.7.1 Prevention and control

CMS-QTL (quantitative trait loci) smolts, expected to have lower viral load and morbidity, less cardiac lesions and lower mortality than standard bred Atlantic salmon, are available on the market [69-71] . According to a survey performed by NVI in 2020 [16], 16% of respondents replied that they were used extensively while 27% of respondents replied that they were used to some extent. Functional feeds, to improve cardiovascular health, increase stress tolerance and promote dietary uptake during CMS disease, have been developed by several commercial feed companies. Although vaccine development is ongoing, a vaccine against CMS is not currently available [16].

# 2 Aims of study

The overall objective of this study was to increase the knowledge about the pathogenesis of cardiomyopathy syndrome in Atlantic salmon, *Salmo salar* L..

The specific aims of the project were to:

1. Investigate the potential experimental transmissibility of CMS and establish a challenge model for CMS in Atlantic salmon to characterize development of CMS lesions in affected hearts in a long-term longitudinal study.

2. Characterize a natural outbreak of CMS by a longitudinal study, describing the pathological lesions and the development of the disease, including detection of PMCV in several organs at different time points of the outbreak.

3. Further characterisation of the pathogenesis of CMS in a challenge trial, including more comprehensive analyses of lesions and virus load in various samples from each individual, including non-lethal sampled mucus and blood. Improve the detection of PMCV specific RNA in tissue by establishing a new, more robust and sensitive ISH method for PMCV detection, and compare to the different diagnostic methods of today's CMS diagnostics, including histopathology, real-time RT-PCR and IHC.

# 3 Summary of papers

### PAPER I: Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*

Fritsvold, C., Kongtorp, R. T., Taksdal, T., Orpetveit, I., Heum, M., & Poppe, T. T. (2009).

DOI: 10.3354/dao02123

CMS was experimentally transmitted to Atlantic post-smolts by intraperitoneal injection of tissue homogenate of hearts and kidneys from CMS diagnosed farmed Atlantic salmon. The first histopathological lesions in accordance with CMS could be observed in the experimental fish six weeks post-injection (p.i.), as focal to multi-focal endo- and myocarditis in the atrium, which proceeded to the ventricle. The lesions developed further to severe endocarditis and myocarditis with mononuclear cell infiltration of the atrium and, in a more moderate degree, the ventricular spongiosum. From 33 weeks p.i., similar, focal cardiac lesions were also observed in the ventricular compactum, located close to focal epicarditis, consistent with histopathological findings of more severe field outbreaks of CMS. Our results strongly indicated an infectious aetiology of CMS.

### PAPER II: Characterization of an outbreak of cardiomyopathy syndrome (CMS) in young Atlantic salmon, *Salmo salar* L.

Fritsvold, C., Mikalsen, A. B., Poppe, T., Taksdal, T. & Sindre, H.

DOI: 10.1111/jfd.13521

Young Atlantic salmon were diagnosed with CMS only six months after sea transfer at a fish farm in the southwestern part of Norway. As this was uncommonly early, and the levels of detected PMCV specific RNA was unusally high (Ct <10), the population was followed for almost 10 more months, until slaughtering, resulting in five additional samplings. Of these, two more extensive samplings of the study cage were performed at 3 and 39 weeks post first CMS diagnosis, (wpd), with special emphasis on the cardiac compartments atrium, ventricular spongiosum and ventricular compactum. No difference was observed in PMCV specific RNA-load between the

three examined cardiac compartments, but this could be due to the overall high load and severity of the cardiac lesions, and may be different in cases with more sparse lesions, i.e. early in infection. Both prevalence and load of PMCV specific RNA remained high throughout the study period. Clinical observations, autopsy findings and histopathological lesions were in accordance with previous descriptions of CMS in all sampling, and were accompanied by a slightly increased mortality continuing until slaughtering. CMS was not detected in the other five cages of the site, until diagnosed one of two remaining cages was diagnosed with CMS 10 weeks post slaughtering of the study cage (49 wpd). The PMCV of the site was sequenced, and was similar to earlier observed PMCV variants of Norwegian field outbreaks.

### PAPER III: Characterisation of early phases of cardiomyopathy syndrome (CMS) pathogenesis in Atlantic salmon (Salmo salar L.) through various diagnostic methods

Fritsvold, C., Mikalsen, A. B., Haugland, Ø, Tartor, H. & Sindre, H.

Manuscript ready for submission

Pre-smolt Atlantic salmon were challenged with PMCV by i.p. injection in a CMS challenge trial, and samples were secured from the heart, mid-kidney, skin/muscle, blood and mucus at 0, 10, 20 and 52 days post challenge. Histopathology of CMS related tissue lesions and detection of PMCV specific RNA were compared using various previously used and wellknown methods and the not previous published method RNAscope *in situ* hybridisation. Samples from three different cardiac compartments, mid-kidney and skin/muscle tissue were compared to non-lethal parallel samplings of blood and mucus. Detection of PMCV specific RNA in plasma indicated a viremic phase preceding the cardiac lesions. Mid-kidney also showed a relatively high virus load at early time points. In contrast, the viral load in mucus was significantly lower. As previously observed, cardiac tissue have the highest amount of PMCV specific RNA when cardiac lesions have manifested. Compared to immunohistochemistry, the RNAscope *in situ* hybridisation method was considerably more sensitive and robust, and represents a new and more reliable method for detection of PMCV specific RNA in relation to CMS lesions of the heart. Collection of

plasma and mid-kidney for RT-PCR detection, and inclusion of the RNA scope *in situ* hybridisation method for PMCV detection, may significantly improve CMS diagnostics, especially in early screening, cases of untypical lesions or mixed infections.

# 4 Materials and Methods

## 4.1 Field samplings

Clinical and pathological descriptions of field outbreaks of a health problem usually constitute the background for the first attempts of identifying the problem as a disease, to understand disease processes and explain them [63, 72], and CMS is no exception. Case studies can provide answers to how a disease propagate and evolve in both the population and in the individual fish, and give valuable information on handling of disease outbreaks, and in some cases, prevent or reduce the impact of future outbreaks, or even prevent them.

Field material of farmed Atlantic salmon gives a genuine picture of how the observed disease appears and progress in natural settings in the studied farmed fish, with all known and un-known impacting factors of a fish farm. Increasing the number of samplings and samples adds more information and strengthens the study. However, field samples also have disadvantages: in the natural environment on a fish farm, the number and type of possible factors impacting the fish, their response to the disease and how the disease develops, are numerous and can be difficult or impossible to identify, track, control and measure the effect of. A major problem are the lack of control of other concurrent diseases, which makes identification of, and differentiation between similar or early, sparse pathological lesions more challenging. Studying different populations and disease outbreaks at several time points of the production can to some degree reduce this, as will knowledge of other plausible causes of the observed pathology, and suitable diagnostic tools to differentiate between them.

Paper II is based on studies of material received from a natural outbreak of CMS. The material received made up a longitudinal case study starting at the time of disease outbreak. Clinical observations, both macroscopic and microscopic pathological findings, and microbiological analyses were included, and in two comprehensive samplings, a selection of clinically healthy fish were included to act as a control group to the dead fish with CMS. The main challenge of this study was the detection of PRV

in the study cage shortly after the first CMS outbreak, and detection of SAV later in the production period. Both detections were followed by suspected, but not histopathologically confirmed, cases of HSMI and PD. Another challenge was the variations in sample sizes and, that selection criteria of fish, and choice of tissues sampled, differed a little between the samplings, as some samplings were performed by the fish health service at the farm initiated by clinical findings and/or increase in mortality indicating disease, and the two more comprehensive samplings were performed at the NVI. An overview of the sampled material in paper II is presented in Table 1 below.

**Table 1** Overview of sampled material for all papers. G = gill, Pb= Pseudobranch, C= heart, H = liver, P = Pyloric cecae with exocrine pancreas, S = Spleen, R = Kidney, Skin/M = Skin with adjacent red and white skeletal muscle.

| **Fixative:** | **Formalin** | **RNAlater** | **Lysis buffer** | |
|---|---|---|---|---|
| **Method(s)** | Histopathology | Real-time RT-PCR | Real-time RT-PCR | |
| | IHK | Sequencing | | |
| | ISH | | | |
| **Paper I organs** | G, Pb, C, H, P, S, R, Skin/M | C, R | | |
| **Paper II organs** | G, C, H, P, S, R, Skin/M | G, C x3*, H, P, S, R, Skin/M | | |
| **Paper III organs** | G, C, H, P, S, R, Skin/M | C x3*, R, Skin/M | Blood | Whole blood** |
| | | | | Plasma |
| | | | | Pelleted blood cells |
| | | | Mucus | Pectoral fin |
| | | | | Lateral line |
| | | | | Anus |

*The three compartments sampled separately: Atrium, ventricular spongiosum and ventricular compactum.

**Sampled in heparinised tubes

## 4.2 Experimental challenge trial

Establishing a challenge model is an important step to determine if a newly discovered disease of farmed fish is transmissible and if it can be transmitted by water. Recognition of transmission route(s) are principal knowledge essential for effective control measures against a disease.

In experimental trials, potential effects of an intervention introduced by researchers are studied under controlled conditions, reducing the environmental factors that can influence the outcome to a minimum, or keeping them at a constant, and in some cases, measurable level. Experimental challenge trials, also called infection experiments, are experimental trials suited to study if a suspected infectious disease is transmissible i.e. to naïve fish. In addition to studies of transmissibility, the resulting pathology and other physiological effects in the fish, the experimental conditions enable studies of tissue tropism and effect of different treatments on the diseases. Several variables might affect the outcome of experimental challenge trials: Study design, origin and pre-treatment of the challenge material, route of infection and susceptibility of the experimental fish. Fish may differ in susceptibility to a pathogen due to seasonal physiological changes, physiological stage, genetic differences between fish strains and differences in stress level.

Use of *in vivo* experiments should always be used with caution, and be limited to experiments without better alternatives, and where the benefits of the potentially increased knowledge outweighs the negative sides of sacrificing experimental animals, consistent with the three R's: Replacement, Reduction and Refinement [73, 74].

The experimental challenge trials describes in paper I and paper III, were both approved by the Norwegian Animal Research Authority, and performed in accordance with applicable legislation and ethical guidelines for the use of experimental animals.

For the inoculum in paper I, cardiac and kidney tissue of six Atlantic salmon from a field outbreak of CMS were pooled and homogenated. Of these individuals, four were found dead, and were diagnosed with severe CMS, and two were clinically normal,

with only mild cardiac inflammation. This selection was made to increase the probability of including possible infectious agents, as the aetiological agent was unknown at the time; hence, there was no available methods to test for the presence.

The inoculum for the challenge experiment in paper I was deliberately not filtrated, as it was not known whether the causal agent could be bacterial or viral, or if it could be bound/attached to large particles than would be excluded if filtrated. However, the supernatant of the tissue homogenate used as challenge inoculate, was pre-treated with Gentamycin in a 50 µg mL-1 concentration, to ensure that at least gentamycin sensitive bacteria were excluded from the inoculate.

The design of the challenge trial in paper I is schematically presented in figure 12. In short, about 200 post-smolt Atlantic salmon, divided in two tanks, were injected i.p. with 0.2 ml tissue homogenate of heart and mid-kidney of CMS diseased fish from a field outbreak. Two control tanks were also included, and the control fish were injected i.p. with 0.2 ml L-15 – medium. Every 3rd week, ten fish were sampled for histopathology (gills, pseudobranch, heart, liver, pylorus with cecae, spleen, mid-kidney and skin/muscle), and mid-kidney for bacteriology. An overview of the sampled material in paper I is presented in Table 1.

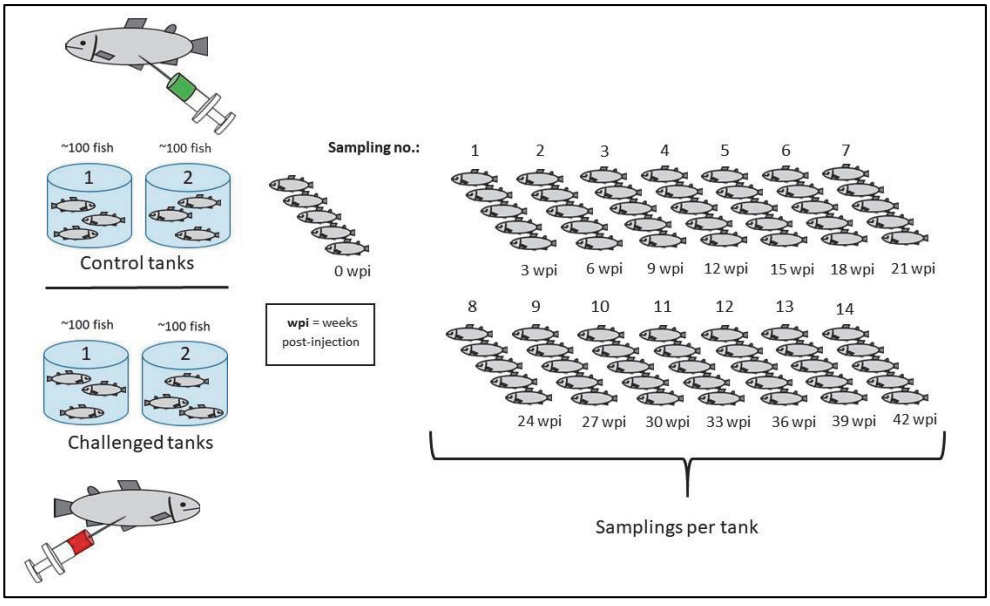


**Figure 12** Schematic presentation of the design of challenge trial in paper I.

Below is a schematic presentation of the design in the challenge trial described in paper III (Figure 13). 270 Atlantic salmon post-smolts were i.p. injected with 0.1 ml tissue homogenate from spleen of CMS diseased fish from a field outbreak. Sampling of 15 fish were performed at 10, 20 and 52 days post-injection (dpi). Heart, mid-kidney and skin/muscle were sampled for histopathology, immunohistochemistry (IHC) and *in situ* hybridisation (ISH). Heart (atrium, ventricular spongiosum and ventricular compactum), mid-kidney, skin/muscle, mucus and blood were sampled for real-time RT-PCR. An overview of the sampled material in paper III is presented in Table 1.

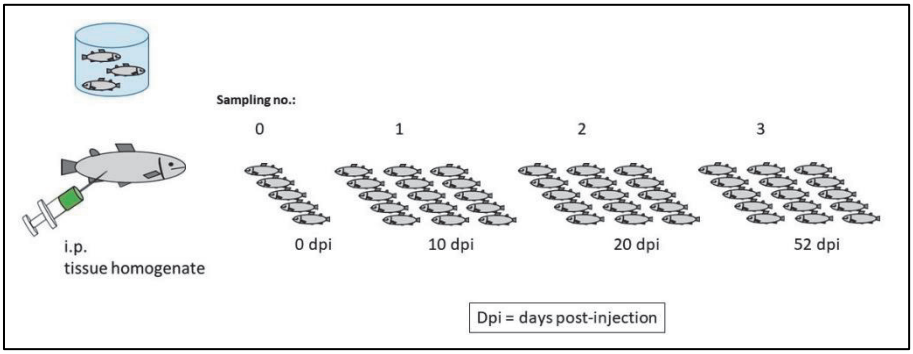


**Figure 13** Schematic presentation of the design of challenge trial in paper III.

At the time of the challenge trial described in paper I was performed and described (2007-2009), the causative agent of CMS, PMCV had not yet been described, hence tissue material from a field outbreak was the best option available. Even today, more than a decade later, there has not been published a description of any cell culture resulting in successful propagation of PMCV to high titres in any known fish cell culture, and various tissue homogenates are still used as inoculum in PMCV challenge trials, including the challenge trial described in paper III. Unfortunately, this may potentially introduce not only the intended, causative agent, but also other co-existing infectious agents of the individuals used. Hence, the almost ubiquitous presence of PRV in Norwegian farmed Atlantic salmon makes it challenging to use field material for inoculum in experimental trials with PMCV, especially as PRV can cause HSMI, one of the most important differential diagnosis of CMS. It is therefore important that field material intended for use as inoculum in challenge trials are tested for all known viral (and bacterial) agents by PCR.

## 4.3 Histopathology

A CMS diagnosis is currently based on standard light microscopy histo-pathological examination of tissue slides only, but is often combined with a PCR detection of PMCV specific RNA.

The word “histopathology” is made from the greek word “histos”, meaning “tissue”, “pathos” meaning “suffering” and “logia” meaning “study of”, and describes the microscopic examination of very thin tissue samples to evaluate the manifestations of disease [75].

As the macroscopic changes of dead and diseased fish at autopsy often are rather unspecific, evaluating samples of the same fish by histopathology enables a more detailed examination of the pathological lesions in tissues, making it possible to differentiate between diseases with similar clinical and/or macroscopic presentation.

Processing for histopathology examinations starts at autopsy, where tissue samples are cut and placed in a fixative, most common 10 % neutral buffered formalin (formaldehyde in water solution), to prevent decay. Cross-linking of protein molecules stabilizes the tissue during fixation, which progress with approximately 1 mm into the tissue pro 24 hours, hence samples for histopathology should be cut in relatively thin slices and kept in formalin for at least 24 hours before further preparation. In successive stages, water is removed from the sample by the use of increasing concentrations of alcohol, and in the last step xylene, as the molten paraffin wax used to permeate the tissue in the last stage is not soluble in alcohol. When the properly orientated paraffin-embedded tissue has cooled, the specimen is solid, and thin microtome sections can be cut and mounted on a glass slide. To reveal cellular components and provide contrast enabling light microscopy viewing, the sections are usually stained with one or more different dyes. A combination of hematoxylin and eosin (abbreviated H&E or HE) is the most commonly used stain, resulting in blueish nuclei and pinkish red (eosinophilic) cytoplasm and extracellular matrix like collagen and keratin. After staining, thin, protective cover slides are mounted on top of the tissue sections, which then is ready for viewing by a light microscope.

All histopathological slides of the three papers were stained by H&E, before evaluated by ordinary light microscopy, but a majority of them have later also been scanned for digital evaluation and imaging.

Histopathological evaluations are subjective: As focus of the pathological description of a disease are the deviations from normal tissue, it requires time and experience by the pathologist, not to miss or misinterpret minor changes, and to be able to differentiate between normal variations, autolytic changes and other concurrent diseases or abnormalities in an individual. Usually only one slide of each organ are examined, and the cut surface through a single level of i.e. the heart, presents a two-dimensional picture of a three-dimensional reality. Hence, the information of one slide is restricted and do not represent the complete reality, as lesions can be missed if the cutting level passes higher or deeper in the tissue sample, or findings in the level of the slide may not be representative for the organ as a whole. To reduce investigator bias, one can use blinding and randomisation of the samples, and/or samples can be evaluated either by repeated scorings by the same pathologist, or by using more than one pathologist to evaluate the samples.

All slides from the experimental challenge described in paper I, were first evaluated non-blinded when ready prepared for light microscopy after every sampling. Inflammatory lesions of all cardiac slides were graded according to Table 2 (histoscores), with a score ranging from 0 to 4, where 0 was no pathological lesions and grade 4 was extensive, severe cardiac inflammation, respectively (see Table 2 for details). For intermediate changes between these scores, steps of 0.5 were used in some cases (especially in paper II and III). Then the slides were re-evaluated semi-blinded (no information about challenge group of the individuals) by a senior pathologist and also discussed, to reduce investigator bias.

The slides from paper II and paper III were in general evaluated non-blinded and graded according to Table 2, including use of intermediate steps of 0.5 when necessary, and some of them were re-evaluated and discussed with a senior pathologist.

**Table 2** Grading of cardiac CMS lesion (paper I, II and III).

| Score | Description |
|---|---|
| 0 | No pathological findings, or slightly increased number of leukocytes |
| 1 | One or a few focal lesions, increased number of leukocytes |
| 2 | Several distinct lesions and small to moderate increase in number of leukocytes |
| 3 | Multifocal to confluent lesions and moderate to severe increase in number of leukocytes |
| 4 | Severe confluent lesions comprising >75 % of the tissue and massive leukocyte infiltration |

## 4.4 *In situ* methods

The latin term «*in situ*» means "in place", and is used to describe methods detecting either infectious agents, tissue epitopes (antigens), DNA or RNA, and visualising their precise placing in the examined tissue. There are three groups of in situ methods: Immunohistochemistry, which detect native antigen (protein or carbohydrate) by antibodies, *in situ* hybridisation localising specific segments of DNA- or RNA-sequences and lectin- or virushistochemistry, where specific carbohydrates are detected by protein binding. The two first methods were used in the work of this thesis.

### 4.4.1 Immunohistochemistry (IHC)

IHC is based on the binding of specific, primary antibodies to antigens, i.e. surface structures of virus, bacteria, particular proteins, lipids or carbohydrates in tissue sections. The antibody-antigen-binding can be visualised in several ways, usually by using a secondary antigen directed against the primary antigen. The secondary antigen includes a bound enzyme (i.e. alkaline phosphatase) reacting with a substrate (i.e. Fast red chromatogen) [76], and results in development of a coloured product visible with a light microscope.

The detecting primary antibodies can be polyclonal or monoclonal. Polyclonal antibodies are isolated from whole serum of a laboratory mice or rabbit, and consists

of a heterogenous mixture of antibodies recognizing different epitopes of the originally injected molecules or agents of interest (virus, bacteria, proteins etc.). Monoclonal antibodies have specificity for one epitope only.

Although the antibodies show preferential avidity for their specific epitope(s), they may show cross-reactivity in the form of weak or partial, unspecific bindings to other antigens in the tissue. Too high levels of such background staining can mask detection of the target antigen, and can be prevented by incubating the samples with protein rich buffers blocking these epitopes, i.e. normal serum, bovine serum albumin (BSA) or non-fat dry milk, as in paper II and III. Other methods to reduce disturbing background staining are to dilute the primary or secondary antibodies, adjust temperature or time for incubations, or change to another secondary antibody or detection system. Poor primary antibody potency or poor enzyme activity of the visualisation system can cause too weak positive signals. To enhance contrast and make it easier to evaluate, the tissues of IHC slides are often counterstained by i.e. hematoxylin.

An IHC for PMCV using polyclonal antibodies directed towards the protein expressed by PMCV ORF3 (kindly provided by M. Rode (Pharmaq)), was used for heart tissue of paper II and paper III of this thesis. The ORF3-related antigen used for preparation of these antibodies were a truncated part of the ORF3-expressed protein, but details have not been available. Both positive and negative control slides were included in the IHC runs, to ensure the specificity of the antibody used. Other quality control possibilities include the use of non-immune tissue slides, where the primary antibody has been omitted or absorbed. Western blot can also be used to validate antibodies.

However, even with good specific antibodies and low levels of background staining, detection of the target antigen/epitope can be difficult, even though the agent or antigen (in search) are present. The level of targeted antigen in the tissue could be below the detection limit of the method (some IHC requires a relative high localised concentration), epitopes may be masked by i.e. formalin-fixation (preventing primary antibody binding) and cellular membranes and extra cellular matrix can block access to binding sites for antibodies. Demasking techniques using proteolytic enzymes (i.e. trypsin), surfactants and citrate boiling in microwave oven have been developed to

de-mask epitopes hidden by formalin fixation, but too long fixation time can cause irreversible antigen masking.

### 4.4.2 *In situ* hybridisation (ISH)

Hybridization in molecular biology is defined as the annealing of a single stranded deoxyribonucleic acid (DNA) or a ribonuclic acid (RNA) to form a complementary DNA or RNA, respectively [77].

In the traditional *in situ* hybridization technic, a probe made of a single stranded DNA or RNA sequence, hybridize (i.e. forms complementary base pairs) with complementary DNA or RNA if present in a tissue sample, hence detects the specific DNA or, as with PMCV, RNA sequence complementary to the probe. A probe is usually labelled with radioactivity-, fluorescence - or enzyme linked antigen allowing for visualisation, quantification and precise localisation of the specific DNA- or RNA probe in the histologic tissue section. The most commonly used probes today are complementary RNA (riboprobes). An *in situ* hybridisation method can be singleplex (detecting one transcript), duplex (detecting two different transcripts simultaneously in the same sample) or multiplex.

Samples for ISH are usually pretreated to fix the target transcripts in place and to increase access of the probe. High temperature facilitates the hybridization of the probe, and after hydrolysis of unhybridised, excess RNA probe by an RNase, excess probe is washed away. To ensure that only exact sequence matches will remain bound, solution parameters like temperature and concentration of salt and/or detergent can be adjusted.

In the experimental study reported in paper III, a singleplex RNAscope ISH protocol was used for detection of PMCV ORF1 RNA in non-stained sections of a selection of hearts, representing all sample points prepared for histopathological evaluation. At optimal conditions, the assay can visualise single RNA molecules in a cell, and can be performed without the RNA-free environment traditional ISH requires[78].

In study III, the RNAscope *in situ* hybridisation assay was used to confirm the virus load indicated by the real-time RT-PCR, to study cell tropism and distribution of PMCV in the cardiac tissues, and for comparison with IHC for PMCV ORF3. A set of 14

probe pairs targeting a 707 nt part of the ORF1, encoding the capsid of PMCV, were designed according to [78]. Every PMCV target probe consists of 18 to 25 bases, connected to a 14-base unique "tail" sequence. After a probe binds immediate next to another probe on the target RNA, the tail sequences of the probe pair are joined to form a 28 base site for hybridisation with the preamplifier (Figure 14). Hence, only target specific signals are amplified. The preamplifier has 20 binding sites for the amplifier, and each amplifier have 20 binding sites for the chosen label probe, resulting in, theoretically, 8000 lables per target probe pair [78]. The label probe can be labelled for fluorescence microscopy or linked to i.e. an alkaline phosphatase (as used in IHC) for visualisation with Fast red for standard light microscopy (Figure 14). This technique, increasing target-specific signals without concurrently increasing the background noise from nonspecific hybridizations, and the possibility for viewing by standard bright-field microscopy, make the RNAscope ISH an easy to read, highly specific and much more robust method compared to the traditional ISH assays using a single probe [78].

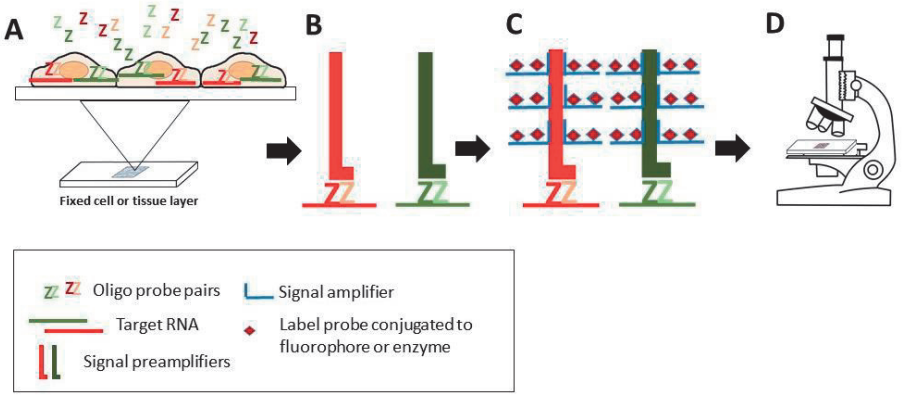


**Figure 14** Schematic overview of the procedure of an RNAscope ISH.
**A:** Fixation and permeabilisation of cell- or tissue samples (formalin fixated paraffin embedded slides), making target RNA sites accessible for the specific target probes.
**B:** Specific target probes (Z) hybridises juxtapositioned to another specific target probe, forming probe pairs (ZZ), with a shared, single hybridisation site for the signal preamplifiers (red/green Ls).
**C:** Signal amplification, either using a fluorophore (for visualisation by a fluorescence microscopy) or an enzyme (for visualisation by standard light microscopy).
**D:** Detection of positive signals using either an epifluorescent microscope or a standard light microscope, dependent on which label probes were selected in step C.

## 4.5 Bacteriology

In the first challenge experiment (paper I), samples of mid-kidney of two fish of each sampled fish group were cultivated in accordance with standard isolation of the most common pathogenic fish bacteria: On blood agar plates with and without 2 % NaCl at +15° and +22°C, respectively for at least 6 days. If the fish presented with skin or fin ulcerations at sampling, both mid-kidney and skin lesions were sampled. Standard procedures for identification of isolated bacteria were followed.

## 4.6 Virology

Detection of viruses or viral components can be performed by documenting cytopathic effect in a cell culture, by molecular methods like PCR, real-time RT-PCR, enzyme immunoassays, and by serological methods like hemagglutination or hemagglutination inhibition. In this thesis, real-time RT-PCR was used for detection of viral specific RNA in all three papers, and sequencing of the viral genome was performed on one individual in paper II.

### 4.6.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) uses the natural process of DNA replication to rapidly make a large number of copies of a specific DNA sequence. A PCR procedure consist of multiple repetitions of the three steps denaturation, annealing and extension of DNA. In the first step, a temperature of about + 95°C denature and separate all double-stranded DNA into two single strands. Temperature is then lowered to + 50-70°C, in a step where the specific DNA primers anneal to the template, single DNA strands, one primer at each strand. The specific temperature used is dependent on length and nucleotide composition of the specific primers used. Increasing the temperature again in the final step to + 72°C allows the heat-stable DNA polymerase of the assay to add nucleotides to the specific primer, using the single stranded DNA strand as template, extending the second strand to make a new double-stranded DNA copy of the sequence starting where the primer was annealed. The result of one cycle of denaturing, annealing and extending DNA, is a doubling in number of the DNA target sequence starting at the specific primer. These three steps are repeated for 25-40 cycles in a typical PCR run, allowing for a very high number of

copies of the studied sequence, if present, after i.e. 40 cycles. The products are usually visualized through size separation by electrophoresis in an agarose gel. DNA primers are usually preferred for better stability. As for the specific probes of in situ hybridisation, DNA primers targeting a specific DNA region with known sequences are synthesised and available on the commercial marked.

### 4.6.2 Reverse transcriptase PCR (RT-PCR)

A further modification of the original PCR reaction is reverse transcriptase PCR (RT-PCR), using a reverse transcriptase enzyme to convert mRNA and RNA to cDNA (complementary DNA), which is then possible to use as a template for standard PCR amplification. RT-PCR can be used to detect viral RNA or whether a specific gene was expressed in a sample. RT-PCR was used in paper II, to produce a product for further study by sequencing (4.6.4.).

### 4.6.3 Real-time RT-PCR/ Quantitative PCR (qPCR)

Another modified PCR variant is the real time, or quantitative, PCR (qPCR). In this method, fluorescence makes it possible to measure the increase in number of double-stranded DNA during a PCR reaction, which is not possible by the standard PCR or the RT-PCR. The results are often given as a Cycle threshold (Ct) (or Cycle quantification (Cq), depending on the software) value, which is the number of cycles run needed before presence of the target in a sample results in a copy number high enough to reach a specific fluorescence threshold set in the analysis software. Real-time PCR is a widely used method in diagnostics and surveillance in fish farming.

A real time RT-PCR, detects genomic RNA, mRNA from production of proteins, defect genomic RNA or post-infectious RNA remnants. It therefore does not differentiate between intact infectious virus particles and incomplete or inactivated virus. Consequently, there is not a direct correlation between the detected amount of viral RNA and virus amount. It is important to be aware that the result cannot be used as an exact measure of the level of replicating (hence potentially infectious) virions in the sample, however, it is reasonable to assume a relationship between a lower Ct value and an increasing viral load.

### 4.6.4 Sequencing and phylogentic analyses

In 1972, Frederick Sanger developed the Sanger DNA sequencing method, also known as the chain termination method. In short, the method is a DNA replication with a single-stranded template, initiated by a DNA primer, using a DNA polymerase and a mixture of the regular deoxynucleotides (dNTP) combined with some molecular labelled dideoxynucleotides (ddNTPs). The incorporation of a ddNTP instead of a regular dNTP terminates the elongation of the double-stranded DNA of that particular strand, and since this happens at random, the result are strands of replicated DNA at lots of different lengths because of different termination points. Running the mixture of replicated DNA trough gel electrophoresis, generates a ladder of different sized bands, in accordance with the different termination points of the replication. Today, specific fluorochrome labelling of the different ddNTPs and the use of fluorescence spectroscopy, makes it possible to produce the nucleotide sequence of the template strand analysing all the different ddNTPs in one run.

In paper II, Sanger sequencing was performed as described earlier [54] on one individual at the 17 wpd sampling. Similar PCR and sequencing procedures were used in combination with two primer sets amplifying overlapping products of the UTR between ORF2 and ORF3, and the 5' end of ORF3 to study a possible variation between strains in several individuals at one time point, and over time during the CMS outbreak followed. For this purpose, samples from 4, 3 and 3 individuals, respectively, of the 0, 3 and 39 wpd sampling, were selected.

# 5 Results and discussion

## 5.1 Transmissibility

In 2007-2008, when the challenge experiment described in paper I was performed, the aetiological agent of CMS was still unknown, but a viral aetiology was suggested and suspected, although attempts of virus isolation from CMS diseased fish had not yet succeeded. The choice of heart and kidney tissue of CMS diseased fish of a field outbreak of CMS for the challenge inoculate was based on the experience that heart was the organ manifesting the most consistent and severe histopathological lesions, in addition to the results of the then recently performed successful transmission experiments of HSMI using hearts [60], a disease resembling CMS both in occurence and pathology. Kidney tissue is considered a suitable tissue as inoculum in experimental trials, based on the fact that infectious agents like IPNV, SAV and ISA are typically detected in the kidney throughout the infection, and kidney material can easily be harvested fairly sterile [6, 58, 79].

However, as noted in section 4.2., the use of tissue homogenate increases the risk of introducing other infectious agents in the challenge material, especially the widespread virus PRV-1. For the first experimental trial (paper I), the causative agent of HSMI was not yet identified, thus presence of this virus in the challenge material could not be ruled out prior to challenge. The observed cardiac lesions in the experiment were very similar those described for field cases of CMS, and the development pattern differed from previous challenge trials of HSMI. For the experimental trial in paper III, the challenge material was tested negative for important known fish viruses of Norwegian aquaculture before use.

As propagated virus was not available for either the challenge trial in paper I or paper III, i.p. or i.m. injection of a tissue homogenate has been the option of choice to challenge the fish in largescale experiments. These challenge routes differ from natural infection, which is believed to be either through water or by direct fish-to-fish contact. However, the observed pathology and the development of CMS (paper I and III), was in accordance with what is observed in clinical cases of CMS in the field [11,

18, 22] (paper II). Low (paper I) or no mortality (paper III) was observed in the challenge trials, which may be connected to unnatural transmission route [80], the early life stage of the experimental fish, insufficient observation period or lack of unknown contributing factors to disease development.

The tissue homogenate used as inoculum for i.p. injection was deliberately not filtered before use, in case the causative agent would bind to larger particles, but was treated with 50 µg/ml gentamycin, an antibiotic.

As injection of tissue homogenate from farmed fish diagnosed with CMS induced histopathological lesions consistent with CMS, we demonstrated for the first time in paper I that CMS is a transmissible disease under the experimental conditions used, hence a functioning challenge model for CMS was established.

In addition, we demonstrated that CMS could be transmitted and occur in very young, unvaccinated post-smolt Atlantic salmon (average weight 35 g when challenged), in contrast to the common findings of CMS in large salmon closer to slaughter in field outbreaks of CMS. In parallel with our challenge trial, Bruno et al. [32] showed transmissibility in 1 kg Atlantic salmon. In the case study described in paper 2, these findings were confirmed in a field study, where 1 kg vaccinated Atlantic salmon at a sea farm, presented with severe CMS lesions as described for larger fish in late sea water phase.

A horizontal transmission route could not be confirmed in paper I, as no cohabitants were included, but horizontal transmission of CMS has been demonstrated in later challenge studies [34, 43]. Whether transmission of PMCV is through water or direct fish to fish contact, is not yet known. Vertical transmission has been discussed and studied [64, 65], but no evidence has yet been published [81].

Only one of the six cages other than the study cage described in paper II were diagnosed with CMS, 10 weeks post slaughter of the studied cage. This indicates a lower risk of transmission within a farm than seen with other viral salmonid diseases like PD, ISA and VHS [58, 59, 82-84].

The hypothesis of a viral aetiology was finally confirmed a short time after publication of paper I, as the causative agent piscine myocarditis virus (PMCV) was detected and

described [34, 35]. Versions of our challenge model is still in use for CMS challenge trials, as a well-functioning cell culture for PMCV has yet to be found.

## 5.2 Mortality, clinical and autopsy findings

In general, the clinical and autopsy findings of CMS are signs of heart failure, circulatory disturbances and general congestion: oedema of skin scale-pockets, ventral petechiae, ascites, liver discolouration, liver haemorrhages with and without necrosis, thrombi of various sizes in lumen of the heart, fluid in the pericardial cavity and/or the pericardial sac causing a cardiac tamponade. In terminal phases, rupture of the thin-walled atrium or sinus venosus, caused by weakening of the wall due to massive inflammation, degeneration and necrosis of the myocardium can be observed.

Haemorrhagic liver necrosis, fibrinous pseudomembranes on the liver surface, increased peritoneal fluid and various discolouration of the liver may be related to circulatory failure [21], and could be observed in both comprehensive samplings of the field study (paper II), and was also observed in the study cage in the samplings performed by the fish health services. In the first trial (paper 1), 2 challenged fish presented with fibrinous pseudomembranes on the liver at 12 wpc, and various discoloration of the liver was seen in a total of 14 fish from 12 wpc, and in particular, the last 6 samplings.

In the first experimental challenge (paper I), an IPN outbreak resulted in high mortality in one of the two control groups and some mortality in the other control group and both challenged groups from 3 to 11 wpc. After the IPN outbreak had passed, 12 challenged fish died sporadically throughout the remaining study, probably related to CMS. In addition to the liver pathology (at autopsy) described above, from 12 wpc a total of 12 challenged fish had distended atriae, some of them with blood clots and most of them registered from 24 wpc and onwards. In general, this is considered to be a low degree of CMS related clinical disease and/or mortality.

No mortality was observed in our last challenge trial (paper 3), as expected: the study period was relatively short (52 days), the challenge was performed at an experienced experimental lab, the fish were documented free of the most significant diseases of

Norwegian Atlantic salmon farming pre-challenge and mortality is, in general, difficult to reproduce in challenge trials [80]. The reason of this is probably complex, but the limited observation time in trials, the use of young, healthy fish, the lower general stress level than in a conventional fish farm and a route of infection differing from the natural infection route may all contribute. As CMS is characterised as a chronic inflammatory diseases developing over time, the relatively short observation time in challenge trials may be a major reason to no or low mortality. A single fish had an enlarged atrium at the 52 dpc sampling, probably related to CMS.

However, the mortality of the study cage in our field study (paper II) was moderately increased from the first diagnosis, continued to be higher than in the neighbouring cages and persisted at this elevated level until slaughtering 39 weeks post first CMS diagnosis (wpd). No moribund fish were registered before 28 wpd, and after this time point, the majority of moribund fish was probably related to a concurrent SAV-infection in the cage. Dead fish (paper II) presented with typical CMS pathology: some degree of oedema of skin scale-pockets and sparse to moderate ventral petechiae were observed, while signs of general congestion, like ascites, fibrinous casts on the liver, red or light discoloured liver and yellowish fluid and/or blood clots in the pericardial cavity, were found in various degree. All fish sampled as suspected CMS fish, had a cardiac tamponade, where blood and blood clots filled the pericardial sac and/or the cardiac cavity.

## 5.3 Histopathological findings

Detailed characterisation of pathology is important for the understanding of disease development and host-agent interactions, and is the basis for selection of the best suitable sampling material and diagnostic methods.

Heart is the obvious tissue for histopathology examination for CMS. Still, evaluation of a selection of other organs of the individual fish is important to differentiate possible CMS lesions from different stages of, or co-infections with, other diseases causing of cardiac lesion, like PD, HSMI and ISA.

In our first two studies (paper I and II), a standard diagnostic tissue selection was sampled and formalin fixed for histopathology (Table 1). In the last challenge study

(paper III), the formalin fixed samples were limited to heart, mid-kidney and skin/skeletal muscle, as the fish were thoroughly tested for the most important salmonid viral diseases pre-challenge (see paper III) and to focus these organs (based on the results of paper II).

In accordance with previous descriptions of CMS [12, 18, 21, 26, 36, 85], mononuclear leukocytes dominating the initial inflammation were observed in all three studies, in addition to foci of degeneration and necrosis of spongy myocardium in both atrium and ventricle. In the early stages (paper I and III) and in less affected parts of the spongious cardiac tissue (paper II), subendocardial monocyte infiltration could be seen in a "beads on a string"-like appearance, (Figure 15 a)). In more severely affected foci, loss of myocardium caused hypertrophic endocardial cells to look like "empty stockings" of hypertrophic endocardial cells with nothing but inflammatory cells inside (Figure 15 b)). In later, more sever stages (paper II, and to some extent in the last samplings described in paper I), an additional mixture of inflammatory cells including lymphocytes and plasmacells infiltrate the cardiac lesions, and most of the spongy myocardium, especially of the atrium, was affected.

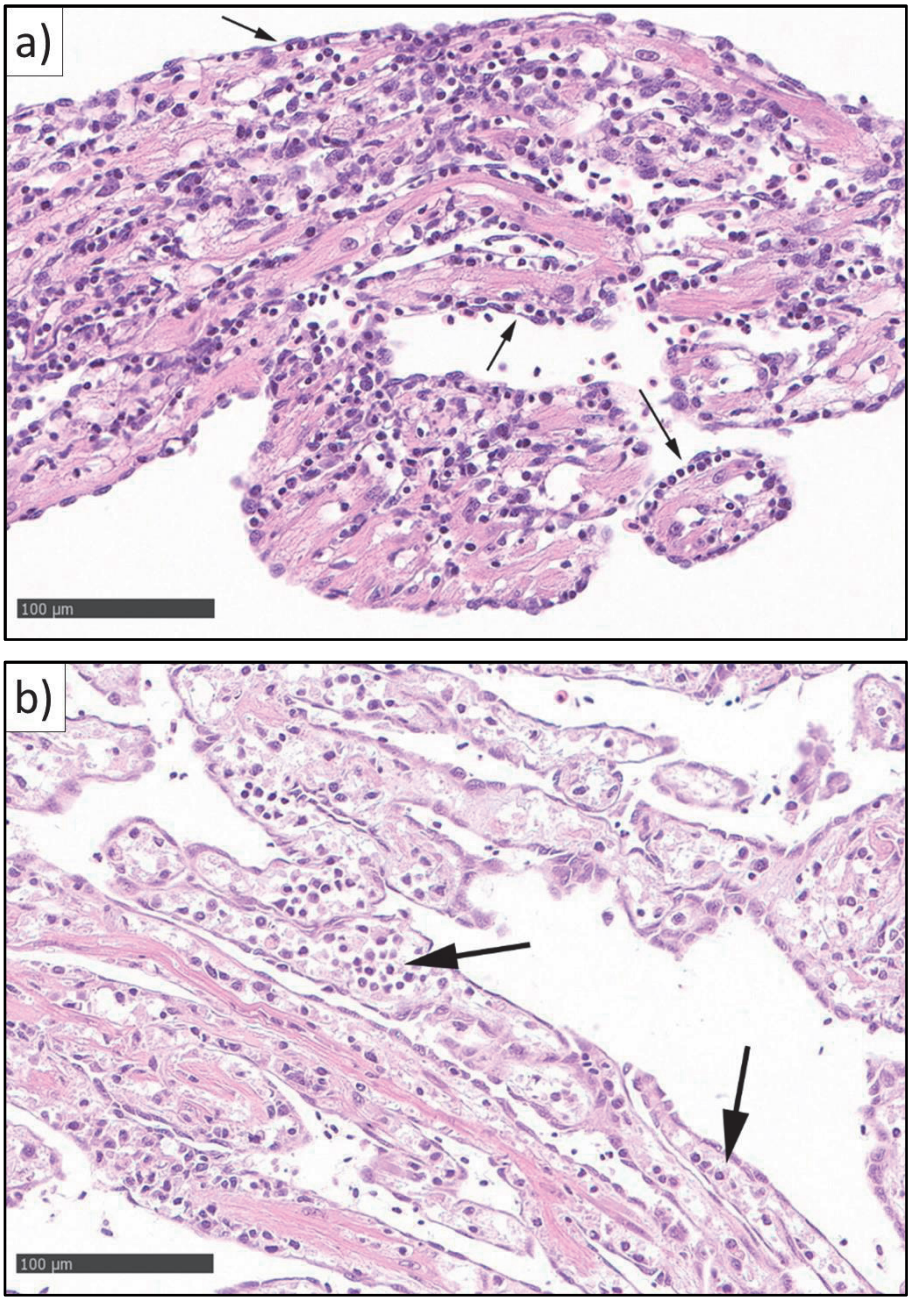


**Figure 15 a)** Subendocardial monocyte infiltration seen like "beads on a string" (arrows). **b)** Loss of myocardium causes hyperthophic endocardial cells to look like "empty stockings" of hypertrophic endocardial cells with nothing but inflammatory cells inside (arrows). (Both photos, HE, 400 x magnification).

Minor thrombi were seen in the lumen of both atrium and ventricle, in increasing numbers with increasing severity (all papers). Myocyte karyomegaly (enlarged nucleus of myocardial cells), possibly resulting from compensatory hypertrophy [26], multinucleated giant cells and "nest-like" aggregates of multiple nuclei have been observed in the myocardium of CMS fish [26, 36], and could occasionally be seen in severe lesions of the field outbreak (paper II).

From the 24 wpc sampling in the first experimental trial (paper I), melanin deposits were observed subendocardially and on the surfaces if the atrium in some challenged fish. This was not seen in hearts of the experiment described in paper III. Melanin is suspected to be of importance in the salmonid inflammatory responses [86], and the melanisation was suggested to have an active part in the local, late stage inflammatory reaction of these CMS hearts [87].

## 5.4 Progression of the cardiac inflammation

A similar pattern of emerging and development of cardiac lesions was observed in the two challenge experiments (paper I and III), even though the last study (paper III) had a much shorter duration, less samplings and shorter sampling intervals (3 weeks (paper I) vs 10, 10 and 32 days (paper III)) (details in Figure 13).

Inflammatory lesions of the atrium appeared a few weeks before lesions of the ventricular spongiosum, with a moderate delay before some individuals with concurrent focal epicarditis and focal myositis of the ventricular compactum could be found (Figure 16, paper I). Atrial lesions in general were more severe compared to lesions of the ventricular spongiosum, also in the same individual, which may be a result of a more advanced atrial disease compared to the ventricle.

By 6 weeks, mild and sparse inflammation was observed in atrium (paper I). At 7.5 wpc (52 dpc, paper III), all fish had mild to moderate lesions in the spongy ventricle, which is resembling the time point when the first lesions in ventricular spongiosum were observed (9 wpc) in the first challenge experiment (paper I).

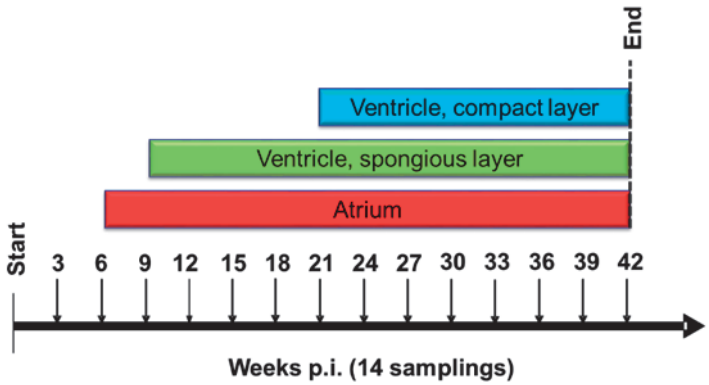


**Figure 16** Appearance of histological cardiac lesions (as observed by light microscopy) over time post-injection in paper I (Figure from paper I, Fritsvold et al. 2009).

The severity of the atrial lesions in paper I peaked at 9 wpc, 6 weeks after the first lesions appeared, with a delayed, but similar peak for severity of ventricular compactum lesions. The level of severity was constant throughout the study, resembling the observations in the field study (paper II).

A very sparse to sparse epicarditis was observed in about half the fish in both studies, but the number of fish with epicarditis decreased with time (paper I and III). After roughly 5 months, the first observations of mild, focal compact layer lesions were seen in the first study (paper I) (Figure 16), and 2.5 months later, a slight increase in number of fish/per sampling and severity of these compactum lesions were observed and continued for the last 12-15 weeks of the study (paper I). All fish with compact layer lesions had lesions in the ventricular spongiosum of similar or higher severity, and a majority of them also presented with an associated focal epicarditis. As the last experimental trial (paper III) lasted 52 days only, it was not surprising to observe very mild and sparse focal lesions of the ventricular compactum only, in 1 of 15 sampled fish at each of the two last sampling points.

In the field study (paper II), the clinical, autopsy and histopathological observations at the time point of the first diagnosis and all succeeding samplings, were in accordance with previous descriptions of typical, severe CMS.

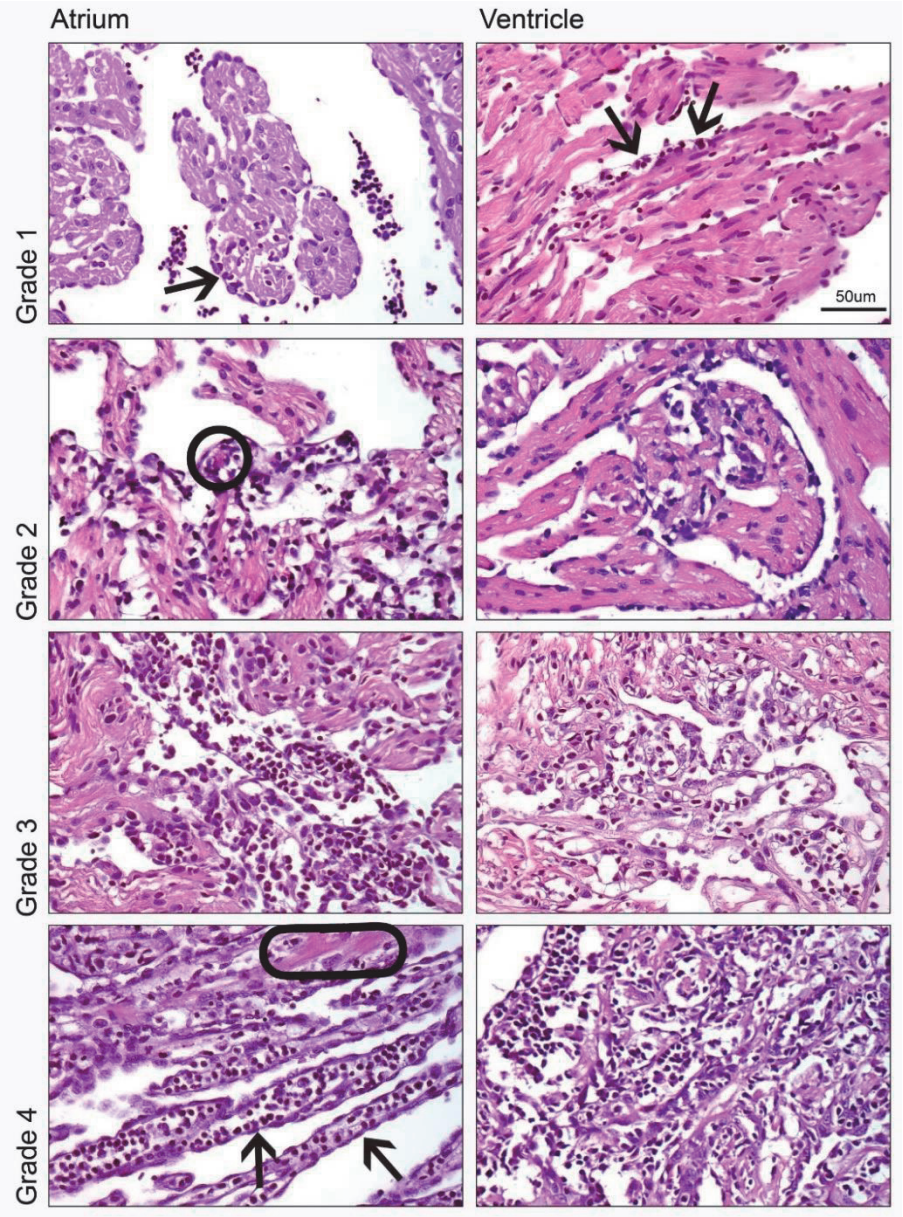


**Figure 17**. Histological classification of lesions in cardiac atrium and spongy ventricle, in accordance with the grading described in Table 2, section 4.3. Grade 1: arrows indicate minor inflammatory lesions consisting of sparse, focal subendocardial infiltration by mononuclear leukocytes and some degree of subendocardial vacuolisation, in both atrium and ventricle. Grade 2: several distinct lesions with small to moderately increased number of leukocytes. Myocyte degeneration and necrosis are encircled in the atrium. Grade 3: Multifocal to confluent lesions with moderate to severe leukocyte infiltration. Grade 4: Arrows indicate hypertrophic endocardial cells in the atrium forming empty tubes where almost all muscle fibres have been replaced by inflammatory cells, dominated by small mononuclear lymphocyte-like cells. Myocyte degeneration and necrosis are encircled in the atrium. Ventricular example is from a focal Grade 4 lesion. Haematoxylin and eosin staining. Scale bar (applies to all panels) = 50 µm (Figure 1, paper I, Fritsvold et al. 2009).

## 5.5 Pathogenesis

The results in paper III indicate that kidney is an early target organ for PMCV infection (Figure 18a) and high levels of PMCV specific RNA in plasma at 10 to 20 dpc indicate a viremic phase in this period, preceding the manifestation of cardiac lesions (Figure 18b), both in agreement with unpublished results from NMBU, Aquamedicine unit and Pharmaq AS [88]. The decrease in PMCV specific RNA load in all blood sampled at 52 dpc could indicate that the fish mount an immune response limiting the PMCV infection in the targeted organs of the early phases. Previous CMS challenge experiments have documented some early phase immune responses and possible trends of healing of lesions [43]. In contrast to this, no indications of healing or recovery was seen in the 42 weeks of our first challenge experiment (paper I). In the challenge trial in paper III, the short duration made it impossible to evaluate if healing occurred.

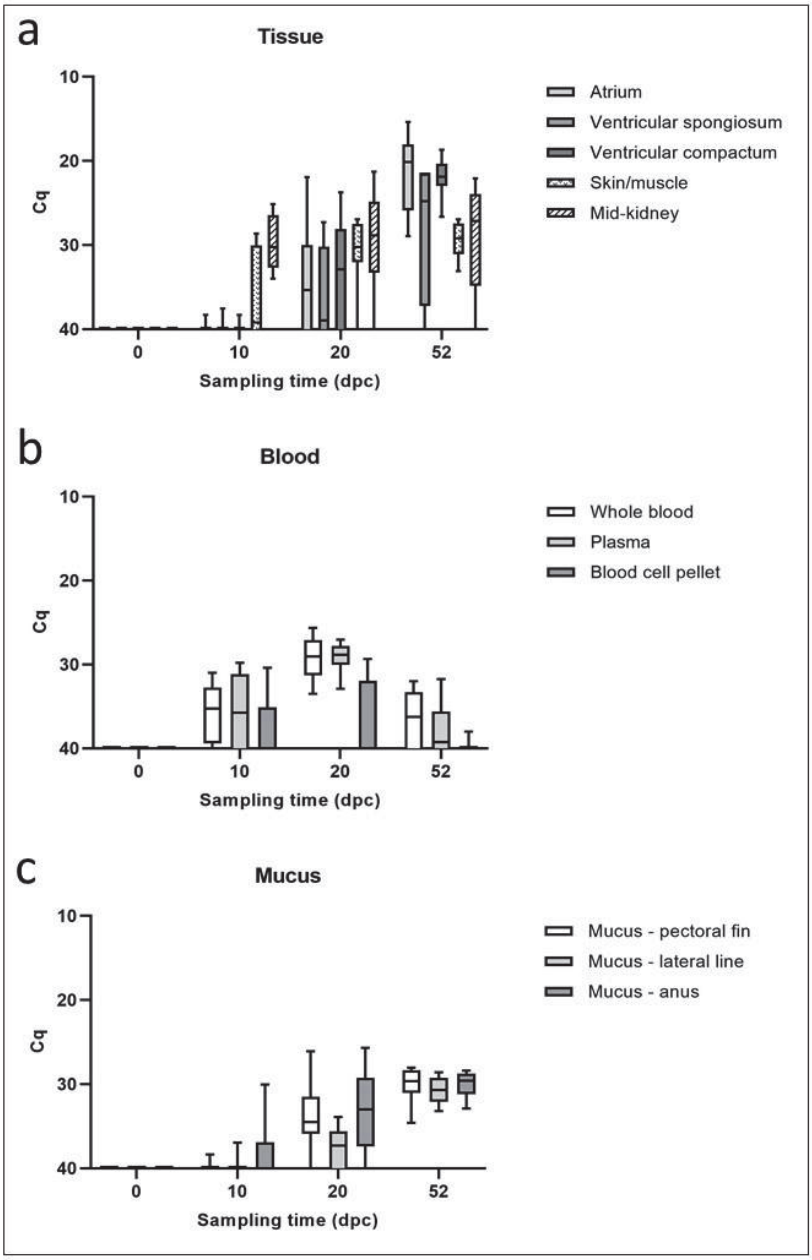


**Figure 18** Levels of PMCV specific RNA given as Cq values, for all sampling time points, in days post challenge (dpc) in three heart compartments, mid-kidney and skin/muscle and blood components and surface mucus. **a)** Tissue samples of cardiac atrium, ventricular spongiosum, ventricular compactum, mid-kidney and skin/muscle. **b)** Parallel samples of blood: whole blood, plasma and blood cell pellet. **c)** Mucus samples at three different locations of the surface of the fish: Pectoral fins, lateral line and anus. Results are presented as box plot with whiskers representing minimum and maximum values in the individual data set. n=4 at 0 dpc and n=15 at other time points, excluding ventricular spongiosum at 52 dpc where n=4. Note the inverted numbering of the Y-axis. (From paper III, Figure 3).

The immune response indicated from the reduced virus levels in plasma in paper III is interesting, as the inflammatory response induced by PMCV in cardiac lesions of later CMS phases do not seem to significantly inhibit or limit the infection, or perhaps more precise: the inflammation. CMS in farmed Atlantic salmon has since the first observations been described as a chronic, inflammatory disease, and do not seem to heal in field cases [42]. In accordance with this, severe cardiac CMS lesions and a high PMCV specific load were seen from the first diagnosis 5-6 months after sea-transfer until slaughtered almost 10 months later (paper II), and the fish seemed unable to eliminate the virus, limit the inflammation or recover from the disease. Inflammatory processes with its release of cytokines, lysosymal content and other harmful factors can cause extensive tissue destruction, leading to a prolonged inflammatory condition [41]. Hence, the infection with PMCV may initiate the disease process, but the development into a chronic condition may, to a certain extent, be self-sustained by the immune response of the fish, where the immune response itself become the most damaging factor of the disease process. This side-effect of inflammation is commonly seen in diseases causing chronic inflammation [41, 89], and could be part of a possible explanation of the long term and severe inflammation observed in CMS fish.

In infections with cytopathic viruses, necrosis is a typical initial finding, preceeding an inflammatory response, as seen in cardiomyocytes in early phases of PD [59]. In contrast, for infections with viruses with less cytopathic effect, inflammation is an important mechanism of pathogenesis [90]. This could indicate that PMCV is not a highly cytopathogenic virus, but the lack of a functional cell culture model prevents studying this aspect.

In contrast to what is observed with PD, HSMI and ISA, the inflammation of CMS tends to be restricted to the spongiosum of atrium and ventricle, with a very distinct border between (in general) normal ventricular compactum and severe inflammation of the ventricular spongiosum in late phases of CMS. As discussed in paper II, this coincide with the pattern of the cardiac blood supply, where spongious myocardium, dependent on oxygen diffusion across the endocardium, receives venous blood of lower oxygen content than the ventricular compactum, receiving freshly oxygenated

blood by the coronary vessels. Both subendocardial monocyte infiltration and hypertrophy of endocardium may also contribute to lower oxygen supply to the myocardium beneath, by increasing the distance of diffusion, possibly affecting myocardic, and therefore, cardiac function.

In the late stages of CMS, high levels of PMCV specific RNA was detected in the three cardiac compartments sampled (paper II). Compared to other viral cardiac diseases, like PD and ISA [80] (Pers.comm. H. Sindre, NVI) the detected PMCV specific RNA levels indicate a much higher virus load in the heart at severe and terminal CMS. Cardiac tissue contained approximately 100-1000 times more PMCV specific RNA compared to the other organs, like kidney, of these fish. No difference in PMCV RNA load between the three cardiac compartments sampled was observed, and these findings were supported by the results of the latest sampling point of the experimental study in paper III. Both prevalence and load of PMCV specific RNA persisted until slaughtering (paper II).

To infect a fish, all viruses must cross a mucosal barrier. Early replication of ISAV has been shown in several mucosal surfaces, like the gills, the pectoral fin, the skin and the GI tract of Atlantic salmon [91]. To our knowledge, paper III represents the first investigations of the presence of PMCV in surface mucus. PMCV specific RNA was detected in swabs of mucus, sampled at the pectoral fin, ventral line and anus, in increasing amounts towards the end of the study (Figure 18c). This could be an indication of a high level of viral shedding in mucus at this stage of infection. These findings are supported by previous experimental studies where virus was transmitted from i.p. injected fish to cohabitants [34]. Due to the limited sampling time points in this study (paper III), the precise time frame for shedding is not possible to define. Depending on virus subtype, shedding of SAV is detected in mucus from 2-5 wpc [92]. However, as the outer surface of the fish is exposed to seawater, virus shedded to the water by other infected individuals may contribute to the detection of PMCV in mucus. In addition, *in situ* investigations in paper III by RNAscope did not reveal any specific PMCV staining in skin tissue. Also, a putative cell receptor for PMCV has not yet been described, hampering investigation of skin as

point of entry or for shedding. The possible role of mucosal surfaces in transmission of PMCV, needs further investigations.

## 5.6 Diagnostic methods and non-lethal sampling

CMS diagnoses are traditionally based on histopathological findings alone. With an increasing number of co-infections with other viral diseases targeting the heart and atypical cases of CMS, a wider selection of diagnostic tools are required for differentiation and correct aetiological diagnoses. In addition, the slow cage to cage transmission in field outbreaks and the fact that fish of the same cage can have different stages of CMS at the same time, makes differentiation between CMS and its differential diagnoses challenging.

In paper III, well established diagnostic methods like histopathology of CMS lesions, detection of PMCV in tissue samples by real-time RT-PCR and the less used method immunohistochemistry, were compared with the not previous published method RNAscope ISH. In addition, the potential of non-lethal samples of blood and mucus for use in diagnostic purposes were investigated.

Our results in both paper II and III confirms that as soon as inflammatory cardiac lesions manifest, heart tissue is the material of choice for both for histopathology examination, PMCV detection by real-time RT-PCR and ISH. Compared to IHC for ORF3, RNAscope ISH showed higher sensitivity and robustness, and can be included as a supplementary diagnostic method when required (paper III).

Inclusion of mid-kidney and plasma samples for real-time RT-PCR, may increase the probability to detect infection with PMCV in the early phases of CMS (paper III). Also, the high prevalence of virus detection (100%) seen using plasma samples at early infection phase concurrently with lower prevalence and more interindividual variation in viral RNA levels detected in both target heart and other samples tested is interesting for regular non-lethal screening purposes if the interest is to detect introduction of virus to a farm or salmon population.

Future CMS diagnostics will probably continue to rest heavily on proper histopathological evaluations of hearts. However, these examinations should be supported by at least one PMCV specific method, most ideally an *in situ* method, uniting high specificity of PMCV RNA detection of cardiac tissue, to the observed cardiac lesions *in situ* in early, untypical or challenging cases with co-infections.

# 6 Main conclusions

**Paper I**

- A functioning challenge model was established for CMS.
- CMS is a transmissible disease under the experimental conditions in this study.
- Histopathological lesions consistent with CMS were induced in Atlantic salmon post-smolts after injection of tissue homogenate from farmed fish diagnosed with CMS.
- These results indicate that CMS has an infectious aetiology and should be treated as a potentially contagious disease.

**Paper II**

- CMS in young Atlantic salmon had typical clinical and autopsy signs, and histopathological cardiac lesions as described for larger fish in late sea phase, similar to previous descriptions of typical CMS.
- The prevalence and load of PMCV specific RNA in the fish remained high until slaughtering, with similar amounts in all sampled cardiac compartments.
- No fish from the other five cages at the site were diagnosed with CMS, until fish sampled from the last cage at the site were diagnosed 10 weeks after slaughtering of the study cage (49 wpd).
- Sequence analysis of the PMCV on the site showed that the outbreak virus was similar to PMCV variants previously sequenced from Norwegian field outbreaks.
- The CMS fish do not seem able to eliminate the viral infection, heal the cardiac lesions or recover from the disease.
- In severe cases with PMCV specific RNA levels like our case in paper II, there was no different between the cardiac atrium, spongy or compact ventricle regarding choice of tissue for viral detection.

**Paper III**

- The development of pathological cardiac lesions observed in the experiment was in accordance with previous descriptions of CMS.
- Our results indicate a viremic phase 10 to 20 dpc preceding the cardiac lesions.
- In this early, possible viremic phase, virus could also be detected in relatively high amount in mid-kidney by real-time RT-PCR. Plasma and/or mid-kidney samples may therefore be candidates to screen for early phase PMCV infection.
- The RNAscope in situ hybridisation method, showed higher sensitivity and robustness compared to the immunohistochemistry, and may be a valuable support to histopathology in CMS diagnostics, especially in cases of untypical lesions or mixed infections.

# 7 Future perspectives

The knowledge of CMS and PMCV has increased substantially since the first descriptions in 1985, especially after transmissibility was confirmed in 2009 and the causal agent described in 2010/2011. However, there are still significant pieces of the puzzle lacking to better understand CMS.

Knowledge on the pathogenesis of CMS is a key to understanding the disease, and forms the basis for defining efficient measures to control the disease. A natural continuation of these studies would be more detailed studies of the infection dynamics in a fish cage and between cages within a farm: how fast the infection spreads, what proportion of the fish is infected, and if all infected fish develop classical, severe CMS. Further investigations of the pathogenesis should include more extensive studies of cell and tissue tropism and more detailed identification of cells, tissues and other structures involved in the pathology of the cardiac lesions. The role of the immune response in the development of CMS pathology needs to be characterised, including the apparent failure of the response to eliminate the infection in most fish. Other important questions to answer is whether fish can survive CMS, if the cardiac lesions can heal and if some fish can eliminate PMCV.

Identification of a putative receptor for PMCV entry of cells is important for investigations of viral uptake mechanisms and cell/tissue tropism. Mechanisms and duration of virus shedding should also be described further, as this is important for designing preventive strategies. The function of various gene products of PMCV also needs further characterisation.

The possible existence of other, yet undescribed, determinants of disease than the well-known stress related trigging events should be investigated. In addition, mechanisms of importance for the large variation in time span from detection of PMCV in a farm to clinical CMS is diagnosed, and the transmission routes of CMS, especially horizontal vs. vertical transmission, requires further studies. Although some work has been done to investigate a possible marine reservoir of PMCV, a

potential presence of the virus in other species and environment has not been extensively examined.

To establish an *in vitro* model for PMCV propagation in cell culture is important for further virus characterisation, like evaluation of the biophysical properties, including resistance to various disinfectants, and investigations of cytopathogenic effects of PMCV. In addition, using purified virus as challenge material in experimental models will reduce the risk of contamination of the challenge inoculum with infectious agents and other components with possible negative effect on an experiment. Finally, PMCV susceptible cell cultures can be a useful tool for CMS diagnostics. There is also a need for more sensitive and specific antibodies towards viral proteins for use in both diagnostics and research.

Non-lethal sampling methods are increasingly in demand both for surveillance and diagnostic purposes, and the potential of both mucus, blood and environmental samples like water for early detection of virus, needs to be further developed.

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# 9 Articles/Papers

**Enclosed papers**

**I** **Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar***

**II** **Characterization of an outbreak of cardiomyopathy syndrome (CMS) in young Atlantic salmon, *Salmo salar* L.**

**III** **Characterisation of early phases of cardiomyopathy syndrome (CMS) pathogenesis in Atlantic salmon (*Salmo salar* L.) through various diagnostic methods**

# 10 Scientific papers I-III

# Paper I

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# Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*

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ABSTRACT: Cardiomyopathy syndrome (CMS) is a disease of unknown aetiology, having significant economic impact as it primarily affects large, farmed Atlantic salmon *Salmo salar* L. in seawater, close to harvest. In the present study, we have demonstrated that CMS is a transmissible disease under experimental conditions. Histopathological lesions consistent with CMS were induced in Atlantic salmon post-smolts after injection of tissue homogenate from farmed fish diagnosed with CMS. Six weeks post-injection (p.i.), experimental fish started developing focal to multi-focal lesions in the atrial endo- and myocardium, with subsequent progression to the ventricle. This proceeded into severe endocarditis and subsequent myocarditis with mononuclear cell infiltration of the atrium and, to a lesser degree, the spongy layer of the ventricle. These lesions were consistent with histopathological findings in field outbreaks of CMS. From Week 33 p.i., lesions also appeared in the compact myocardium, with focal epicarditis adjacent to focal myocardial lesions. In conclusion, these results indicate that CMS has an infectious aetiology and should be treated as a potentially contagious disease.

KEY WORDS: Atlantic salmon · *Salmo salar* · Cardiomyopathy syndrome · CMS · Experimental transmission · Myocarditis · Pathology · Cardiomyopathy · Transmission



## INTRODUCTION

Cardiomyopathy syndrome (CMS) is a severe cardiac disease of unknown aetiology in farmed Atlantic salmon *Salmo salar*. It was first diagnosed in 1985 in Norway, and later in Scotland and the Faeroe Islands (Amin & Trasti 1988, Bruno & Poppe 1996, Rodger & Turnbull 2000). Suspicious cases have also been reported from Canada (Brocklebank & Raverty 2002). Outbreaks of CMS in farmed fish occur along most of the Norwegian coast, with the highest number of affected sites in mid-Norway (Kongtorp et al. 2006a). Most CMS cases are diagnosed during the late seawater phase; typically from 14 to 18 mo after sea transfer. Mortality may be moderately elevated over a long period, or suddenly high without prior symptoms (Ferguson et al. 1990, Brun et al. 2003). As CMS generally affects large fish, the economic losses may be substantial, even though the cumulative mortality may be low or moderate (Brun et al. 2003, Østvik & Kjerstad 2003).

Diagnosis of CMS is based on clinical findings, autopsy and histopathology. At autopsy, fish with CMS typically show skin haemorrhaging, raised scales and exophthalmia. Ascites and fibrinous casts on the liver surface are also common. The atrium and sinus venosus are usually enlarged, sometimes ruptured, and blood or blood clots often fill the pericardial cavity (Bruno & Poppe 1996). Histopathologically, CMS is characterised by inflammation and necrosis of endocardium and spongy myocardium in the atrium and ventricle. Cellular infiltrates consist mainly of mononuclear cells, most probably lymphocytes and macrophages. The compact myocardium is usually not affected, but epicardial cell infiltrates may extend into the compact layer along branches of the coronary vessel (Ferguson et al. 1990). Lesions may progress to such a state that the wall of the atrium or sinus venosus

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weakens or ruptures, with resultant haemopericardium and death. Previous studies indicate that CMS is a chronic disease developing over a period of several months prior to the terminal clinical phase (Ferguson et al. 1990, Kongtorp et al. 2006a).

Several hypotheses on the cause of CMS have been put forward, including environmental, immunological and microbiological factors (Kongtorp et al. 2005). Most of these hypotheses have not been studied further. Cardiac lesions in CMS may resemble pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI), although the diseases are histopathologically distinguishable in typical cases (Kongtorp et al. 2004b, 2006b, McLoughlin & Graham 2007). PD is caused by salmonid alphavirus (McLoughlin et al. 1996, McLoughlin & Graham 2007). HSMI is experimentally transmissible and thought to be of viral aetiology (Kongtorp et al. 2004a). Both diseases cause severe myocardial inflammation and necrosis. Due to similarities with PD and HSMI, and the widespread occurrence of CMS, a viral aetiology has been suggested, although attempts to isolate virus from tissue sampled from fish with CMS have not yet been successful (Kongtorp et al. 2005). The aim of the present study was therefore to investigate the transmissible nature of CMS under experimental conditions.

## MATERIALS AND METHODS

**Experimental fish.** A total of 496 healthy Atlantic salmon smolts *Salmo salar* of a wild strain were used as experimental fish. The fish had been hatched and grown in a fresh water cultivation facility (Hellefoss) in eastern Norway, geographically isolated from the Norwegian population of farmed fish. The fish were not vaccinated, and the cultivation facility had no history of disease. The experiment was performed at the Norwegian Institute of Water Research (NIVA), Solbergstrand, Akershus. Both the cultivation and research facilities are situated in an area with no commercial fish farms, physically and geographically well separated from the endemic area of CMS in Norway. The research facility was approved for challenge experiments with unknown, suspected infectious agents by the Norwegian Food Safety Authority. All effluent water was filtrated (filter pores ≥300 µm) before being treated with hypochlorite, resulting in a total concentration of chlorine in effluent water of at least 35 mg $l^{-1}$ 30 min after treatment.

The fish were transported to the research facility (approximately 3 h transport time) in a water tank with oxygenation 10 wk before commencement of the experiment. There was no transport-related mortality. The fish had not been exposed to seawater, but most fish had lost their parr markings and had a silvery appearance. The average length and weight were 17 cm and 35 g, respectively, a fish size which fit well with the capacity at the facility.

**Husbandry.** At the research station, the fish were housed indoors in a fibreglass tank. Osmoregulatory capacity was tested after 2 wk of acclimatisation by exposing 7 fish to full salinity seawater for 24 h. After sedation in chlorobutanol (300 mg $l^{-1}$), blood was sampled from the caudal vein of these fish, the fish were euthanized by decapitation and the chloride concentration was measured (Central Laboratory, Norwegian School of Veterinary Science) (Eisenman 1967, Tietz 1995). As the results indicated smoltification, the experimental fish were transferred to seawater 7 d later. After another 5 d, the fish were transferred to the experimental tanks.

During the experiment, the fish were kept in cylindrical fibreglass tanks with a conical bottom and central drainage, containing approximately 1.35 $m^3$ of water. Water flow was 300 l $h^{-1}$, providing a complete water exchange every 4.5 h. Seawater was pumped from a depth of 60 m. Mean temperature was 8.5°C (range: 7.1 to 10.2°C) and mean salinity 33.8‰ (range: 32.7 to 34.4‰). The fish were fed a commercial pelleted feed with automatic feeders, at a feeding ratio of approximately 1% body weight $d^{-1}$. A photoperiod regime of 10:14 h light:dark was used. Mortalities were registered, collected and stored at −18°C, and fish showing aberrant behaviour were killed by a blow to the head and decapitation before collection and similar storage.

**Preparation of inoculates.** ***Tissue homogenate:*** Samples of cardiac and kidney tissue homogenates from 6 Atlantic salmon collected during 2 field outbreaks of CMS were pooled and used for inoculation. Four of these fish were found dead in the cages and displayed severe inflammation and necrosis of endo- and myocardium in spongy tissue of the atrium and ventricle on histopathological examination. The other 2 fish showed normal swimming behaviour and were caught live from the same cage as 2 of the dead fish. These fish had only mild inflammation in cardiac tissue. Tissue samples were diluted 1:10 in Leibowitz L-15 cell culture media, homogenised and centrifuged at 2500× *g* for 7 min. The supernatant was further diluted 1:2 in L-15 supplemented with gentamycin (final concentration: 50 µg $ml^{-1}$) before inoculation.

***Negative control inoculate:*** Leibowitz L-15 cell culture media, supplemented with gentamycin (50 µg $ml^{-1}$) was used as the negative control inoculate.

**Challenge.** The study was initiated after a 2 wk acclimatisation period in the experimental tanks. Experimental fish were randomly allocated to 4 groups of

approximately 100 fish (range: 92 to 102), each group in a separate tank. Injection of inoculates was performed after sedation in chlorobutanol (300 mg $l^{-1}$). Duplicate groups of challenged fish (denoted Challenges 1 and 2) were injected intraperitoneally (i.p.) with 0.2 ml supernatant from tissue homogenate. Similarly, duplicate groups of negative control fish (Controls 1 and 2) were injected i.p. with 0.2 ml of negative control inoculate.

**Sampling.** Samples for histology, real-time reverse transcription-polymerase chain reaction (RRT-PCR) and microbiology were collected from 5 experimental fish before commencement of the study. Post-challenge, sampling was performed every 3 wk for a period of 42 wk, resulting in a total of 14 samplings. At each sampling, 5 experimental fish were collected from each group. Sampled fish were anesthetised in chlorine butanol (300 mg $l^{-1}$) and killed by decapitation. Samples for RRT-PCR and cell culture were stored at –80°C until results from the histopathological examination had been finalised.

As the number of fish in Control Group 1 was greatly reduced by an outbreak of infectious pancreatic necrosis (IPN), sampling from this group was not performed from Week 15 to Week 33. The last fish in this group were sampled 36 wk post injection (p.i.).

**Histopathology.** Tissue samples from gill, pseudobranch, heart, liver, pyloric caeca with pancreas, mid-kidney, spleen and skeletal muscle were fixed in 10% neutral phosphate-buffered formalin and prepared by paraffin wax embedding and standard histological techniques (Bancroft & Stevens 1990). Sections were stained with haematoxylin and eosin (H&E).

Sections of cardiac tissue were classified histologically based on the presence of mononuclear endo- and myocarditis, degeneration and necrosis in the spongy layer of the ventricle and atrium (Ferguson et al. 1990). The atrium, epicardium, compact and spongy layers of the ventricle and the endocardium of both cardiac compartments were examined and evaluated. The findings were graded from 0 to 4 according to the criteria in Table 1 (see also illustrations in Fig. 1).

**Microbiology.** ***Bacteriology:*** Swabs from mid-kidney of 2 fish in each sampled group were cultivated on blood agar plates with and without 2% NaCl at 15 and 22°C, respectively, for at least 6 d. Similar cultivation was also performed on samples from both mid-kidney and skin lesions from fish with skin ulcerations. Standard procedures for identification of isolated bacteria were performed.

***RRT-PCR:*** After completion of the experiment, 76 fish were examined for piscine nodavirus, salmonid alphavirus 3 (SAV3) and infectious pancreatic necrosis virus (IPNV) by RRT-PCR. The fish examined were those used for inoculate preparation, all fish from the pre-challenge sampling, a selection of mortalities, all fish from 3 scheduled samplings and some fish with severe heart lesions from scheduled samplings (see Table 2). Total nucleic acids were extracted from heart and kidney tissue using the NucliSens® easyMAG™ on-board protocol (bioMerieux) according to the manufacturer's instructions. The nucleic acid concentrations were determined using a Nanodrop ND-1000 (NanoDrop Technologies). Detection of nodavirus by RRT-PCR was performed according to Grove et al. (2006), but without quantification. RRT-PCR for detection of SAV (Jansen et al. 2007) and for detection of IPNV (Ørpetveit et al. 2007) was also performed. All RRT-PCR reactions were performed on a Stratagene Mx3005P.

Table 1. *Salmo salar*. Histological classification of lesions in endo-, epi- and/or myocardium

| Score | Description |
|---|---|
| 0 | No pathological findings, or slightly increased number of leukocytes |
| 1 | One or a few focal lesions, increased number of leukocytes |
| 2 | Several distinct lesions and small to moderate increase in number of leukocytes |
| 3 | Multifocal to confluent lesions and moderate to severe increase in number of leukocytes |
| 4 | Severe confluent lesions comprising >75% of the tissue and massive leukocyte infiltration |

***Cell culture:*** Post-experiment, heart and kidney tissue from 6 challenged fish sampled at 12, 18, 24 and 27 wk p.i., all with moderate to severe (Grade 3 or 4) CMS-like histopathological atrial lesions and at least Grade 2 lesions of the spongy myocardium of the ventricle, were examined in cell cultures according to routine procedures at the National Veterinary Institute, Oslo, Norway. The tissue samples were homogenised in cell culture medium (w/v 10%), and the homogenates were cleared by low-speed centrifugation. As IPNV is ubiquitous in Norwegian salmon farming (Melby et al. 1991, Jarp et al. 1995, 1996), the homogenates were treated with a mix of polyclonal neutralising antibodies against IPNV serotype Sp and serotype Ab. The homogenates were then inoculated onto cell cultures from bluegill fry fibroblast (BF)-2 cells (Wolf & Quimby 1966), epithelioma papulosum cyprini (EPC) (Fijan et al. 1983), rainbow trout gonad (RTG)-2 (Wolf & Quimby 1962), chinook salmon embryo (CHSE)-214 (Lannan et al. 1984) and Atlantic salmon head kidney (ASK) (Devold et al. 2000). Inoculated cells were incubated for 1 wk at both 15 and 20°C in parallel and were regularly investigated with an inverted microscope for the occurrence of a cytopathic effect (CPE). After 1 wk, the supernatants were pas-

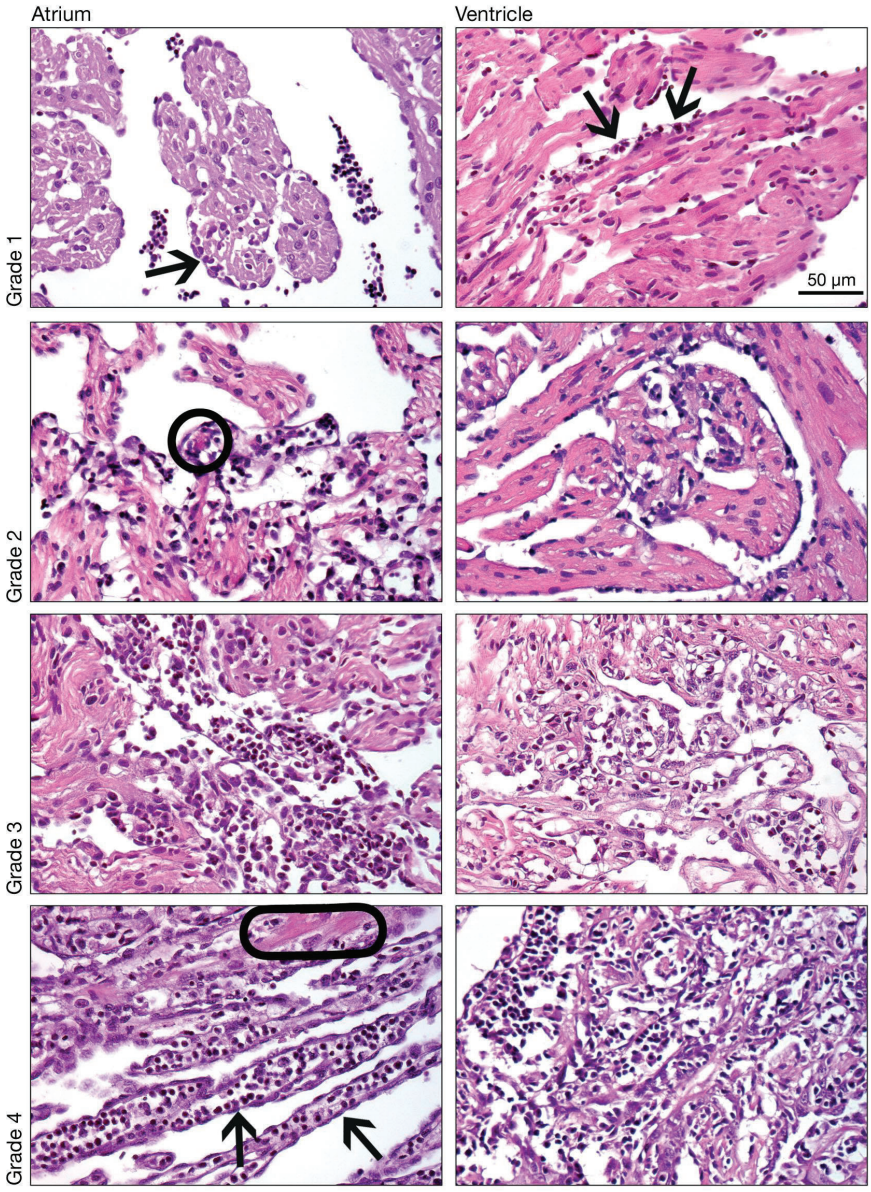


Fig. 1. *Salmo salar*. Histological classification of lesions in cardiac atrium and spongy ventricle, in accordance with the grading described in Table 1. Grade 1: arrows indicate minor inflammatory lesions consisting of sparse, focal subendocardial infiltration by mononuclear leukocytes and some degree of subendocardial vacuolisation, in both atrium and ventricle. Grade 2: several distinct lesions with small to moderately increased number of leukocytes. Myocyte degeneration and necrosis are encircled in the atrium. Grade 3: Multifocal to confluent lesions with moderate to severe leukocyte infiltration. Grade 4: Arrows indicate hypertrophic endocardial cells in the atrium forming empty tubes where almost all muscle fibres have been replaced by inflammatory cells, dominated by small mononuclear lymphocyte-like cells. Myocyte degeneration and necrosis are encircled in the atrium. Ventricular example is from a focal Grade 4 lesion. Haematoxylin and eosin staining. Scale bar (applies to all panels) = 50 µm

saged onto corresponding cells and incubated for a further week and investigated as described above.

## RESULTS

### Clinical signs

Only 2 fish showed clinical signs. One challenged fish was observed lying on the bottom of the tank at the first scheduled sampling and was included in the sampling. Another lethargic challenged fish was sampled 12 wk p.i.

**Mortality.** A total of 132 fish (26.6%) died during the experiment. Half of these fish were from Control Group 1, which experienced an IPN outbreak. In the period from 3 to 6 wk p.i., the 2 challenged groups and Control Group 1 displayed a distinct increase in mortality (15, 23 and 17 mortalities, respectively). A few weeks later (9 to 11 wk p.i.), the mortality in Control Group 1 peaked again and another 41 fish died during an IPN outbreak. The mortality of Control Group 2 followed a similar pattern (Fig. 2), but was lower, of shorter duration, and most likely related to fin and skin ulcerations (Table 2). There was no mortality in the control groups after Week 11 p.i., while an additional 12 challenged fish died sporadically throughout the study period, probably related to CMS.

**Autopsy.** Twelve fish from the challenged groups, the majority sampled 24 wk p.i. or later, had distended atria with or without blood clots. In every sampling from Week 24 p.i., sparse amounts of melanin-like deposits were visible in the atrium of 1 or 2 challenged fish.

Two challenged fish (12 and 24 wk p.i.) had fibrinous casts on the liver surface. From 12 wk p.i., and particularly in the last 6 samplings, a pale (9 fish), discoloured (6 fish) and/or yellowish (4 fish) liver was observed in a total of 14 challenged fish. No gross liver findings were observed in the control fish before the 2 last samplings, where 10 control fish displayed a pale (8 fish), yellowish (3 fish) and/or discoloured (6 fish) liver.

Throughout the study, most of the experimental fish suffered sparse, diffuse scale loss. In the first 24 to 27 wk, approximately half of the experimental fish also had mild to moderate skin lesions on one or both pectoral fins, with a varying degree of haemorrhage. Some of these fish had similar lesions on other fins and the tail, as well. Such lesions were not observed after Week 24. However, scarred fins and skin were seen in previously affected fish throughout the study. Four fish in Challenged Group 1 showed mild, uni-, or bilateral central cataract at the samplings 36 and 42 wk p.i.

Mortalities from Weeks 21 (2 fish) and 33 (1 fish) all displayed similar changes: distended atria with blood clots and varying degree of haemopericadium, in addition to fibrinous casts on the liver surface, a yellowish liver and ascites.

**Histopathology.** The fish injected with material from diseased fish developed lesions consistent with CMS

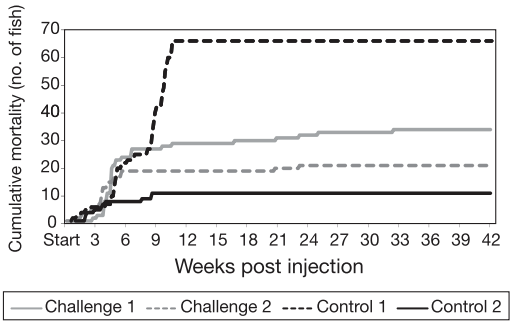


Fig. 2. *Salmo salar*. Cumulative mortality of Atlantic salmon after being intraperitoneally (i.p.) injected with tissue homogenate from Atlantic salmon diagnosed with cardiomyopathy syndrome (Challenged Groups 1 [grey solid line] and 2 [grey dotted line]) and after being i.p. injected with negative control inoculate (Control Groups 1 [black dotted line] and 2 [black solid line]). Total number of dead fish in each group to the right of the graph

Table 2. *Salmo salar*. Results of real-time RT-PCR examinations, p.i.: post-injection

| Time sampled | Category of fish | No. of fish tested | IPNV+ |
|---|---|---|---|
| Pre-challenge | Fish from field outbreak of CMS (6 fish), material for inoculate | 6 | 3 |
| Pre-challenge | Experimental fish | 5 | 0 |
| 3 wk p.i. | Scheduled sampling | 20 | 0 |
| 6 wk p.i. | Scheduled sampling, challenged fish with heart lesions | 1 | 1 |
| 8.5 wk p.i. | Mortalities, Control Group 2 | 2 | 0 |
| 9–10 wk p.i. | Mortalities, Control Group 1 | 8 | 8 |
| 12 wk p.i. | Scheduled sampling | 20 | |
| | *Challenged fish* | 10 | 8 |
| | *Control Group 1* | 5 | 4 |
| | *Control Group 2* | 5 | 0 |
| 15 wk p.i. | Histopathology + Challenged Group 1 | 1 | 1 |
| 24 wk p.i. | Scheduled sampling, incl. 5 fish from Control Group 2 only | 15 | |
| | *Challenged fish* | 10 | 9 |
| | *Control Group 2* | 5 | 0 |
| 27 wk p.i. | Histopathology + Challenged Group 1 | 2 | 2 |

(Ferguson et al. 1990). Detailed results are presented in Figs. 3 & 4, and findings according to time are shown in Fig. 5. Focal to multi-focal inflammation became evident in the atrium 6 wk p.i., with subsequent progression to the spongy myocardium of the ventricle 9 wk p.i. The inflammation in both the atrium and ventricle was dominated by mononuclear leukocytes, in addition to necrosis of spongy myocardium and endocardium.

Early atrial lesions observed at 6 wk p.i. were mild to moderate. The number of affected fish increased from 9 wk p.i. Severity of atrial lesions peaked at 12 wk p.i., and remained at this level for the rest of the study. The most extensive atrial lesion (Grade 4) was recorded in a single fish sampled 24 wk p.i. Grade 1 to 4 atrial lesions were found in at least 60% of the challenged fish at all samplings from 9 wk p.i., except at 33 wk p.i. Melanin-like deposits as observed at autopsy were located subendocardially in atrial lesions and in degenerated atrial myocardium. In the most severe foci, the deposits were fairly large (Fig. 6).

Mild lesions in the spongy layer of the ventricle (Grade 1) were initially detected in 3 fish with atrial changes at 9 wk p.i. The first fish with more moderate spongy layer lesions (Grade 2) was registered at 12 wk p.i., and, in the following samplings, the number of fish with Grade 2 lesions remained fairly constant. Ventricular spongy layer lesions of Grades 1 to 3 were found in at least 50% of the challenged fish at all samplings from 12 wk p.i., except at 27 and 39 wk p.i. The most severe inflammatory changes observed in this tissue were Grade 3, occurring in a few fish at 30, 33 and 42 wk p.i.

In the epicardium, a slightly increased number of leukocytes were observed in 34 of the 130 challenged fish, but in accordance with Table 1, these were graded 0. A total of 23 challenged fish displayed Grade 1 lesions in the epicardium, and 2 fish displayed Grade 2 lesions. The number of fish with epicardial changes decreased towards the end of the study.

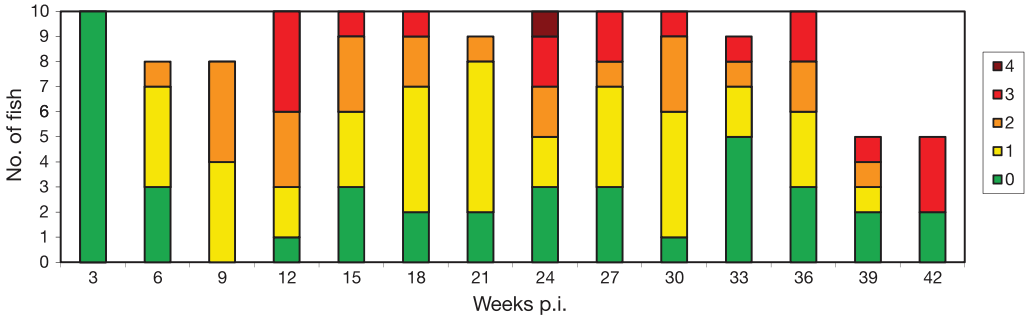


Fig. 3. *Salmo salar*. Histological findings in the atrium of the challenged fish. The lesions were classified from 0 (normal) to 4 (severe changes) according to Table 1. No or very little atrial tissue was present in the histological samples of 1 or 2 fish at 6, 9, 21 and 33 wk post-injection (p.i.) and could, therefore, not be evaluated in these fish. Due to high mortality in Challenge Group 1, the last fish in this group were sampled 36 wk p.i.

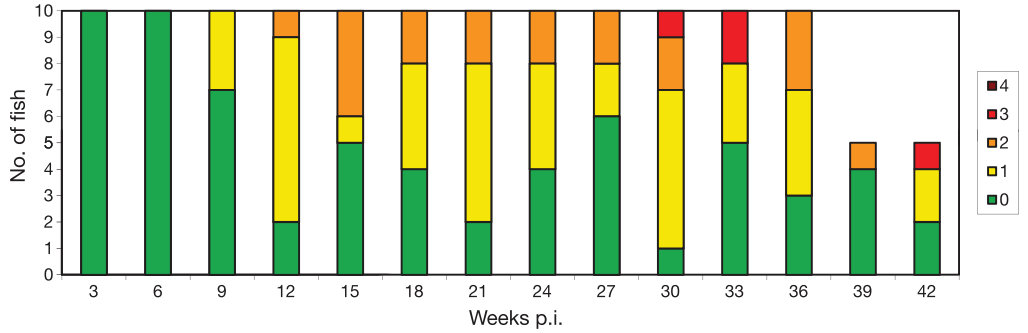


Fig. 4. *Salmo salar*. Histological findings in the spongy layer of the ventricle of the challenged fish. The lesions were classified from 0 (normal) to 4 (severe changes) according to Table 1. Due to high mortality in Challenge Group 1, the last fish in this group were sampled 36 wk post-injection (p.i.)

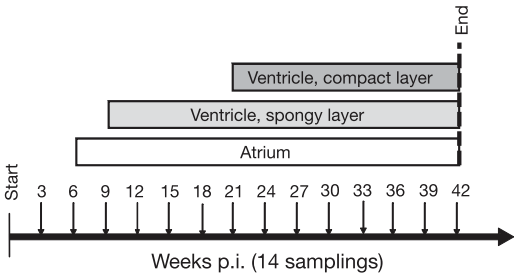


Fig. 5. *Salmo salar*. Appearance of histological cardiac lesions (as observed by light microscopy) over time post-injection (p.i.)

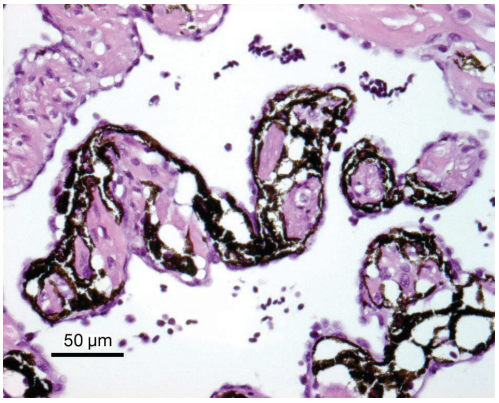


Fig. 6. *Salmo salar*. Melanin deposits in atrial lesions of experimental fish (36 wk post-injection) with a diagnosis of cardiomyopathy syndrome

Mild (Grade 1) compact layer lesions were first registered in a single fish at 18 wk p.i. and in 3 fish at 21 wk p.i. Thereafter, 1 or a few well defined foci of inflammatory cells in the compact layer, ranging in severity from Grade 1 to 2, were observed in 1 to 3 of the fish sampled at 30, 33, 36 and 42 wk p.i. All 12 fish with lesions in the compact layer also had inflammatory lesions in the spongy layer of the ventricle of identical or greater severity, while 7 of them also displayed additional focal Grade 1 or 2 lesions in the epicardium.

No CMS-like lesions were identified in the 5 fish sampled prior to challenge. None of the control fish had pathological changes in the compact layer of the ventricle, and only non-specific and sparse findings graded 0 or 1 according to Table 1 and Fig. 1 were detected in the hearts of a few fish. During the first distinct mortality period, IPN was diagnosed by histopathology and IPNV immunohistochemistry in both challenged groups and Control Group 1, but not in Control Group 2.

### Microbiology

**Bacteriology.** Bacteria were cultivated from 8 sampled fish (4 challenged) and 1 dead fish (control). The bacterial growth was dominated by a mixed flora considered to be of little or no significance. In only 2 fish (3 wk p.i.) were bacteria, subsequently identified as *Vibrio* sp. (dead, control fish) and sparse mixed flora (sampled, control fish), isolated from both kidney and skin or fin lesions.

**RRT-PCR.** All examined samples were negative for nodavirus and SAV by RRT-PCR. Three of the 6 fish used for inoculation of challenged fish had low to moderate levels of IPNV (Table 2). This was also the case in all examined fish from both challenged groups and Control Group 1. During the period of high mortality, moderate to low amounts of IPNV were found in all mortalities examined from the 2 challenge groups and Control Group 1. Fish tested for IPNV in Control Group 2 were negative throughout the study.

**Cell culture.** No cytopathic effect was detected in any of the cell cultures at the chosen incubation temperatures.

## DISCUSSION

In the present study cardiac lesions consistent with CMS were successfully transmitted to naïve Atlantic salmon *Salmo salar* post-smolts following i.p. injection of tissue homogenate from diseased fish. Both characteristics and severity of histopathological changes were reproduced in experimental fish. In addition, typical clinical signs and gross lesions were observed in some fish (Ferguson et al. 1990, Poppe & Ferguson 2006). These results indicate that CMS may be caused by an agent present in cardiac or renal tissue.

The first histopathological lesions appeared in the atrium from 6 wk p.i., while lesions in the spongy layer of the ventricle did not appear until 9 wk p.i. Generally, ventricular lesions were not seen in experimental fish without associated, and mostly more severe, atrial lesions. The explanation for this is uncertain, but the same pattern is typically seen in fish sampled from field outbreaks (T. T. Poppe unpubl. data). In such field material, atrial lesions are generally more severe than ventricular lesions, indicating a more advanced stage of the disease process in the atrium. The sequential occurrence of cardiac changes observed in the present study emphasizes the importance of sampling both atrial and ventricular tissues for histopathology. Evaluation of both tissues is a clear advantage in histopathological diagnostics, in order to differentiate between CMS and similar heart diseases.

Typical gross lesions, including enlarged atrium, fibrinous casts on the liver surface and ascites were seen in several challenged fish. These were mostly associated with histological findings of moderate to severe atrial lesions. Other typical autopsy findings in field cases of CMS indicating circulatory collapse, e.g. skin haemorrhage, raised scales and exophthalmia (Bruno & Poppe 1996), were not seen. This may be associated with the low degree of clinical disease and/or mortality in the present study. Most experimental fish showed normal swimming and feeding behaviour, in spite of severe cardiac lesions. These findings are in accordance with naturally occurring CMS, in which extensive cardiac lesions do not necessarily result in clinical disease (T. T. Poppe unpubl. data). Cardiac lesions similar to those seen in CMS have been observed in wild Atlantic salmon in Norway, but have not been associated with clinical disease (Poppe & Seierstad 2003). Several factors may influence the outcome of CMS under natural conditions. For instance, vaccination status, physiological stage, feeding regime, water temperature, oxygen levels, concurrent infections, parasite burden and other stressors may contribute to the development of clinical disease. Also, infection pressure, transmission route and factors related to pathogen exposure may be of significance. The almost simultaneous occurrence of increased mortality in all fish groups during the first weeks of this experiment could indicate exposure of the fish to unidentified environmental stressors in addition to the bacterial skin and fin infections. The markedly higher number of dead fish in 3 of 4 groups appears to be linked to the IPN diagnosis in these groups. Both the relative importance of infectious and environmental components in CMS-associated mortality and the potential for horizontal transmission should be further studied.

The experimental fish differed in at least 3 ways from the farmed fish typically affected in field outbreaks of CMS: (1) they were from a wild stock, (2) they were smaller and (3) they were unvaccinated. Experimental fish from a geographically isolated wild Atlantic salmon strain were chosen to reduce the risk of prior exposure to a possible causative agent present in the population or environment of farmed fish, and to avoid other factors associated with fish farming that may be of significance for the development of CMS. The results generated indicate that injection of tissue homogenate alone is sufficient for the development of histopathological changes typical of CMS. Additionally, the low age of experimental fish did not appear to hinder the development of cardiac lesions. Under natural conditions, CMS is commonly observed in large salmon (>2 kg) the second year in seawater, causing losses among large specimens of the population (Brun et al. 2003). The present study showed that post-smolts were capable of developing the same type and severity of lesions as larger salmon. However, the size of the fish may partly explain the limited number of fish showing clinical signs in the present study. The heart/body volume ratio decreases with increasing fish size, and large fish may therefore have a lower cardiac capacity compared to smaller fish (Agnisola & Tota 1994, Gamperl & Farrell 2004), rendering them more vulnerable to the effects of severe cardiac lesions.

The use of unvaccinated experimental fish in the present study may have contributed to the successful transmission of CMS in young fish. At 30 to 40 g weight prior to seawater transfer, all farmed salmon in Norway are routinely vaccinated i.p. with oil-adjuvanted vaccines. These vaccines may induce both specific and non-specific immunity (Poppe & Breck 1997, Koppang et al. 2008), which could interfere with susceptibility to infections. However, little is known about the immunological processes involved in the development of CMS. In some challenged fish, melanin deposits were observed on atrial surfaces and/or subendocardially from 24 wk p.i. Such deposits have also been observed in field cases of CMS, but not as a consistent finding (C. Fritsvold pers. obs.). Melanin is suspected to be of importance in the inflammatory responses of salmonids, but its exact role or effect is not fully understood (Thorsen et al. 2006).

Although transmissibility was demonstrated in the present study, a causal agent was not identified. RRT-PCR indicated that neither nodavirus nor SAV3 contributed to the development of CMS in the present study. No cytopathic effect was found in cell cultures inoculated with tissue material from challenged fish with moderate to severe (Grade 3 to 4) atrial lesions, indicating that other known fish pathogenic viruses were not present. Likewise, bacteria known to grow on blood agar were sparse, and probably did not contribute significantly to the development of cardiac changes. In addition, any prospective causal agent present in the transmitted material would have to be resistant to gentamycin at the concentrations used.

A viral aetiology for CMS has been previously suggested (Grotmol et al. 1997). Filtration of the inoculate was deliberately not performed in the present experiment, to ensure that possible causative bacteria were not eliminated from the inoculate before challenging the fish. In future studies, filtration of homogenates prior to injection may be used to investigate the possibility of a viral aetiology. The virological procedures used were intended to rule out the presence of known viruses, and may not have been optimal for detecting unknown viruses. In the further search for an etiological, possibly viral, agent, a range of different cell lines and/or growth conditions should be tested. As CMS

appears to be a relatively slowly developing disease (>3 wk for development of initial histopathological changes in experimental fish), the relatively short incubation periods for the cell cultures may have been too short to allow for CPE to develop in the present study.

IPNV was identified by PCR in fish used in inoculate preparation, as well as in both challenged groups and 1 of the 2 control groups. This may explain why challenged fish experienced an IPN outbreak. The source of IPNV infection in 1 of the 2 control groups is unknown. Fish may have been infected by IPNV at the cultivation facility or at the research facility, as neither of them routinely disinfected their inlet water.

Postviral endo- and/or myocarditis are well known in human medicine (Eriksson & Penninger 2005). This may also be a mechanism in the development of CMS, for instance triggered by an infection with IPNV. A possible association between IPNV and CMS has previously been suggested (Brun et al. 2003). However, the presence of IPNV and the outbreak of IPN in one of the control groups did not induce CMS-like lesions in those fish. In addition, a similar experiment with CMS has recently been performed by Bruno & Noguera in Scotland, with findings parallel to those observed in the present study. IPNV was not found in that study (D. W. Bruno pers. comm.). It therefore seems unlikely that IPNV played a significant role in the development of CMS in the present study. However, an interaction between IPNV and a possible causal agent of CMS cannot be ruled out.

Focal pathological changes appeared in the compact myocardium of 8 fish from 30 wk p.i. Compact layer involvement is uncommon in field outbreaks of CMS, but has been observed in some fish with extensive epicarditis (Poppe & Ferguson 2006). Strict focal epicarditis was associated with the compact layer lesions in the present study, but these changes were only mild to moderate. Absence of lesions in pancreatic tissue and red skeletal muscle indicate that the myocardial lesions in our study were not associated with HSMI or PD. In support of this, failure to detect SAV3 appears to exclude PD. Myocarditis in the compact layer is particularly pronounced in HSMI (Kongtorp et al. 2004a), which is experimentally transmissible by a model similar to that used in the present study. The aetiology of HSMI is not yet fully understood. A possible presence of the HSMI agent in the inoculate or the experimental fish used cannot be excluded. However, only a few fish showed mild to moderate focal changes in the compact myocardium in the present study, while this tissue is severely affected in infection experiments with HSMI (Kongtorp et al. 2004b, Kongtorp & Taksdal 2009). The most common cardiac lesions in the present study were very similar to those seen in field cases of CMS, and the development of cardiac changes also differed from experiments with HSMI. Furthermore, the time from challenge to appearance of lesions in the compact layer in the present study was considerably longer than in experimental transmission of both HSMI (Kongtorp et al. 2004b, Kongtorp & Taksdal 2009) and PD (McLoughlin et al. 1996, Christie et al. 2007).

In conclusion, cardiac changes consistent with CMS and, to a certain extent, clinical disease were transmitted to experimental fish. The results indicate that CMS may develop independently of PD and HSMI. Further studies are needed to establish the aetiology and pathogenesis of CMS and to develop disease-specific diagnostic tools.

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RESEARCH ARTICLE

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# Characterization of an outbreak of cardiomyopathy syndrome (CMS) in young Atlantic salmon, *Salmo salar L*

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## Abstract

Cardiomyopathy syndrome (CMS) is the most common viral cardiac disease in Norwegian Atlantic salmon farming and typically affects large, market size fish. Only six months after seawater transfer, Atlantic salmon were diagnosed with CMS at a fish farm in the south-western part of Norway. Due to the unexpected young age and the remarkable large amounts of virus-specific RNA (Ct <10), the fish group was monitored with five additional samplings until slaughtered almost 10 months later. At three weeks after the first CMS diagnosis (weeks post-diagnosis, wpd) and at slaughter (39 wpd), more comprehensive samplings were performed of the study cage, with specific focus on three different cardiac compartments. The clinical, autopsy and histopathological findings at first diagnosis and at all succeeding samplings were similar to previous descriptions of typical CMS. A slightly elevated mortality was observed in the cage with diseased fish at the time of the first CMS diagnosis and continued throughout the study. The prevalence and load of PMCV-specific RNA in the fish remained high until slaughtering, with similar amounts in all sampled cardiac compartments. No fish from the other five cages at the site were diagnosed with CMS, until fish sampled from the last cage at the site were diagnosed 10 weeks after slaughtering of the study cage (49 wpd). Sequence analysis of the PMCV on the site showed that the outbreak virus was similar to PMCV variants previously sequenced from Norwegian field outbreaks. In conclusion, CMS in young Atlantic salmon had clinical signs and histopathological cardiac lesions typical for the disease, and diseased fish could be found in the study cage until slaughtering.

**KEYWORDS**

1 kg Atlantic salmon, Atlantic salmon, cardiomyopathy syndrome, CMS, histopathology, piscine myocarditis virus, PMCV, real-time RT-PCR, *Salmo salar L*., sea water, sequencing

## 1 | INTRODUCTION

CMS is a contagious cardiac disease in farmed Atlantic salmon (*Salmo salar* L.), causing significant losses to the fish farming industry in Norway. In recent years, the disease has also been found in other salmon farming countries such as Scotland (Rodger & Turnbull, 2000), Ireland (H. D. Rodger et al., 2014) and the Faroe Islands (Poppe & Seierstad, 2003). A disease with similarities to CMS has also been reported in Canada (Brocklebank & Raverty, 2002).

CMS has been observed in Norwegian aquaculture since 1985 (Amin & Trasti, 1988). Since 2012, the yearly number of sites diagnosed with CMS by the Norwegian Veterinary Institute (NVI) has been about 100. As CMS is not a notifiable disease in Norway, and other commercial laboratories do histopathological diagnosing, the actual number of CMS cases has probably been higher. In 2020, 111 sites were diagnosed with CMS. This number is considered more reliable, as NVI and the three largest commercial histopathological laboratories in Norway for the first time cooperated in counting cases, eliminating the potential of counting a site twice.

Clinical CMS manifests by severe macroscopic and microscopic circulatory disturbances, caused by chronic heart failure, or sometimes even cardiac tamponade, resulting from severe, chronic endo- and myocarditis. Histopathologically, CMS is characterized by subendocardial inflammation, myocarditis and, in severe cases, degeneration and necrosis of spongious myocardium (Bruno et al., 2013; Ferguson et al., 2006).

Initial cardiac CMS lesions are most often observed in atrial spongiosum, followed by ventricular spongiosum, starting in a multifocal pattern, which in severe and late stages include all spongiosum of both cardiac chambers. Usually, the inflammation is more widespread and severe in atrial spongiosum compared with ventricular, but this may represent an 'in-between', moderate stage before the inflammation has evolved to the most severe stage with equal and complete distribution of lesions between these compartments. Compact myocardium is usually not affected, but a cellular epicarditis can sometimes be observed in moderate-to-severe cases, and is probably the origin of the perivascular inflammation occasionally seen along coronary vessels in the ventricular compactum (Bruno et al., 2013; Ferguson et al., 2006).

Sparse subendocardial leucocyte infiltration of the early stages progresses to the spongious myocardium as the disease reaches more severe stages. Mononuclear cells dominate the inflammatory process, together with lymphocytes, monocytes and macrophages, and occasional granulocytes (Bruno et al., 2013; Wiik-Nielsen et al., 2012). Endocardial cells initially become hypertrophic, then hyperplastic as the disease progresses, with increasing severity and extent of the inflammation. In severe stages of CMS, the intense cellular infiltration and the endocardial changes weaken the natural thin walls of the cardiac atrium and the sinus venosus, which may eventually rupture when strained, that is by stress, resulting in haemopericardium and sudden death (Ferguson et al., 1990). Even if the walls do not rupture, the intense cellular infiltration of severe CMS entails heart failure and severe congestion, often causing secondary lesions in other internal organs, in particular liver and spleen (Ferguson et al., 2006).

In atypical cases, in early stages and in cases where more than one causative agent is present at the same time, it can be challenging to distinguish between the cardiac lesions of CMS and its most important differential diagnoses: pancreas disease (PD), heart and skeletal muscle inflammation (HSMI) and infectious salmon anaemia (ISA) (Kongtorp et al., ,,2004, 2006) (McLoughlin & Graham, 2007) (Evensen et al., 1991). However, background information of the fish group, clinical signs, pathological lesions or absence of such in other organs, combined with detection of, and evaluation of the amount of, causative agent, can help differentiating between these diseases.

In 2009, in parallel with another group, we confirmed that the disease was of infectious nature (Bruno & Noguera, 2009; Fritsvold et al., 2009) and the causative agent, *piscine myocarditis virus* (PMCV), was identified in 2010 (Haugland et al., 2011; Lovoll et al., 2010).

PMCV was initially found to share homology with members of the family *Totiviridae* and has recently been found to have closest relation to three other piscine toti-like viruses found in wild golden shiner (*Notemigonus crysoleucas*), common carp (*Cyprinus carpio*) and farmed lumpfish (*Cyclopterus lumpus*) (Sandlund et al., 2021). None of these are yet classified into a virus family. The non-enveloped, approximately 50 nm, spherical PMCV virions have a double-stranded (ds) RNA genome of 6,688 base pairs with three open reading frames (ORF1, ORF2 and ORF3). The proteins encoded by ORF1 and ORF2 are believed to represent the coat protein and an RNA-dependent RNA polymerase, respectively. Several roles for the ORF3-encoded protein have been suggested, and research is ongoing, but final conclusions are yet lacking (Haugland et al., 2011; Nibert & Takagi, 2013; Sandlund et al., 2021).

CMS is typically diagnosed in fish of weight about 3–4 kg, about 1.5 years post-seawater transfer, quite close to harvest. Although experimental trials have shown that small salmon can be infected and develop CMS lesions both in sea water (Fritsvold et al., 2009; Hansen et al., 2011; Hillestad & Moghadam, 2019; Martinez-Rubio et al., 2014; Timmerhaus et al., 2011) and even in fresh water (Pers. comm. A. B. Mikalsen), it is only in the last few years that an increase in atypical cases affecting younger fish, shortly time after sea transfer has been observed (Fagerland et al., 2013; Fritsvold & Bang Jensen, 2019; Svendsen et al., 2019; Wiik-Nielsen et al., 2016). Whether this new trend is caused by changes in management, host susceptibility or PMCV virulence factors is not yet known. In this work, we describe the pathology, virus distribution and load in young, 1 kg fish, and monitor the fish group from the first detection of CMS until slaughtering of the site.

## 2 | MATERIAL AND METHODS

### 2.1 | Origin of samples

The samples originate from an aquaculture farm with Atlantic salmon (*Salmo salar* L.) in south-west of Norway (Figure 1). The site

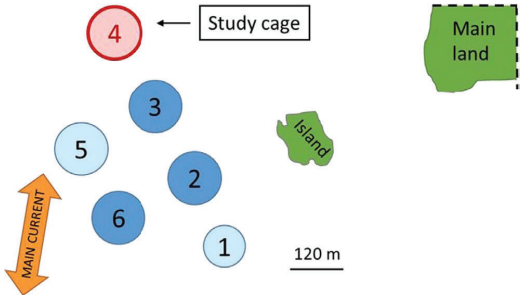



**FIGURE 1** Overview of the cages at the monitored fish farm. All cages were 38 m deep and ring-shaped. Direction of main sea current is indicated by thick arrow, and study cage marked with red ring. Distance from farm to mainland is about 350 m. Sea depths at the site vary between approximately 70 and 165 m. Cages 1 and 5 (light blue) were hatched in early spring 2012, in contrast to the other groups, hatched in late autumn 2012 (see detailed production data in Table 1)

was stocked with smolts of equal origin, but with small differences in hatching time, that is spring of 2012 (cages 1 and 5) and late autumn of 2012 (remaining cages).

All fish were vaccinated in the freshwater stage in the summer of 2013, using intraperitoneal injection of an inactivated virus vaccine against pancreas disease (PD), and an inactivated combination vaccine against *Aeromonas salmonicida* subsp. *salmonicida*, *Listonella anguillarum* serotype O1, *Listonella anguillarum* serotype O2a, *Vibrio salmonicida*, *Moritella viscosa* and *infectious pancreatic necrosis virus (IPNV) serotype* Sp 0. After subsequent smoltification, a total number of about 970 000 smolts were put to sea in the 6 ring-shaped cages at the site, at three different time points with 5-week intervals, during the summer and early autumn of 2013 (Table 1).

The first CMS diagnosis at the site was confirmed in cage 4, six months after transfer to sea water during a routine health service control. This cage was selected as the main study cage and was further monitored with a total of five additional samplings over a period of almost 10 months (Figure 2). Three of the samplings were performed by the fish health service at random time points and submitted to NVI for routine diagnostics, and two more comprehensive samplings were performed at the NVI on submitted whole fish. Additionally, the fish health service submitted samples from two other cages (cages 3 and 6), at two time points, as autopsy findings and/or increased mortality indicated disease outbreaks. The fish of the study cage were slaughtered out 39 weeks post-first CMS diagnosis, in late autumn of 2014, while the remaining fish at the site were slaughtered at 15 weeks later (Figure 2).

## 2.2 | Sampling and preservation of samples

All fish were evaluated clinically when sampled. Autopsy was performed at the site, except for the two comprehensive samplings. At all samplings, a standardized set of organs consisting of gill, heart, liver, exocrine pancreas, spleen, kidney and skin with underlying red and white skeletal muscle were sampled and fixed in 10% neutral buffered formalin for histopathological examination.

At the regular samplings, parallel samples of head kidney and the apex of the heart were fixed in RNA*later*® (Ambion) for subsequent real-time PCR analyses.

At the two comprehensive samplings, 3 and 39 weeks post-first CMS diagnosis (wpd), an increased number of fish were submitted and a wider number of tissues under selection were sampled in the laboratory. Each of these samplings consisted of both recently dead fish, with clinical findings indicating a CMS diagnosis, and apparently healthy fish (see Figure 2 for details). To reduce the impact of autolysis, gills were sampled and fixed in formalin and RNAlater at the farm site. Autopsy and all other tissue sampling were performed at the NVI, after expressing transport of whole fish on ice. The cardiac compartments atrium, ventricular spongiosum and ventricular compactum were sampled separately for preservation on RNAlater, in addition to inclusion of spleen tissue. In contrast to the regular samplings, mid-kidney was chosen for kidney samples on RNAlater, in accordance with the NVI's recommendation for viral tissue sampling, to better represent both secretory and haematopoietic kidney tissues, and to match tissue sampled for histopathology.

## 2.3 | Histopathology

All formalin-fixed tissue samples were embedded in paraffin wax, sectioned and prepared for histology by standard procedures (Bancroft & Stevens, 1990), resulting in 3-μm-thick tissue slides examined by light microscopy after haematoxylin and eosin (H&E) staining. For the two more comprehensive samplings (3 and 39 wpd), severity and distribution of pathological changes in cardiac atrium, compact and spongiform myocardium of the cardiac ventricle, and the epicardium, were scored from 0 (no lesions) to 4 (severe, typical lesions) according to Fritsvold et al. (2009) (Table 4). In addition, all tissue samples of exocrine pancreas and skeletal muscle with attached skin from these two time points were evaluated histopathologically. All relevant pathological changes in the tissue samples received from regular samplings were evaluated and described in accordance with standard diagnostic histopathology routines of the NVI, without scoring of cardiac tissue.

## 2.4 | Immunohistochemistry

All heart samples resulting from the first CMS diagnosis (0 wpd), the following, first comprehensive sampling (3 wpd) and the last comprehensive sampling (39 wpd) at slaughtering were included for virus-specific immunohistochemistry (IHC) for PMCV.

IHC was performed in accordance with a protocol developed previously (Gulla et al., 2012) and further optimized for research purposes at NVI. In short, 3-μm-thick dewaxed and dehydrated cardiac

**TABLE 1** Selected production data for all cages of fish at the monitored fish farm

| Cage | Diameter of cage | Time ofseawater transferin 2013 | Weight (grams)at seawater transfer | No. of smolts(rounded)at seawater transfer | Weeks, seawater transfer to slaughter | Mortality, all cages:**Early February 2014–end of November 2014 (= Last 10 months of sea phase for study cage, cage 4)** | |
|---|---|---|---|---|---|---|---|
| | | | | | | % | No. of fish |
| **1** | 120 m | Late June | 86 | 101 000 | 60 | 2.0 | 2 010 |
| **2** | 160 m | Early September | 98 | 183 000 | 80 | 3.2 | 5 890 |
| **3** | 160 m | Early August | 68 | 175 000 | 68 | 7.3 | 12 565 |
| **4** | 160 m | Early August | 68 | 175 000 | 67 | 12.4 | 21 526 |
| **5** | 160 m | Late June | 73 | 162 000 | 65 | 1.9 | 2 956 |
| **6** | 160 m | Early August | 68 | 174 000 | 82 | 2.5 | 4 343 |

*Note:* CMS was diagnosed in the study cage, cage 4 (pink marking), about 6 months post-seawater transfer. Fish of cages 1 and 5 (light blue marking, also in Figure 1) hatched in the spring of 2012, while fish put to sea in all other cages, including the study cage, hatched in November 2012.

tissue slides was incubated with 5% BSA in a Tris buffer for 20 min at room temperature, followed by incubation for 90 min with a 1:2000 primary rabbit polyclonal antibody dilution, based on recombinant proteins from ORF3 (ΔORF3, ZN01101, Kanin 062, PMCV), a kind gift from M. Rode, Pharmaq AS. Subsequently, slides were incubated for 30 min with a 1:500 secondary antibody dilution (biotinylated goat anti-rabbit Ig; DAKO E0432, DAKO), followed by a 30-min incubation with Streptavidin alkaline phosphate in a 1:500 dilution (Streptavidin-AP, Vector SA-5100). Finally, the slides were incubated for 20 min with Fast Red as signal substrate (10 mg Fast Red TR Salt (Sigma F2768), 2 mg Naphthol AS-MX fosfat, 0,2 ml N,N-dimethylformamide, 0,01 ml 1 M levamisole in 9,8 ml 0.1 M Tris buffer pH 8.2). Cardiac tissue samples from salmon that tested negative by real-time RT-PCR for PMCV, PRV and SAV were used as negative controls to cover for the most relevant differential diagnoses.

### 2.5 | PCR and sequencing

RNA was extracted from all RNAlater-preserved tissue samples, and a real-time RT-PCR targeting the ORF1 of PMCV was performed on 500 ng extracted total nucleic acids from each sample (Lovoll et al., 2010).

All hearts from all samplings, except 0 wpd, were investigated for the presence of salmonid alphavirus (SAV) and piscine orthoreovirus (PRV-1) with real-time RT-PCR (Hodneland & Endresen, 2006; Jansen et al., 2010; Lovoll et al., 2012). A real-time RT-PCR targeting the VP3 region of infectious pancreatic necrosis virus (IPNV) (Orpetveit et al., 2010) was performed to rule out the presence of this virus in the comprehensive sampling 3 wpd.

Sanger sequencing was performed as described previously (Wiik-Nielsen et al., 2013) on individual 3 from 17 wpd sampling with resulting sequence of ORF1 and ORF3 reading frames, also including parts of the UTRs in 5' and 3' end of ORFs. To study a possible variation between strains in several individuals at one time point and over time during the outbreak, similar PCR and sequencing procedures were used in combination with two primer sets amplifying overlapping products of the UTR between ORF2 and ORF3 (F-primer 5'-CGCGCTCATAGAAGAAAAGAAC and R-primer 5'-ACAATCCCTTCCCCTACAC, personal gift Cheng Xu, Norwegian University of Life Sciences) and the 5' end of ORF3 (PMCV ORF3-1 primer set from Wiik-Nielsen et al. 2012)(Wiik-Nielsen et al., 2012).

## 3 | RESULTS

### 3.1 | Clinical signs, viral infections and corresponding diagnoses of all samplings

In the early spring of 2014, a fish farm in the south-west of Norway experienced increased mortality in 1 kg Atlantic salmon (*Salmo salar* L.) in one of the 6 cages at the site, only 6 months after seawater transfer. Clinical findings and autopsy indicated a circulatory heart disease. Histopathological examinations of the samples revealed cardiac lesions and lesions in other organs (i.e. liver) typical for CMS, and a CMS diagnosis was confirmed in this cage. The site was further monitored until complete slaughter about 12 months later, with two comprehensive samplings of the cage initially diagnosed (study cage) at 3 weeks post-diagnosis (wpd) and at slaughtering, 39 wpd, including all material from regular fish health service samplings of all cages received at NVI since the first CMS diagnosis. This resulted in material from a total of 8 different time points, 6 of them of the study cage (Figure 2).

No significant drop in appetite was observed in the study cage in the observational period, the sampled fish were all in good

condition, but the mortality rate was moderately increased from 0 wpd. Mortality with typical CMS pathology, both clinically and histopathologically, continued to be higher for the study cage than for the other cages, persisting until slaughter 39 weeks later (Table 1). The mortality of the study cage was not reported to be associated with handling, stress or any treatments. For the last 5 months before slaughter (4 time points of registration) the daily mortality varied between 0.37% (mid-summer) and 1.47% (2 weeks before slaughter), resulting in a 12.4% cumulative mortality since the first CMS diagnosis. No other significant causes of the mortality were found in the fish of the study cage during the observational period of 39 weeks post-first CMS diagnosis. Moribund fish was not observed before the 28 wpd and following samplings.

All examined fish from all samplings of the monitored cage were positive for PMCV-specific RNA, and large amounts of viral RNA were detected in the hearts of the CMS-diagnosed fish at all time points (Table 3, Figure 6). PMCV ORF3 protein products were detected by immunohistochemistry staining not only in the cardiac lesions of all CMS-diagnosed fish at the first sampling, but also in those of CMS fish at 3 and 39 wpd (Figure 7).

PRV-1 RNA was detected in the study cage in low and moderate levels for most of the fish (Table 3 and Figure 6) from the second sampling (3 wpd) and onwards, but without typical or severe histopathological manifestations of HSMI. At 19 wpd, in cage 3, next to the study cage, an increase in mortality was found to be observed, and clinical observations, autopsy findings and histopathological lesions strongly indicate an HSMI outbreak, but an HSMI diagnosis was not given, as the lesions were too unspecific. As expected, all 5 sampled fish were positive for PRV-1, but, interestingly, negative for PMCV.

At 28 wpd, all five fish sampled of the study cage were diagnosed with CMS; in addition, HSMI was suspected in four of them, but no SAV was detected. In the next sampling (37 wpd), SAV3 RNA was detected in hearts of the study cage fish (Figure 3 and Table 2) with mean Ct-value 27.6, and similarly, it was found in samples taken at slaughter (39 wpd). As these fish lacked histopathological lesions typical for PD, a PD diagnosis could not be concluded. SAV3 RNA was also detected at similar Ct-values in fish from cage 6, sampled close to emptying of the site (49 wpd) (not shown). These fish were diagnosed with severe CMS, but, like the examined individuals at 37 wpd in cage 4 (study cage), also lacked pathognomonic signs of PD. No IPNV was found in the 15 fish examined at the first comprehensive sampling (3 wpd).

A majority of the fish sampled at both comprehensive samplings had intestinal tapeworms (*Eubothrium crassum*). The presence of *Paramoeba perurans* at the site was confirmed by PCR at another commercial laboratory (Ct-value 27.2) about 30 wpd, but without clinical disease. At 35 wpd, clinical amoebic gill disease (AGD) was present in two cages (no. 2 and 3), and at 37 wpd, also in a third cage, no. 6. At this time point, the average gill score in the study cage was 1 (out of maximum 5), as defined previously (Taylor et al., 2009).

### 3.2 | Detailed characteristics from the two comprehensive samplings

The study cage was subjected to two more comprehensive studies at an early (3 wpd) and a late time point (at slaughter, 39 wpd). An increased number of individuals were sent as whole fish to NVI for clinical observations, autopsy, histopathological evaluation and in situ detection of PMCV antigens by immunohistochemistry in relation to detected pathological cardiac lesions. Differences in viral load in various organs were also investigated, with specific emphasis on the three cardiac compartments the atrium, ventricular spongiosum and ventricular compactum, and results were compared with the distribution and severity of cardiac histopathological lesions (Figure 6, Table 3 and 5).

### 3.3 | Clinical observations

Regarding size, shape and weight, all fish of both comprehensive samplings were in the expected normal range; hence, there was no obvious differences between fish selected for sampling by clinical signs indicating CMS or apparently healthy fish regarding these measures.

Fish selected for sampling as presumed CMS fish were all fresh morts and had an overall similar presentation of a few typical clinical signs: some degree of oedema of skin scale pockets, and sparse-to-moderate, ventral petechiae. It was difficult to find moribund fish until 28 wpd, when the number of moribund fish slightly increased, most likely related to introduction of SAV to the fish group: PD was suspected at 28 wpd, and SAV was detected by PCR at 37 wpd, but never with histopathological findings confirming PD.

### 3.4 | Autopsy observations

In general, fish suspected to suffer from CMS, selected for sampling based on clinical observations, had very similar autopsy findings in both the early and late comprehensive samplings. A majority of them presented different degrees of typical signs of general congestion, such as ascites and fibrinous casts on liver surfaces, red or light discoloured liver, blood-coloured or clear, yellowish fluid and/or blood clots in the pericardial cavity (Figure 4).

All suspected CMS morts had enlarged, blood-filled atria at both samplings. All morts at the 39 wpd sampling, as well as the majority of the morts at the 3 wpd sampling, had a cardiac tamponade with blood clots filling the pericardial sac and/or cardiac cavity. Two presumed healthy fish (fish 6 and 9) at the 39 wpd sampling had a cardiac abnormality (situs inversus), and one fish (fish 9) had a grossly multifocal gill inflammation, present as 'untidy' gill lamellae with white foci on several gill filaments. The gender ratio was close to 50/50 in both samplings. Intestinal tapeworm (*Eubothrium crassum*) was a common finding in both comprehensive sampling, regardless

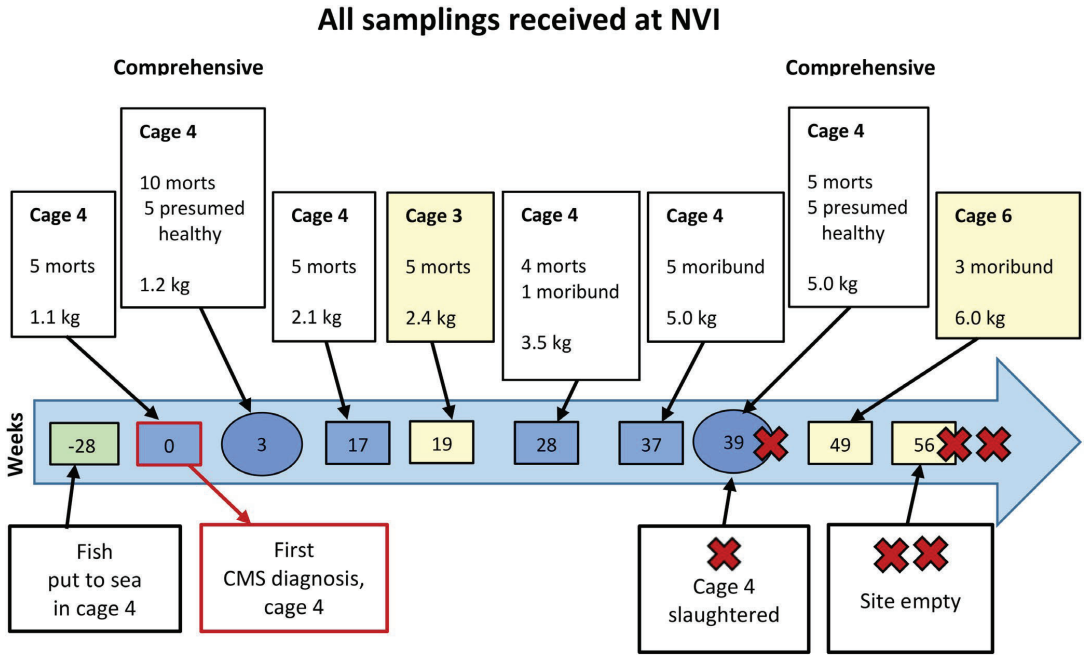



FIGURE 2 Overview of relative time points for seawater transfer, first CMS diagnosis and slaughtering of cage 4 (study cage), relative to all sampling time points of the site. In addition, number and mean weight of sampled fish, and whether they were morts or moribund. Study cage sampling time points in blue, samplings of other cages in yellow

of clinical CMS. With these exceptions, fish sampled as healthy fish presented normal findings at autopsy.

### 3.5 | Histopathological findings

H&E-stained tissue samples of heart, exocrine pancreas and skeletal muscle (red and white) with attached skin from all fish, and in addition, gill, liver, spleen and mid-kidney from the presumed CMS fish, were evaluated histopathologically.

The histopathological cardiac lesions were similar to descriptions of typical CMS in larger, market size fish, both in presentation and in distribution (D. W. Bruno et al., 2013; Ferguson et al., 2006). They presented as a multifocal-to-diffuse cellular myocardial and subendocardial inflammation in the majority of the 15 fish selected for sampling by clinical signs indicating CMS at both 3 wpd and 39 wpd, confirming the suspected CMS diagnosis (details for 3 wpd fish are given in Table 5). As expected in fish with CMS, the most severe cardiac lesions were seen in the atrium and spongiosum of the cardiac ventricle, while the compactum of the ventricle had no or only focal and relatively sparse inflammatory lesions, possibly slightly more severe at 39 wpd than at 3 wpd (Figure 5). At 3 wpd, three of the fish sampled as possible CMS fish based on clinical signs did not have cardiac pathology in accordance with a CMS diagnosis: very few and sparse cardiac lesions in fish 4 and 9 could be indications of very early HSMI or CMS, while fish 7 had an almost completely normal heart with very few inflammatory cells, not indicating any specific cardiac disease (Table 3a). All fish selected for sampling as possible CMS fish at 39 wpd sampling had histopathological cardiac lesions resulting in CMS diagnoses. Of the clinically healthy fish in this last sampling, the cardiac changes of fish 6, 7 and 9 were in accordance with a sparse-to-moderate, and for fish 8, a moderate, HSMI and/or PD diagnosis. Fish 10 had more sparse cardiac lesions, possibly indicating very early HSMI and/or CMS (Table 3b).

In some of the CMS-diagnosed fish, the few, minor foci of myositis observed in the cardiac compact layer of the ventricle were located close to the epicardium or the spongiosum of the ventricle. A few CMS fish presented with a sparse, multifocal perivascular inflammation in the compact layer of the ventricle. Focal-to-multifocal epicarditis, differing in degree from very sparse to focally severe, was observed in the CMS fish with the most severe lesions in spongious cardiac tissues (Table 5). In an increasing number of fish per sampling point, a possible slight tendency of an increase in distribution and severity of the cardiac lesions, from multifocal to more diffuse, and from moderate to severe, to more severe, was observed towards the end of the production period, and no indications of regeneration or healing of cardiac tissue were observed.

Signs of circulatory disturbances in the form of dilated liver and renal sinusoids, small cardiac thrombosis in cardiac cavities and fibrinous layers on liver surfaces were observed in a majority of the fish. Apart from this, all examined samples of exocrine pancreatic tissue, red and white muscle with attached skin, gills and mid-kidney were normal. All fish had some degree of granulomatous peritonitis on surfaces of the internal organs, in some fish causing adhesions between organs or between organs and the abdominal walls.

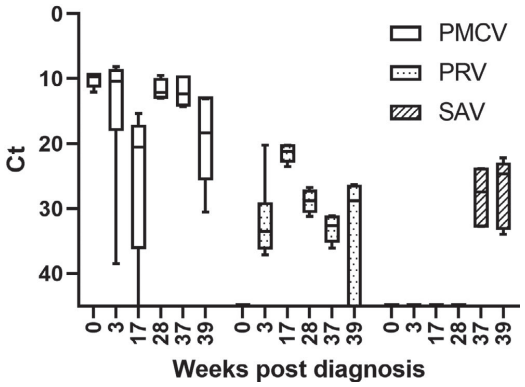



**FIGURE 3** Comparison of real-time RT-PCR results of PMCV, PRV and SAV for all heart samples of study cage fish sampled as presumed diseased fish based on clinical signs indicating circulatory disturbances and heart disease. As the fish health services sampled apex of the hearts for viral detection in their ordinary samplings, ventricular compactum was chosen as the equivalent cardiac tissue of the two comprehensive samplings, 3 and 39 wpd. Ct-values are given as box plots, where median is marked as a vertical line on the box, which shows the interquartile range, while maximum and minimum values are presented as the top and bottom lines of the box

### 3.6 | Viral levels in heart compartments and other organs

At the first comprehensive sampling at 3 wpd, PMCV RNA was detected in all 15 investigated fish (Table 3, Figure 6) from cage 4. The highest load of viral RNA was detected in cardiac tissue, with Ct-values below 10 in all CMS-diagnosed fish (Table 3a and Figure 6a). The Ct-values indicate approximately 100–1000 times more virus in the heart compared with other organs tested for these fish. There was no significant difference in obtained Ct-values for the three investigated cardiac departments. PMCV RNA was also detected in the hearts of the dead fish without CMS diagnosis (fish 4, 7 and 9), although with higher Ct-values (Table 3a) and at lower levels in the 5 clinically healthy fish. Only one of the clinically healthy fish was found to be PMCV-positive in all three cardiac compartments, and only two individuals sampled as healthy had a positive spleen or kidney (Figure 6a).

At the 39 wpd sampling, PMCV RNA could still be detected in all sampled organs of all fish, except for 4 individuals with one negative tissue sample each (Figure 6b). In the 5 fish with CMS diagnosis, the heart was still the organ containing the most viral RNA, compared with other organs (Table 3b and Figure 6b). PMCV RNA was also detected in all, but one, cardiac tissue samples of the 5 fish without clinical signs of CMS and no CMS diagnosis, but in lower amounts (Table 3b).

In both samplings, individuals with CMS also had high levels of PMCV-specific RNA in spleen and mid-kidney, but at a lower level compared with cardiac tissue (Figure 6). The high levels of PMCV-specific RNA in cardiac samples remained high throughout the production period, until slaughter almost 10 months later (Figure 6 and Table 3).

Including both comprehensive samplings, the correlation between heart lesions (histopathological score) and viral levels (Ct-values for detected RNA) was high for all cardiac compartments. The highest correlation coefficient was found for atrium and spongiosum

**TABLE 2** Overview of causes triggering samplings of the study cage, prevalence of fish diagnosed with CMS and prevalence of PMCV, SAV and PRV virus RNA detection

| Weeks post-first CMS diagnosis | 0 | 3 | 17 | 28 | 37 | 39 |
|---|---|---|---|---|---|---|
| ***Cause of sampling*** | Suspected HSMI | Suspected CMS | Suspected CMS | Suspected CMS +PD | Suspected AGD, CMS +PD | Suspected CMS |
| ***Type of fish*** | Morts | Morts | Morts | 4 morts, 1 moribund | Moribund | Morts |
| ***No. of fish with CMS**** | 4/4 | 7/10 | 3/5 | 5/5 | 5/5 | 5/5 |
| ***No. of fish PCR PMCV+*** | 4/4 | 10/10 | 3/5 | 5/5 | 5/5 | 5/5 |
| ***No. of fish PCR SAV+*** | Not tested | 0 | 0 | 0 | 5/5[‡] | 5/5 |
| ***No. of fish PCR PRV +*** | 0 | 10/10[†] | 5/5 | 5/5 | 5/5[§] | 5/5 |

*Note*: CMS was suspected in both comprehensive samplings (3 and 39 wpc), while observations and clinical findings indicative of different diseases initiated the other samplings, performed by the fish health services. At the comprehensive samplings, the viruses were detected in all three sampled heart compartments of each mort, unless otherwise stated (see also Table 3, incl. details of results from sampled fish presumed healthy)

[†]Three individuals were PRV-negative in atrium, and one individual was negative in both atrium and ventricular spongiosum

[‡]One individual was SAV-negative in the atrium

[§]One individual was PRV-negative in both ventricular spongiosum and ventricular compactum, and one individual was negative in ventricular compactum.

**TABLE 3** Comparison of individual Ct-values in cardiac tissue (COMP V= ventricular compactum, SPONG V = ventricular spongiosum) for PMCV and PRV in the two comprehensive samplings, at an early (3 wpd) and late (39 wpd) time points. (a) Comprehensive sampling 3 wpd. Fish 1–10 selected for sampling based on clinical signs as possibly CMS diseased. Fish 4, 7 and 9 did neither have CMS nor HSMI. (b) Comprehensive sampling 39 wpd, at slaughtering. Fish 1–5 were selected for sampling based on clinical signs as possibly CMS diseased. Fish 6–10 were clinically healthy fish. Histopathologically, fish 7 and 8 were suspected to have a sparse HSMI, possibly masking a very early CMS infection, in combination with cardiac lesions in accordance with those seen in late-stage PD survivors

| | | Fish no. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Suspected CMS | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | **ATRIUM** | 11.3 | 7.9 | 8.9 | 20.3 | 10.4 | 8,7 | 26.4 |
| **PMCV** | **COMP V** | 9.9 | 9.2 | 8.2 | 15.6 | 12.2 | 9,9 | 38.5 |
| | **SPONG V** | 9.0 | 8.2 | 8.1 | 21.7 | 10.0 | 9,0 | 25.6 |
| **Diagnosis:** | | **CMS** | **CMS** | **CMS** | **-** | **CMS** | **CMS** | **-** |
| | **ATRIUM** | 31.3 | 37.0 | No Ct | 23.7 | No Ct | No Ct | 35.0 |
| **PRV** | **COMP V** | 34.3 | 32.3 | 35.7 | 23.9 | 34.3 | 37.1 | 36.0 |
| | **SPONG V** | 34.2 | 29.1 | 28.5 | 24.6 | 34.9 | No Ct | 36.1 |

| | | Fish no. | | | | |
|---|---|---|---|---|---|---|
| | | Suspected CMS | | | | |
| | | 1 | 2 | 3 | 4 | 5 |
| PMCV | ATRIUM | 16.5 | 12.5 | 10.8 | 15.6 | 16.5 |
| | COMP V | 19.9 | 13.2 | 13.1 | 18.3 | 30.5 |
| | SPONG V | 14.9 | 10.1 | 11.7 | 15.8 | 13.2 |
| | **Diagnosis** | **CMS** | **CMS** | **CMS** | **CMS** | **CMS** |
| PRV | ATRIUM | 25.8 | 38.4 | 26.0 | 26.5 | 24.4 |
| | COMP V | 28.8 | No Ct | No Ct | 27.7 | 26.4 |
| | SPONG V | 28.1 | No Ct | 27.5 | 27.2 | 27.7 |

compartments (r = 0.92 and 0.93, respectively), with somewhat lower for compact ventricle (r = 0.72).

Low amounts of PRV-1 RNA were detected in the hearts and other organs of all fish at both early and late comprehensive samplings, usually with highest amounts in the spleen, followed by cardiac tissues. At 3 wpd, Ct-values were typically above 30, not indicative of active, clinical HSMI disease. Three of the fish (no. 4, 9 and 12, all without CMS diagnoses) had higher amounts of PRV-1 (cardiac range 19.23 – 24.55, splenic range 16.2 – 20), although no HSMI diagnoses were assigned to any of them. A similar trend was seen at 39 wpd, but with a little higher average Ct-values than at 3 wpd; that is, average cardiac Ct-value of all fish from both groups was 27.23 (range 24.32–No Ct) for all three examined cardiac compartments, and spleen average Ct-value of all 10 fish was 23.28 (range 21.9 – 27.77).

## 3.7 | Detection of PMCV proteins by immunohistochemistry

Positive IHC staining, of varying but convincing strength, was seen in typical cardiac CMS lesions, most often in the ventricular spongiosum, but sometimes also in atrial tissue, in all fish diagnosed with CMS after histopathological examination. However, the correlation between amount of PMCV RNA, severity of histopathological cardiac lesions, and strength or amount of positive IHC staining was neither obvious nor consistent: CMS-diagnosed individuals with very high levels of PMCV-specific RNA and relatively severe histopathological cardiac lesions sometimes presented with weaker IHC staining than other individuals with lower Ct levels and no CMS diagnosis (Table 5).

## 3.8 | Genomic sequence characteristics

The first sequencing performed on individual 3 from 17 wpd showed nucleotide identity of 99,61% nucleotides (nts) in ORF1 and 100% identity of ORF3 when compared to the reference isolate first described, PMCV AL V708 (GenBank acc. no. HQ339954). Comparison of translated sequences showed 99,42% identical amino acids in the ORF1-encoded protein described as 5 synonymous mutations and 5 non-synonymous mutations (Table 6a), compared with the reference isolate (GenBank acc. no. ADP37186.1). No changes were seen in

| | | | Clinically healthy | | | | |
|---|---|---|---|---|---|---|---|
| **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
| 10.1 | 24.4 | 7.0 | No Ct | 29.5 | 29.5 | 30.5 | No Ct |
| 10.9 | 23.5 | 8.2 | 30.9 | 30.8 | No Ct | No Ct | 30.3 |
| 9.3 | 21.8 | 8.2 | No Ct | 29.4 | 30.6 | No Ct | 28.8 |
| **CMS** | - | **CMS** | **PossiblysparseHSMI** | - | - | - | - |
| 29.2 | 19.2 | No Ct | 28.5 | 19.8 | 36.8 | 34.7 | No Ct |
| 31.3 | 20.2 | 32.8 | 26.2 | 20.4 | No Ct | 31.8 | 34.6 |
| 30.4 | 20.0 | 30,9 | 26.7 | 23.8 | 33.7 | 24.1 | 36.4 |

| **Clinically healthy** | | | | |
|---|---|---|---|---|
| **6** | **7** | **8** | **9** | **10** |
| 33.3 | 18.6 | 33.3 | 31.4 | 30.7 |
| 30.7 | 17.8 | 30.0 | 32.0 | 30.4 |
| No Ct | 16.6 | 31.3 | 33.5 | 21.9 |
| **Moderate HSMI and/ or PD** | **Moderate HSMI and/or PD, possibly also CMS** | **Moderate HSMI and/ or PD** | **Sparse-to-moderate HSMI and/or PD** | **Sparse CMS and/or HSMI** |
| 27.9 | 24.3 | 25.7 | 26.4 | 30.3 |
| 26.5 | 26.8 | 26.1 | 25.2 | 28.7 |
| 25.3 | 25.7 | 27.9 | 26.4 | 27.3 |

the parts of UTRs included in sequenced amplicons. To study potential variation in viral strains between individuals at one sampling time point and over time in the infection progression, two sequence overlapping amplicons were sequenced covering mainly UTR between ORF2 and ORF3 and subsequent first 547nts/182 aa of ORF3/ORF3-encoded protein. The nucleotide sequence of the sequence generated from all the included samples showed 99.63%–100% identity to the reference isolate PMCV AL V708 (GenBank acc. no. HQ339954) (Table 6b).

## 4 | DISCUSSION

In contrast to the traditional appearance of CMS in large fish of at least 3–4 kg, close to slaughter, CMS with typical clinical and histopathological findings and mortality was found in young, 1 kg Atlantic salmon, *Salmo salar* L., as early as 6 months post-seawater transfer.

Throughout the production period, the clinical manifestations in the fish of the study cage were in accordance with descriptions of typical CMS (i.e. mortality of fish of good condition, with ventral petechial haemorrhages, scale pocket oedema and/or ascites), and similar to what was observed in the two initial samplings (0 and 3 wpd). Histopathologic lesions were similar to what is described as typical CMS in larger, market size fish, both in presentation and in distribution, with the most severe lesions in the atrium and spongiosum of the cardiac ventricle (D. W. Bruno et al., 2013; Ferguson et al., 2006).

A PMCV infection was confirmed by a real-time RT-PCR, which revealed large amounts of virus-specific RNA, with Ct-values as low as Ct 7 in cardiac tissue. Levels as low as this are uncommon; however, Ct-values of about 12–15 have been occasionally observed in cases of severe CMS in routine diagnostics at the NVI (personal observations C. Fritsvold).

To verify whether this high viral level was due to a more virulent virus, we performed sequencing of ORF1 and ORF3 of the PMCV strain in one fish sampled in the middle of the outbreak period (17 wpd) for comparison with the reference PMCV isolate AL V708 and other field strains sequenced similarly previously (Haugland et al., 2011; Wiik-Nielsen et al., 2012). Compared with the reference, a few mutations were only found in ORF1 resulting in five amino acid changes. Two of these are shown to be present in almost all (A40V) or all (T773A) later sequences available (Wiik-Nielsen et al., 2012).

**TABLE 4** Grading of cardiac CMS lesion

| Score | Description |
|---|---|
| 0 | No pathological findings or slightly increased number of leucocytes |
| 1 | One or a few focal lesions, increased number of leucocytes |
| 2 | Several distinct lesions and small-to-moderate increase in number of leucocytes |
| 3 | Multifocal-to-confluent lesions and moderate-to-severe increase in number of leucocytes |
| 4 | Severe confluent lesions comprising >75% of the tissue and massive leucocyte infiltration |

Of the three remaining amino acid substitutions, A486P has been seen previously in individuals from six Norwegian field outbreaks and two Irish outbreaks (Tighe et al., 2019; Wiik-Nielsen et al., 2012). The outbreaks including viral strains with this mutation are not described in detail, and it is not known whether these are comparable to the present severe outbreak, and it cannot be concluded if this specific mutation can be linked to a viral strain with higher virulence. The N354D and A774V mutations have not been seen previously. In general, linking specific mutations in the PMCV genome to virulence and severity of CMS outbreaks has been infeasible due to, in general, high homology between PMCV strains sequenced, and also the presence of several strains among individual fish in an outbreak (Wiik-Nielsen et al., 2012)(personal observations A.B. Mikalsen). To study the diversity of PMCV at a specific sampling time point and any possible change in viral genomic sequence over time, we included sequencing of the 5' part of ORF3, also including upstream UTR. Similar to what was found in full ORF3 at 17 wpd, the UTR and part of ORF3 sequenced were equal to the reference isolate AL V708 for all samples at 0 and 3 wpd. At 39 wpd, two of three samples showed one synonymous nucleotide mutations in the small 3' part of ORF2 included and in two ORF3s. Due to the low amount of samples included for sequencing, it cannot be concluded if this is an evolutionary selection favouring strains including these mutations. Also, any role of synonymous nucleotide substitutions in relation to virulence needs to be studied further. What caused the high levels of virus-specific RNA in our case is not known, but some kind of reduced immune competence, that is due to environmental or other stress, may affect viral replication.

An increasing degree of severity of histopathological CMS lesions correlated consistently with an increasing load of PMCV-specific RNA detected by real-time RT-PCR in atrium and spongious ventricle. This is in accordance with a previous study where laser-captured tissue from CMS lesions in ventricular spongious layer was compared with normal, lesion-free samples of the same heart and tissue, showing a high correlation of virus presence in lesions and no detection in closely located healthy tissue (Wiik-Nielsen et al., 2012). As previously characterized for CMS (Ferguson et al., 2006), only sparse or sparse-to-moderate, focal and/or perivascular cardiac lesions were seen in the compact layer of some ventricles, in severe

**TABLE 5** Summary of histopathological evaluation, levels of PMCV-specific RNA and the presence of PMCV ORF3-encoded protein antigen in atrium, ventricular compactum and ventricular spongiosum of hearts of morts sampled as presumed CMS diseased in the first comprehensive sampling (3 wpd), early in the CMS outbreak

| Cardiac compartment | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Atrium | | 11,3(+) | 7,9++ | 8,9+ | 20,3 (-) | 10,4+(+) | 8,7++ | 26,4 +++ | 10,1+(+) | 24,4 ++(+) | 7,00 ++(+) |
| Ventricle | compactum | 9,9 + | 9,2 (+) | 8,2 (+) | 15,6 (+) | 12,2 + | 9,9 + | 38,5 ++(+) | 10,9 + | 23,5 ++ | 8,2 + |
| | spongiosum | 9,1+ | 8,2(+) | 8,1(+) | 21,7 + | 10,0(+) | 9,0++ | 25,6 +++ | 9,3++ | 21,8 +++ | 8,2 + |
| Epicardium | | | | | | | | | | | |
| Histopathology diagnosis | | CMS | CMS | CMS | Neither CMS nor HSMI | CMS | CMS | Neither CMS nor HSMI | CMS | Neither CMS nor HSMI | CMS, possibly early HSMI |

*Note:* Selection was based on clinical findings in accordance with CMS only. For each cardiac compartment, ***grading of cardiac lesions is colour-coded*** in accordance with table 4, ***viral levels are given as Ct-values in numbers*** from real-time RT-PCR, and the ***presence of ORF3-encoded protein detected by immunohistochemistry is given as (-) = no signal, + = sparse, ++ = moderate and +++ = strong positive signals.*** Parenthesis indicates a slightly weaker signal than without.

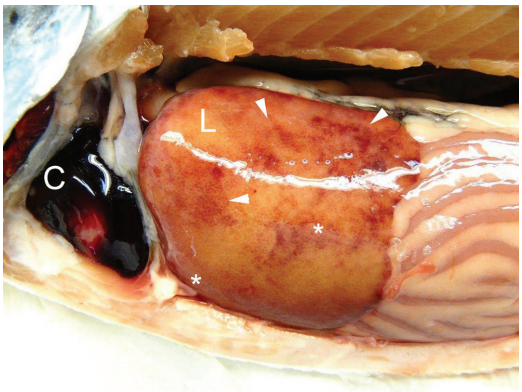



**FIGURE 4** Typical autopsy findings, representative of circulatory disturbances at comprehensive sampling 3 wpd: cardiac tamponade (C), yellowish liver (L) with multifocal sparse-to-moderate haemorrhaging (arrowheads) and a fibrinous layer (*) on the liver surface

or very severe cases of CMS only, but still, the viral levels in compactum were comparable to the load in the ventricular spongiosum and atrium, tissues with more severe and widespread lesions. Why the main lesions characteristic for CMS are seen in atrium and spongious layers, while still comparable viral levels in compact layers, is not known. The compact layer is supplied by freshly oxygenated blood by coronary arteries branching into the compactum, returning venous blood into the bloodstream in the atrioventricular region (Farrell et al., 1990), and in some stages of CMS, viraemia may be detected, rather than targeted viral infection in samples of this cardiac department (personal observation, C. Fritsvold). High viral levels were also detected in the other organs tested (gill, spleen and kidney), which indicates a systemic infection at this stage, but without lesions other than in the cardiac target tissue.

Interestingly, the distribution of the cardiac lesions seen for CMS in general seems to reflect the pattern of cardiac tissue blood supply and blood oxygenation level, as the spongious cardiac tissues of both atrium and ventricle, receiving venous blood of lower oxygen levels and dependent on oxygen diffusion across endocardium, are identical to the tissues where the typical and most severe cardiac CMS lesions are found. If not a coincidence, there may be various explanations to this, and further investigations are needed. The initial subendocardial cellular inflammation and occasional endocardial hypertrophia of CMS may increase the distance for oxygen diffusion to a dysfunctional level, causing anoxia or low oxygen supply to the myocardium beneath, possibly reducing myocardiac, hence cardiac, function. This may also hamper the immune response to the PMCV infection of these spongious tissues, or even accelerate the damage the PMCV induces.

Some of the individuals of the study cage diagnosed with CMS also presented pathological changes neither typical, nor completely unusual, for severe CMS. The findings of few and minor foci of myocarditis and perivascular inflammation of the ventricular compactum, and focally severe epicarditis, were considered as CMS-related due to location, severity of the overall cardiac lesions and the high PMCV RNA levels in those fish. It cannot be excluded that some of these lesions could represent, for example, a double infection with PRV-1, and at 39 wpd even triple infections incl. SAV, resulting in very sparse, and possible early- or late-phase HSMI and/or PD lesions. These individuals did not show clinical HSMI or PD disease, but had low levels of PRV-1. Also, other individuals among those sampled showed high levels of PRV-1 and/or indications of early histological characteristics of HSMI, comparable to ongoing active infection, which could support an early or late phase of HSMI in the fish population. The adhesive and infiltrative granulomatous peritonitis observed in all fish was most likely an adverse effect related to i.p. vaccination.

Surprisingly, the strongest positive signals in slides examined by immunohistochemistry (IHC) were generally seen in individuals other than the fish with the highest viral levels and/or most severe cardiac lesions, in fish with no histopathological cardiac diagnosis, that is fish 4, 7 and 9, sampled 3 wpd (Figure 6a and table 3a). Fish 4 and 9 have PRV Ct-values, which could indicate a very early stage of HSMI, while fish 7 differs by having relatively high Ct-values for both PMCV and PRV, and in accordance with this, only sparse cardiac pathology. This unexpected pattern of positive IHC staining may reflect that the antibody used in this IHC targets the ORF3 product, a protein not necessarily present at the same time as the peak in severity of the cardiac lesions or the peak in the presence of viral RNA. The function, characteristics and kinetics of expression of the ORF3 product are still unknown, and its expression may therefore vary throughout different phases of the virus infection.

HSMI or PD was not confirmed as diagnoses in sampled fish at any time point, although indications of possible early or late HSMI or PD were described from a few individuals and fish positive for both PRV (since 3 wpd) and SAV (since 37 wpd, close to slaughtering) were detected in the study cage. At two time points, disease was suspected in other cages at the site, based on autopsy findings and/or increased mortality. At one of these time points (19 wpd), in cage 3, next to the study cage, an increase in mortality was found to be observed, and clinical observations, autopsy findings and histopathological lesions strongly indicated an HSMI outbreak, but HSMI was not diagnosed, as the lesions were too unspecific. As expected, all 5 sampled fish were positive for PRV-1, but, interestingly, negative for PMCV. An ongoing immune response against a primary viral infection may suppress a secondary viral infection, that is by inducing non-specific immune responses (Lund et al., 2016; Wiik-Nielsen et al., 2016), and may partly explain the lack of PMCV infection in this cage. Another explanation could be that infectious PMCV particles were not shed from the PMCV-positive fish of the neighbouring study cage for the necessary time point or period, or the fish in cage 3 had a somehow different genetic composition making them more resistant to a PMCV infection. None of the SAV-positive fish at 37 wpd had histopathological changes in accordance with a PD diagnosis. This could be a result of the PD vaccine protecting them against PD disease, but not SAV infection.

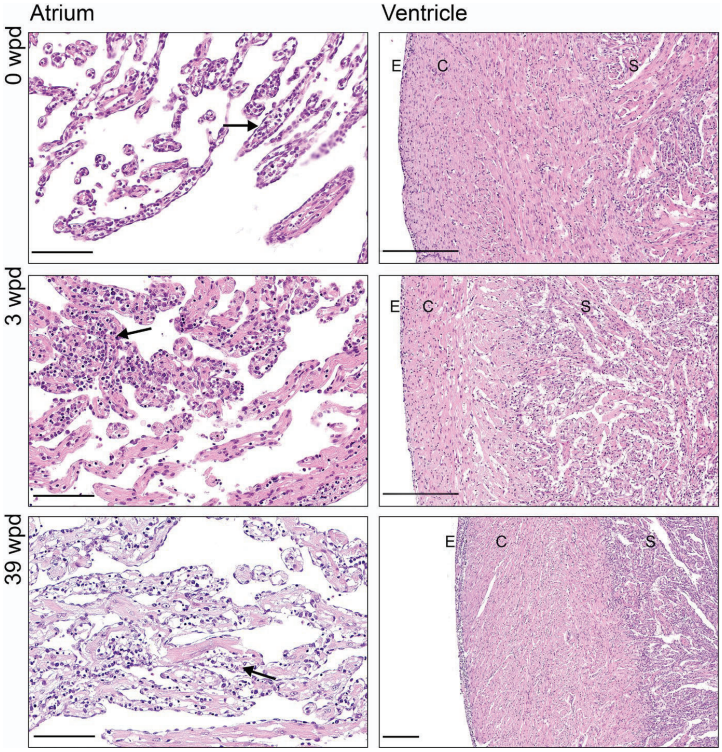
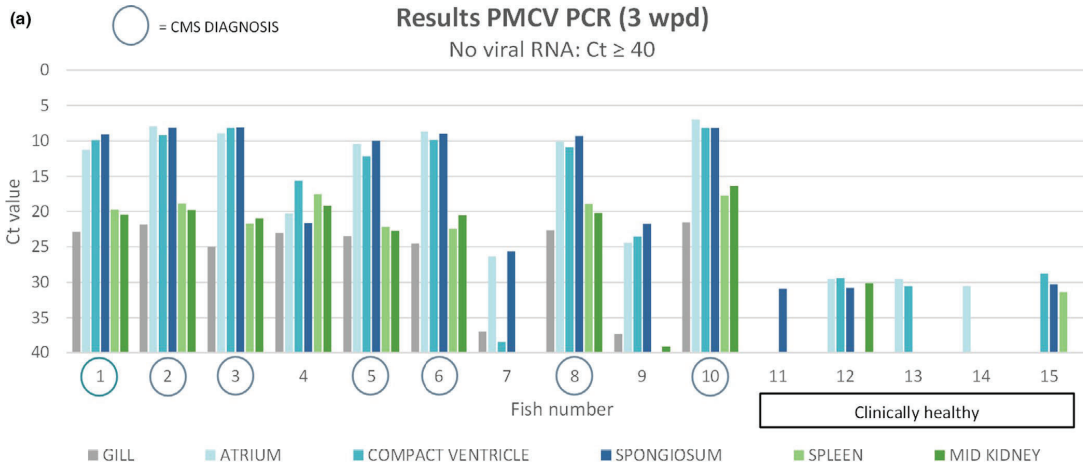



**FIGURE 5** Typical CMS histopathology in cardiac atrium and ventricle at 0, 3 and 39 wpd, respectively. Multifocally large amounts of various inflammatory cells (large arrows) have replaced normal, eosinophilic myocardium. Epicardium (E), ventricular compactum (C) and ventricular spongiosum (S) (H&E stain), atrium scale bars =100μm (200x magnification), ventricle scale bars =250 μm (100x magnification for 0 and 3 wpd, 50x magnification for 39 wpd)

Our study cage was the only cage at the site with a CMS diagnosis until fish of cage 6 was diagnosed at 49 wpd, 10 weeks after slaughtering of the study cage. The two cages with CMS diagnoses were not located next to each other (see Figure 3 a), but the main direction of the sea current is favourable for a possible transmission between them by sea water. In addition, both fish groups originated from the same hatchery and were transferred to sea at the same time in early autumn, most likely arriving at the site by the same well boat. Epidemiological studies have indicated a correlation between time of sea transfer and risk of developing CMS, as studied fish groups stocked in the fall had a significantly higher daily risk rate than both early and late spring stocked fish: fish put to sea in the late fall was found to develop CMS twice as often as early spring stocked fish (Bang Jensen et al., 2020). However, this does not explain why CMS was not detected in any other cage until 10 weeks after slaughter of the study case, almost a year after CMS was first detected at the site.

This observation indicates that despite the high amount of PMCV-specific RNA detected in the hearts and, to some degree, other internal organs of the diseased fish, the horizontal spread of virus beyond the study cage was limited. Whether this was caused by shedding of insignificant amounts of infective PMCV particles to the water, a need for direct fish-to-fish contact for transmission of virus, differences in permissiveness of the fish to CMS or related to location of the cages relative to each other and sea currents, time of sea transfer or other differences between the fish groups is still unknown. In addition, the detected PMCV-specific RNA may not represent infective virus particles or not be proportional to the number of infective PMCV particles as discussed for other viruses (Donald & Isaacs, 1954; Sanjuán, 2018); infective PMCV particles may not travel well in water, may become inactivated and loose infectivity quickly in sea water; or other factors necessary to induce CMS in the fish of the other cages may not have been present. A possible vertical pathway of transmission has been discussed, but does not seem to play a major role in the transmission of PMCV (Bang Jensen et al., 2019; Mikalsen et al., 2020). In addition, the site has a history of CMS disease, and transfer of virus from unknown environmental sources cannot be excluded.

The highest levels of PMCV RNA were detected in the three sampled cardiac compartments, in substantially higher amounts than in any other investigated organ. These results are consistent with previous studies including several organs (Timmerhaus et al., 2011) and support the heart both as the main target for PMCV and as the organ of choice for detection of PMCV RNA. Still, tissue sampling of hearts is often focused on the cardiac apex, which in larger fish may consist of cardiac compactum only, with no spongious myocardial tissue. Our results indicate that sampling from any of the examined cardiac compartments (atrium, ventricular spongiosum and ventricular compactum), for example apex of the heart, will result in similar sensitivity of virus detection. This is in contrast to what may be



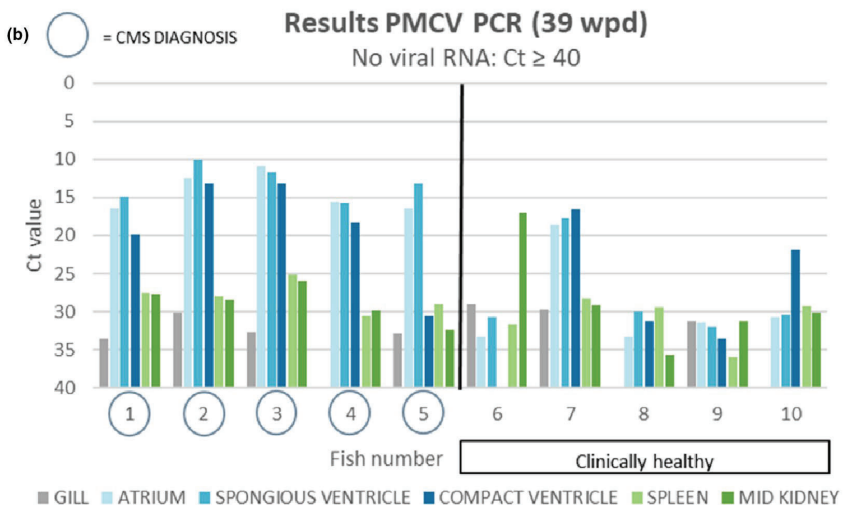




**FIGURE 6** Comparison of viral loads of PMCV between different organs and cardiac compartments in the two comprehensive sampling 3 wpd (a) and 39 wpd (b). Fish with encircled numbers were diagnosed with CMS by histopathology. The other individuals did not have histopathological lesions in accordance with, or severe and/or typical enough lesions to qualify for neither a CMS nor a HSMI diagnosis. (a) Comprehensive sampling 3 wpd. Based on clinical signs, fish 1–10 were selected for sampling as possible CMS diseased. (b) Comprehensive sampling 39 wpd. Based on clinical signs, fish 1–5 were selected for sampling as possible CMS diseased

expected based on the typical location of the cardiac lesions of CMS, as seen in the present outbreak, mostly restricted to the spongious tissues of atrium and ventricle. As the amounts of PMCV-specific RNA in this study in general were at a high level, distribution of the virus in various heart compartments may differ for cases with more sparse amounts of viral RNA, for example in early phases of the infection. This is exemplified by the results of PMCV RNA detection in the healthy fish sampled at 3 and 39 wpd, which showed high Ct-values and more variation in levels of virus between the examined cardiac compartments. In contrast, gill samples generally contained low amounts of PMCV RNA and cannot be recommended for PMCV screening or diagnostic purposes at similar stages of CMS disease. As gills are exposed to the sea water, the presence of virus in gill samples may be caused by virus shed by other individuals, and not necessarily represent active viral infection of the gill tissue sampled. Previous studies have shown comparable amounts of PMCV in gills, relative to other organs, at early time points of infection in experimental challenge studies, indicating that gills may be more involved

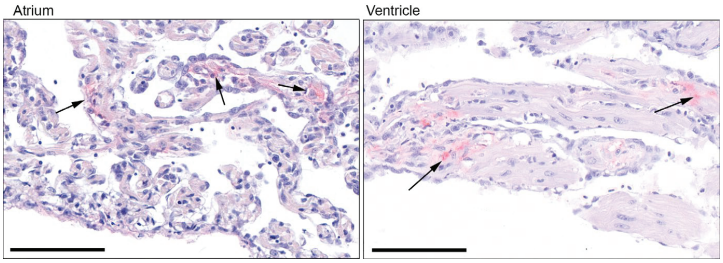



**FIGURE 7** Imm But in the unohistochemistry for PMCV in atrium 3 wpd (left) and ventricular spongiosum 39 wpd (right): positive staining visible as light pink colour (arrows) between inflammatory cells (200 x magnification)

**TABLE 6** Summary of sequencing results. a) Initial sequencing of fish 3 of the 17 wpd sampling for study of mutations in full-length ORF1 and ORF3 nucleotide sequence and encoded proteins. b) Sequencing of 3' end ORF2, subsequent UTR and 5' ORF3

| Time | Fish no. | $ORF1^{full}$ | | $ORF3^{full}$ | |
|---|---|---|---|---|---|
| | | Non-synonymous mutations | Synonymous mutations | Non-synonymous mutations | Synonymous mutations |
| 17 wpd | 3 | A40V, N354D, A486P, T773A, A774V | nts 21, 840, 1,365, 2,289, 2,481 | - | - |

| Time | Fish no. | $ORF2^{nt\ 1882–2181}$ | $UTR^{full}$ | $ORF3^{nt1–547/aa1–182}$ | |
|---|---|---|---|---|---|
| | | Synonymous mutations | Mutations | Non-synonymous mutations | Synonymous mutations |
| 0 wpd | 1 | - | - | - | - |
| | 2 | - | - | - | - |
| | 3 | - | - | - | - |
| | 4 | - | - | - | - |
| 3 wpd | 1 | - | - | - | - |
| | 2 | - | - | - | - |
| | 3 | - | - | - | - |
| 39wpd | 1 | - | - | - | - |
| | 2 | nt 2,100 | - | - | nts 189, 255 |
| | 3 | nt 2,100 | - | - | nts 189, 255 |

*Note:* Synonymous mutations and mutations in UTR are given as position in ORF or UTR. Non-synonymous mutations are given as aa in reference position in aa seq-mutated aa; for example, alanine (A) in reference protein changed to V in position 40 is given as A40V.

at early infection phase, for example as the point of entry after shedding from cohabitants to the water (Hansen et al., 2011). The detection of moderate to high levels of virus in mid-kidney and spleen is similar to what is seen previously in experimental challenges (Timmerhaus et al., 2012) and is probably a natural consequence of a viraemia, as high amount of blood passes through and are continuously filtered in these organs, crucial for immunity, homeostasis, secretion and haematopoiesis.

This paper describes one of the first outbreaks of CMS in young 1.1 kg Atlantic salmon, 5–6 months post-seawater transfer, and is an important reminder that PMCV has the potential to induce CMS with typical clinical and histopathological findings, including increased mortality, in young fish with fairly short time after seawater transfer.

Fish of the affected study cage were diagnosed with CMS about 28 weeks post-seawater transfer, and the disease and related mortality continued until slaughtering 39 weeks later. Clinical and histopathological findings were in accordance with previous descriptions of CMS in larger individuals close to slaughter. Cardiac spongious tissues in both atrium and ventricle had the most severe pathological lesions, and cardiac tissue was by far the most suitable for PMCV-specific RNA detection in this case, with high amounts in atrium, ventricular spongiosum and ventricular compactum. Whether other organs, blood samples, mucus swabs or water samples also could be suited, or even better suited than cardiac samples, for viral detection in other phases of the disease has to be investigated in more comprehensive challenge studies with a reasonable long time span. Another interesting observation was that only one of the other five cages at the site was diagnosed with CMS, as late as almost a year after the first CMS diagnosis at the site.

The detection of typical CMS in the young fish was unusual and unexpected, and at the very beginning of a trend of earlier onset of clinical manifestation of CMS than previously observed (Fritsvold & Bang Jensen, 2019; Svendsen et al., 2019; Wiik-Nielsen, Alarcón, et al., 2016). The majority of CMS cases reported in Norway is still

in larger fish, but the number of cases resembling our study case has been increasing since 2014, and post-smolts as young as 0.3 kg has been reported with typical CMS (Fritsvold & Bang Jensen, 2019), but why these early-onset CMS cases seem to become more frequent or what is initiating them is still unknown.

A shift in the characteristics of CMS from being a late production period problem, to a disease of more prolonged manifestations, appearing at almost any time in the production period, is likely to have large negative impact on the Atlantic salmon farming industry: production-related planning and daily management of a site with an early CMS diagnosis will be affected by limitations in choices of methods, intervals and time points for handling, sorting, lice treatment and slaughtering of the diseased fish group, as stressful experiences are likely to cause CMS outbreaks with increased mortality, which can lead to both increased economic losses and decreased animal welfare.

There is still a knowledge gap on how and when PMCV is shed, how it is transmitted between individuals and groups, what factors are necessary for induction of CMS in PMCV-infected individuals, which mechanisms cause the severe CMS pathology and why diseased salmon apparently do not manage to limit or eliminate the infection, and recover from CMS.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Raw data are stored and backed up according to the Norwegian Veterinary Institute's System for data storage, incl. on institutional servers with regular backup. Access to the data can be obtained by contacting the corresponding author.

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# Paper III

**Manuscript ready for submission:**

# Characterisation of early phases of cardiomyopathy syndrome (CMS) pathogenesis in Atlantic salmon (*Salmo salar* L.) through various diagnostic methods

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## Abstract

Since the first description of cardiomyopathy syndrome (CMS) in Atlantic salmon, in 1985, the disease caused by piscine myocarditisvirus (PMCV) has become a common problem in Atlantic salmon farming, not only in Norway, but also in other salmon farming countries like Scotland and Ireland. In the last years, CMS has been ranged as the most important salmon viral disease in Norway regarding both mortality and economic losses. Detailed knowledge of infection and pathogenesis is still lacking, a decade after the causal agent was first described, and there is a need for a wider range of methods/tools for diagnostic and research purposes.

In this study, we compared detection of PMCV and CMS related tissue lesions using previously used and well-known methods like histopathology and real-time RT PCR to immunohistochemistry (IHC), a less used method, and a new method, RNAscope *in situ* hybridisation. Tissue samples of three different cardiac compartments, mid-kidney and skin/muscle tissue were compared to non-lethal parallel samplings of blood and mucus. The development of pathological cardiac lesions observed in this experiment was in accordance with previous descriptions of CMS. Our results indicate a viremic phase 10 to 20 days post-challenge (dpc) preceding the cardiac lesions. In this early phase, virus could also be detected in relatively high amount in mid-kidney by real-time RT-PCR. Plasma and/or mid-kidney samples may therefore be candidates to screen for early phase PMCV infection.

The RNAscope *in situ* hybridisation method showed higher sensitivity and robustness compared to the immunohistochemistry, and may be a valuable support to histopathology in CMS diagnostics, especially in cases of untypical lesions or mixed infections.

## Introduction

Cardiomyopathy syndrome, CMS, is a viral disease of farmed Atlantic salmon, usually affecting larger fish in their second year in sea water. The causative agent piscine myocarditis virus (PMCV) induces severe cardiac inflammation of the spongious layers of the salmonid atrium and ventricle. The disease has been known in Norwegian fish farming since the mid-1980s, and has since then been described in other countries farming Atlantic salmon: Scotland, Faroe Island and Ireland (T. Poppe & Sande, 1994; T. T. Poppe & Seierstad, 2003; H. Rodger & Turnbull, 2000; H. D. Rodger, McCleary, & Ruane, 2014), in a few cases of wild Norwegian Atlantic salmon (T. T. Poppe & Seierstad, 2003), and a disease resembling CMS has also been described in Canada (Brocklebank & Raverty, 2002). In some cases, the disease period occurs as long-term, with moderately increase in mortality, but sudden outbreaks with high mortality, often induced by stressful events (Ferguson, Bjerkas, & Evensen, 2006). Moribund fish are seldom observed. As CMS can cause considerable mortality in large salmon in good condition close to slaughter, the economic impact is significant, and the disease has for several years been ranked as the most important contagious disease causing mortality of Norwegian Atlantic salmon in ongrowing and broodstock sites (Fritsvold & Jensen, 2021; Garseth, Fritsvold, Svendsen, Bang Jensen, & Mikalsen, 2018).

Several hypotheses about the etiology of CMS were initially discussed, but in 2009, the first experimental challenge experiments succeeded in proving transmissibility of CMS (D.W. Bruno & Noguera, 2009; Fritsvold et al., 2009), and in 2010, PMCV was described as the causative agent of CMS (Haugland et al., 2011; Lovoll et al., 2010). Fish with CMS are normally in good condition and may present with macroscopic changes related to circulatory disturbances, i.e. exophthalmus, abdominal petechial haemorrhaging and raised scale skin pockets due to oedema. An autopsy can reveal ascites, dark or discoloured liver, often with fibrinous cast on the surface and an enlarged, distended atrium and/or sinus venosus, and in severe cases, a ruptured atrium/sinus venosus and pericardial cavity filled with blood clots (cardiac tamponade). Microscopically, the inflammation is usually restricted to the atrium and spongiosum of the cardiac ventricle, sometimes with epicarditis and/or perivascular or focal myositis in the ventricular compactum (D. W. Bruno, Noguera, & Poppe, 2013; Ferguson et al., 2006). PMCV specific RNA is usually found in the highest amounts in cardiac tissues, and in lower levels in kidney, spleen and blood (Fritsvold, Mikalsen, Poppe, Taksdal, & Sindre, 2021; Timmerhaus et al., 2011).

At present, a CMS diagnosis is usually based on clinical observations, autopsy findings and histopathological examination of cardiac tissue (Fritsvold & Jensen, 2020, 2021). A standard set of samples received for fish diagnostics at Norwegian Veterinary Institute, including CMS, usually contain a selection of formalin fixed tissues (gill, heart, liver, exocrine pancreas, mid kidney and skin with attached red and white skeletal muscle), in addition to samples of head or mid kidney and heart, usually the apex. In some cases, the diagnosis is supported by a real-time RT-PCR for PMCV specific RNA or occasionally, by an immunohistochemistry (IHC) for PMCV. A histopathological evaluation of other tissues, especially exocrine pancreas, kidney and skeletal muscle, is usually performed, to distinguish CMS from its most important differential diagnosis pancreas disease (PD), heart and skeletal muscle inflammation (HSMI), and to some degree, infectious salmon anemia (ISA) (Fritsvold & Jensen, 2020).

Initially, CMS was predominantly found in the North-Western part of Norway, but has since spread and can now be found in all 13

production areas along the Norwegian coast. A similar pattern can be observed for HSMI, while PD is predominantly found in production areas in Southwestern- to Mid-Norway. In 2020, there were more than 150 confirmed cases of each of these three viral diseases, all causing cardiac lesions (Fritsvold & Jensen, 2021).

In Norwegian salmonid aquaculture, populations at a fish farm often present with several coexisting infections and diseases, both at site and individual levels. Especially early stages of the cardiac diseases can be difficult to differentiate histopathologically, but the late, post-infection cardiac changes of both PD and HSMI can also be challenging when combined with sparse to moderate CMS lesions. This calls for more than histopathological experience and accuracy to distinguish between different stages of potentially several diseases in one individual, and a wider selection of supporting diagnostic tools is required.

An IHC procedure is available for *in situ* detection of PMCV antigens in relation to the CMS cardiac lesions (Fritsvold et al., 2021), although experiences with the available procedure has shown the method to be less robust and of low sensitivity to be included in routine diagnostics. A traditional *in situ* hybridisation method for detection of PMCV specific RNA has been described (Haugland et al., 2011), but has not been included in routine diagnostics due to a time consuming procedure.

Although initial characterization of PMCV described viral replication in a fish cell culture (Haugland et al., 2011), no cell cultures resulting in efficient replication of PMCV to high titres are described or available. Also, commercially available ELISA-methods or antibody tests for CMS diagnostics have not been described.

An increasing focus on fish welfare and stronger demands for implementation of the 3Rs (replacement, reduction, refinement) (Tannenbaum & Bennett, 2015; Toni et al., 2019) both in farms and in research, makes research on non-lethal sampling methods like blood sampling, mucosal swabs and water samples relevant. None of these methods have at present been tested or are available for CMS diagnostics.

Therefore, the main focus of this study was to characterize the initial phases of CMS pathogenesis of Atlantic salmon in a CMS challenge trial, through comparison of histopathological changes and standard real-time RT-PCR results of three different cardiac tissues, in addition to mid-kidney and skin/muscle tissues, with additional results from non-lethal parallel samplings of blood and mucus. The second goal was to establish a more robust and simple to use *in situ* method for PMCV for use in CMS diagnostic work, and compare this with the immunohistochemistry method for PMCV established at NVI.

## Material and methods

**Experimental fish**

Non-vaccinated Atlantic salmon pre-smolts reared from eyed eggs at the Industrial and Aquatic Laboratory (ILAB) were used as experimental fish in this challenge experiment. The original batch of eggs originated from StofnFiskur, Egghous Vogavik, Iceland, and their parents had been screened and found negative for salmonid alphavirus (SAV), piscine orthoreovirus (PRV), PMCV, infectious pancreatic necrosis virus (IPNV) and infectious salmon anaemia virus (ISAV). The eggs hatched early in May 2017, and the fish were start fed two months later. To verify that the experimental fish were without histopathological cardiac changes and free of the most common pathogens of Norwegian aquaculture, 60 hearts were examined by light microscopy and gills (n= 120), hearts (n =60) and kidneys (n = 60) of the fish group tested by PCR for SAV, PRV, PMCV, IPNV, ISAV and SGPV at 5 g size

(Pharmaq Analytiq AS). The fish group was subject to monthly inspections by a fish health biologist, and no disease related problems were registered before initiating the challenge. Fish included in this study originated from non-vaccinated negative controls in an experimental testing of vaccine candidates against CMS (handled by Pharmaq AS). Marking of fish was performed at the start of the challenge in August 2018 by fin-clipping or shortening of the maxillae. The non-vaccinated control group described in this study had their left maxillae shortened. Before challenging the test groups, 6 fish were sampled at day 0 and found negative for CMS and PMCV by histopathology and PCR (both at Pharmaq Analytic AS and NVI).

**Facilities and husbandry**

The experiment was performed at the Industrial and Aquatic Laboratory (ILAB, Stiftelsen Industrilaboratoriet), in Bergen in early autumn 2018. The 45 fish were kept at 12°C in 500 L tanks of fresh water with in total of 225 additional experimental fish, and fish and tanks were tended and monitored on a daily basis by facility personnel. The fish were fed according to appetite throughout the study, and were taken off feed for a minimum of 24 hours prior to i.p. injection of mock vaccine (see Challenge chapter) and before challenging. Moribund fish were to be euthanized and logged as mortalities, and remarks were to be made if challenged fish displayed typical or atypical signs of disease. Abnormal or unexpected behaviour, loss of appetite, unexpected mortality or signs of disease were to be reported immediately.

**Challenge**

The fish were intraperitoneally (i.p.) injected with a mock vaccine (PBS) seven weeks before challenging. At challenge day, 0 days post challenge (dpc), the fish were i.p. injected 0.1 ml tissue homogenate made from spleen from PMCV positive fish of a field outbreak of CMS. The homogenate was found negative by PCR for SAV, PRV and IPNV (Pharmaq).

**Sampling and preservation**

*Autopsy*

An overview of sampled material at the day of challenge (0 dpc) and at samplings 10, 20 and 52 dpc, is found in Table 1. At sampling, fish were sedated, immobilised and anaesthetised with Tricaine Pharmaq® (tricaine mesilate, "MS222") baths. Sedated fish were inspected for any signs of abnormality before mucus swabs were taken, the fish measured, weighed and blood drawn from the caudal vein *(Vena caudalis*), before they were sacrificed by decapitation and other tissues were sampled at autopsy.

**Table 1:** Overview of use of sampled fish and material per sampling day post challenge (dpc) for histopathology, real-time RT-PCR, Immunohistochemistry (IHC) and *in situ* hybridisation (ISH).

| Method | Material | Organs/material/ location sampled | | Individual fish numbers at each sampling | | | |
|---|---|---|---|---|---|---|---|
| | | | | Day 0 | 10 dpc | 20 dpc | 52 dpc |
| Histo-patho-logy | Tissue | Heart | | 1-6 | 1-15 | 1-15 | 1-5, 7-11, 13-15* |
| | | Mid-kidney | | 1-3, 5-6** | 1-15 | 1-15 | 1-11, 13-15* |
| | | Skin/muscle | | 1-6 | 1-15 | 1-15 | 1-11, 13-15* |
| PCR | Tissue | Cardiac | Atrium | 1 | 1-15 | 1-15 | 1-15 |
| | | | Spongiosum | 1-4 | 1-15 | 1-15 | 1-4*** |
| | | | Compactum | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Mid-kidney | | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Skin/muscle | | 1-4 | 1-15 | 1-15 | 1-15 |
| | Blood | Whole blood | | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Plasma | | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Blood cell pellet | | 1-4 | 1-15 | 1-15 | 1-15 |
| | Mucus | Mucus from pectoral fin | | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Mucus from lateral line | | 1-4 | 1-15 | 1-15 | 1-15 |
| | | Mucus from anus | | 1-4 | 1-15 | 1-15 | 1-15 |
| IHC | Tissue | Heart | | 1,4 | 3,7 | 2,5,12 | 1,4 |
| ISH | Tissue | Heart | | 1,4 | 3,7 | 2,5,12 | 1,4 |
| | | Mid-kidney | | 1 | 3 | 2 | 1 |

*Fish 6 lacks heart for histology, fish 12 lacks all three organs for histology
**Fish 4 lacks mid-kidney
***Spongiosum was sampled of the 4 first fish only

*Blood*
Blood samples were collected in standard heparinised tubes ("Vacuette tube" LH Lithium Heparin, Greiner Bio-One), placed on a gentle mixer during sampling and stored on ice, before 250 µl of heparinised blood was mixed with 750 µl NucliSENS® (bioMéreux SA) lysis buffer. The remaining blood was pelleted for 10 minutes at 2500 (0 and 52 dpc) or 3500 (10 and 20dpc) rounds per minute in a centrifuge. Both 250 µl of the plasma layer and 250 µl of the sedimented blood cells left at the bottom of the tubes were added to separate tubes with 750 µl NucliSENS® each, and left at room temperature for at least 10 minutes before stored at -80°C until analysed.

*Mucus*
Mucus was collected by rolling three separate sterile cotton swabs around the left pectoral fin, along the lateral line of the left side of the fish and over the anus, respectively. The cotton tip of the swab was then cut off and left in suitable tubes with RLT® lysis buffer (Qiagen), and left at room temperature for at least 10 minutes before stored at -80°C until analysed.

*Tissues*
For histological examination and *in situ* methods, heart, mid-kidney and skin with underlying red and white muscle of all fish were sampled and fixed in 10 % neutral buffered formalin. Subsequently, the samples were prepared by standard procedures (Bancroft & Stevens, 1990), including embedding in paraffin wax and sectioned by cutting approximately 3 µm thick sections placed on slides.

For real-time RT-PCR analyses, the three cardiac compartments atrium, ventricular compactum and ventricular spongiosum were sampled separately, in addition to

mid-kidney and skin with underlying red and white muscle, using RNA*later*® (Ambion) for preservation. At the 52 dpc sampling, cardiac spongiosum were sampled of 4 fish only, hence cardiac samples of the remaining 11 fish (fish 5 – 15) consist of atrium and ventricular compactum only at this time point.

**Histopathology**

The formalin-fixed and paraffine-embedded tissue sections were dewaxed and stained with haematoxylin and eosin (HE) after standard procedures (Bancroft & Stevens, 1990).

All slides of all organs were examined by light microscopy, unblinded. All hearts were scored from 0 to 4 according to (Bancroft & Stevens, 1990; Fritsvold et al., 2009) (see Table 2), where 0 refers to no pathological lesions and 4 represents severe, typical CMS lesions. Cardiac atrium, ventricular spongiosum and ventricular compactum were evaluated and scored individually for severity and distribution of inflammation, and scale steps of 0.5 were used to increase the resolution of the scoring table.

**Table 2:** Histopathological grading of cardiac CMS lesions, according to Fritsvold and coworkers (Fritsvold et al., 2009).

| Score | Description |
|---|---|
| 0 | No pathological findings, or slightly increased number of leukocytes |
| 1 | One or a few focal lesions, increased number of leukocytes |
| 2 | Several distinct lesions and small to moderate increase in number of leukocytes |
| 3 | Multifocal to confluent lesions and moderate to severe increase in number of leukocytes |
| 4 | Severe confluent lesions comprising >75 % of the tissue and massive leukocyte infiltration |

**Extraction of RNA and real-time RT-PCR**

Purified total RNA was extracted from the sampled material using a MagNA Pure 96 (Roche) high-throughput robotic workstation following the recommendations from the manufacturer. For the heart and kidney samples, a piece of tissue (≤ 20 mg) was homogenized in 600 µl MagNA Pure lysis buffer. The blood samples and mucus swabs, collected on lysis buffer, were used directly. For extraction, 500 µl of each sample in lysis buffer was used as input with resulting 50 µl eluate. The RNA concentration was then measured on a NanoDrop 8000 (ThermoFisher Scientific) for the heart and kidney samples and 500 ng RNA was used as input for real-time RT-PCR. A fixed amount of 7.5 µl of extracted RNA eluate was used as input for real-time RT-PCR of the mucus swabs and blood samples, to compare the amount of viral RNA per volume of these samples. Real-time RT-PCR was performed with primers and probe targeting a conserved area of the RNA-dependent RNA polymerase gene (ORF2) of PMCV, as described in (Lovoll et al., 2010). All samples of each sampled material were analysed in a single run.

**Immunohistochemistry**

A PMCV specific immunohistochemistry (IHC) protocol employing a polyclonal antibody towards ORF3 was performed on a selection of 9 heart sections from the samplings 10, 20 and 52 dpc, as described previously with some modification (Fritsvold et al., 2021) with some modification. In summary, dewaxed and dehydrated 3 µm slides of cardiac tissue were incubated 20 minutes at room temperature with 5 % BSA in a Tris buffer, followed by a 120 minutes incubation with a 1:2000 primary rabbit polyclonal antibody dilution, based on recombinant proteins from ORF3 (ΔORF3, ZN01101, Rabbit 062, PMCV), kindly donated by M. Rode, Pharmaq AS. Then the slides were incubated for 30 minutes with a 1:500 secondary antibody dilution (biotinylated

goat anti-rabbit Ig. DAKO E 432, DAKO), before another 30 minutes incubation, with Streptavidin alkaline phosphate in a 1:500 dilution (Streptavidin-AP, Vector SA-5100), final visualization with Fast Red. Negative control samples of cardiac tissue from healthy Atlantic salmon, known negative to PMCV, PRV and SAV by real-time RT-PCR were included, and as a positive control, a heart from a clinical field outbreak of CMS with severe lesions and high PMCV specific RNA load was included (Fritsvold et al., 2021).

***In situ* hybridisation**

An RNAscope *in situ* hybridisation assay targeting PMCV was established and used for comparison to confirm the results of PMCV load indicated by the real-time RT-PCR. The results were also used to study viral tropism in infected tissues and in relation to cardiac lesions if present, and for comparison with IHC detecting PMCV antigen in serial tissue sections of hearts. Slides were made of 9 selected formalin fixed cardiac tissue samples, representing all four sampling time points (for details, see Table 1). The selection of these samples was based on their cardiac PCR results, to ensure the inclusion of individuals with the highest amounts of PMCV specific RNA, hence the samples are not representative for all samples at each sampling point. In addition, slides of mid-kidney from one of the fish at each sampling point represented by a cardiac sample, were included.

For this purpose, the RNAscope® 2.5 HD Singleplex Red Chromogenic Reagent Kit (Advanced Cell Diagnostics Inc., Newark, CA, USA) was used according to the manufacturers protocol. A set of 14 pairs of PMCV probes "V-piscine-myocarditis-ORF1" (Advanced Cell Diagnostics Inc., Newark, CA, USA, cat.no. 812021), targeting the PMCV capsid gene area at nt 1050 – 1757 bp on GenBank reference JQ728724.1 were designed by the manufacturer using custom software as described by (Wang F et al. 2012). In addition, probes targeting salmon peptidylprolyl isomerase B (ppib, Cat. No. 494421) and bacterial dapB (dapB, Cat. No. 310043) were included during optimalization. The optimal concentration of PMCV probes was adjusted in a pilot study by testing a dilution series of the probe (1:1 to 1:4 by volume) in 1X RNAscope® Wash Buffer supplied with the kit, and the 1:2 dilution was selected. In short, deparaffination of non-stained serial sections of selected samples prepared as described for histopathology, was performed with xylene baths before several steps of rehydration in alcohol baths. Endogenous peroxidases in the rehydrated slides were blocked by hydrogen peroxidase treatment for 10 min, followed by 15 min boiling of the sections in target retrieval buffer, before a 15 min incubation at 40ºC with a protease. The slides were then hybridised with the same amount of probe on each slide at 40ºC for 2 hours. After probe hybridization, the slides were incubated with signal amplifiers (AMP1 – AMP6) in sequences of duration times and at temperatures recommended by the manufacturer, including separate washing steps. Fast Red chromogen was used to visualise the hybridisation signal, before counterstaining the slides using Mayer's hematoxylin (Chemi Teknik, Oslo, Norway), diluted in distilled water (1:1 by volume), and mounting with cover glasses and VectaMount (Vector Labs, Burlingame, CA).

**Graphics and statistical analysis**

Statistical analysis and graph presentation were performed using GraphPad Prism 9.1.0 (GraphPad Software Inc., La Jolla, CA, USA). Two-way ANOVA followed by Tukey's multiple comparisons test were used to analyse statistically significant differences between real-time PCR Ct-values for each time point per tissue/blood/mucus sample and between sample types per time point. Due to the cut off value at Cq 40 for the real-time PCR procedure, Cq 40 was used as the value for all negative samples and samples with

Cq>40 in the calculations and presentations. Correlation analyses were performed using Microsoft Excel.

## Results

The experimental CMS challenge of Atlantic salmon was set up to compare histopathological changes related to CMS with levels of PMCV specific RNA through PCR studies, in the three cardiac compartments atrium, ventricular spongiosum and ventricular compactum and also mid-kidney and skin/muscle tissues, in the early phase post challenge. Parallel samplings of blood and mucus were also included for evaluation as non-lethal samples and comparison against viral detection in the tissues. No mortalities, clinical signs of disease or observations of atypical signs of disease were observed during the experiment.

**Autopsy**

Average weight of the experimental fish was 48.1 g at mock vaccination (7 weeks days before the challenge), 94.1 g at 10 dpc and 129.1 g at 52 dpc, and they were in normal condition. Only a few fish had macroscopic findings of non-relevant character to the results of the studies, with the exception of fish 8 at 52 dpc, which had an enlarged atrium.

**Development of CMS shown by histopathology**

To study development of disease, six fish were subjected to histopathology studies at 0 dpc, and 15 fish at each of the other three samplings, with a few exceptions of tissues missing from the sample set (Table 1). HE-stained tissue slides of heart (including all three compartments), mid-kidney and skin/muscle were evaluated by light microscopy and cardiac tissues were scored according to Table 2.

Before challenged (0 dpc), the majority of fish showed in general no signs of CMS related lesions (Figure 1 and 2), but a base line finding of one or a few focal lesions of inflammatory cells in the epicardium was observed, and two fish had some lesions with very sparse inflammation of the atrium (Figure 1). All fish had moderate amounts of melanin and/or melanomacrophages in the interstitium of the kidney, most of them light eosinophilic foam-like material in the urinary space of Bowman's capsule and eosinophilic material in lumen of a moderate number of renal tubuli. These were general findings in most fish at all sampling points, but a small increase in the amount of melanin and/or melanomacrophages in the kidneys were seen at the two last sampling points. In red skeletal muscle, about half the fish at the three first samplings presented with a sparse interfibrillar hypercellularity, which was observed in an increased number of fish at the last sampling.

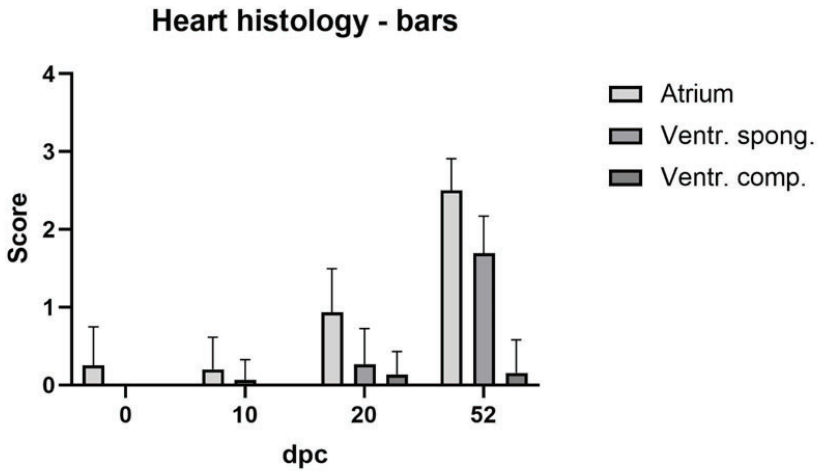


**Figure 1:** Histopathology scores of the three cardiac compartments atrium, ventricular spongiosum and ventricular compactum for all sampling time points, in accordance with Table 2. Average values plus standard deviation of the mean (SD) are shown (n=5 at 0 days post challenge (dpc), n=15 at 10 and 20 dpc and n=13 at 52 dpc).

At 10 dpc, the cardiac findings were comparable to the day of challenge, with exception of one individual with a sparse, focal lesion in the spongiosum of the ventricle. However, 10 days later, at 20 dpc, the number of individuals with sparse atrial lesions increased (Figure 1). The majority of these had a few focal lesions and an increased number of leukocytes (grade 1), but one individual had several distinct lesions also including a higher number of leucocytes (grade 2). A few fish also presented with with a few focal lesions including increased number of leucocytes in the ventricular spongiosum. At this time point, sparse epicardial pathology was also apparent in the majority of fish, and five fish had several epicardial lesions, with a small to moderate increase in leukocyte number. Only one fish had sparse lesions of the compact ventricle at this point.

The most striking difference from the 20 dpc to the 52 dpc sampling, was the increase both in number and severity of inflammatory lesions in atrial and ventricular spongiosum. Atrial changes particularly increased in severity, and ventricular lesions in number (Figure 2). All fish examined at this last sampling had several distinct or multifocal atrial lesions with a moderate to severely increased number of leukocytes. A majority of those with several distinct lesions had a tendency towards the more severe multifocal lesions (Figure 2). Furthermore, all fish had sparse or sparse to moderate lesions in the ventricular spongiosum. Epicardial changes were at the same, or slightly lower level, than at the 20 dpc sampling.

Summarised, lesions of the atrium were recognised before lesions of the ventricular spongiosum. Indications of increasing inflammation in ventricular spongiosum were observed at 20 dpc, and this was more evident at 52 dpc. Epicardial lesions were mostly sparse, with a small increase from 10 to 20 dpc, continuing at the same low level at the last sampling. In general, very few and sparse pathological changes of the compact layer of the cardiac ventricle were observed: only a single fish at each of the two last samplings presented with few focal lesions and an increased number of leukocytes.

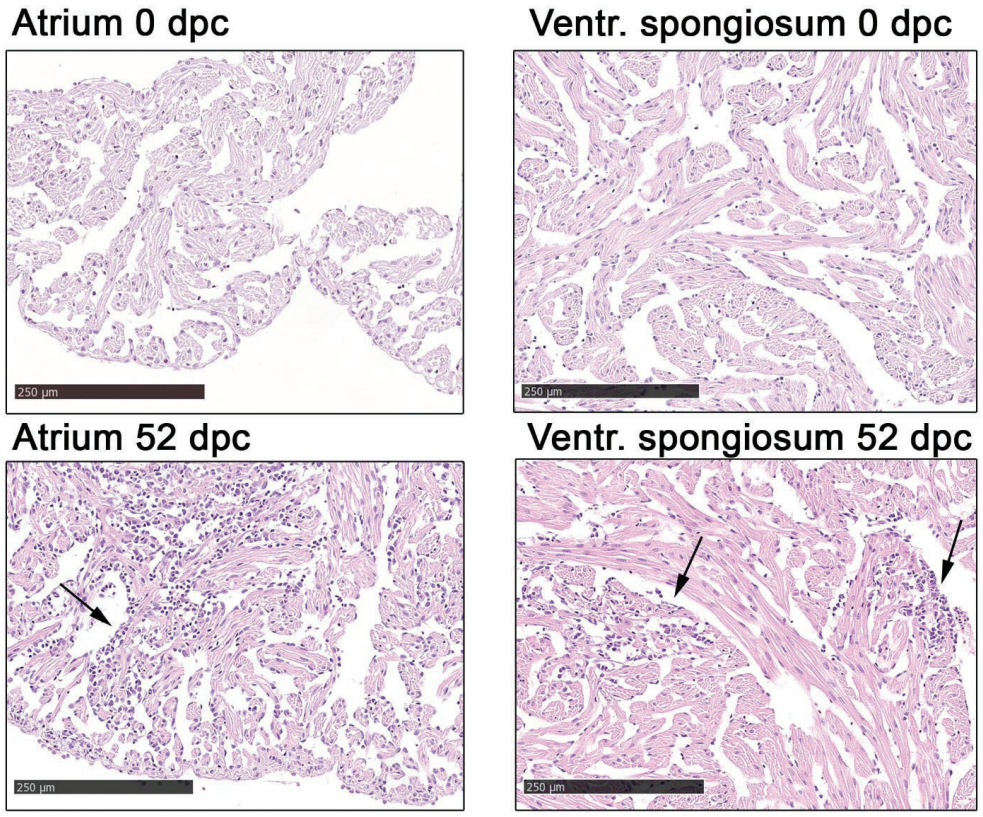


**Figure 2:** Normal atrium and ventricular spongiosum found at 0 dpc (exemplified by fish (F) 1 and 2, respectively), and typical CMS lesions (arrows) in atrium and ventricular spongiosum found at 52 dpc in F9 and 15, respectively. HE-staining and standard light microscopy at 200x magnification (bar = 250 µm).

**Presence of PMCV specific RNA in heart compartments, mid-kidney and skin/muscle**

The various tissues included for detection of PMCV specific RNA revealed some variations in RNA levels in each individual over the course of early phases of infection (Figure 3a). At the earliest sampling after challenge (10 dpc), levels of PMCV RNA were highest in mid-kidney (Figure 3a) and the RNA was detected with a 100% prevalence among the 15 individuals included. Viral RNA was also detected in skin/muscle tissue at a prevalence of 60%, although Cq-values were generally high (spanning from 28.6 - 39.4). At this early time point, PMCV RNA was not detected in samples of any of the cardiac compartments, with the exception of a few samples with Cq-value close to cut off (Figure 3a).

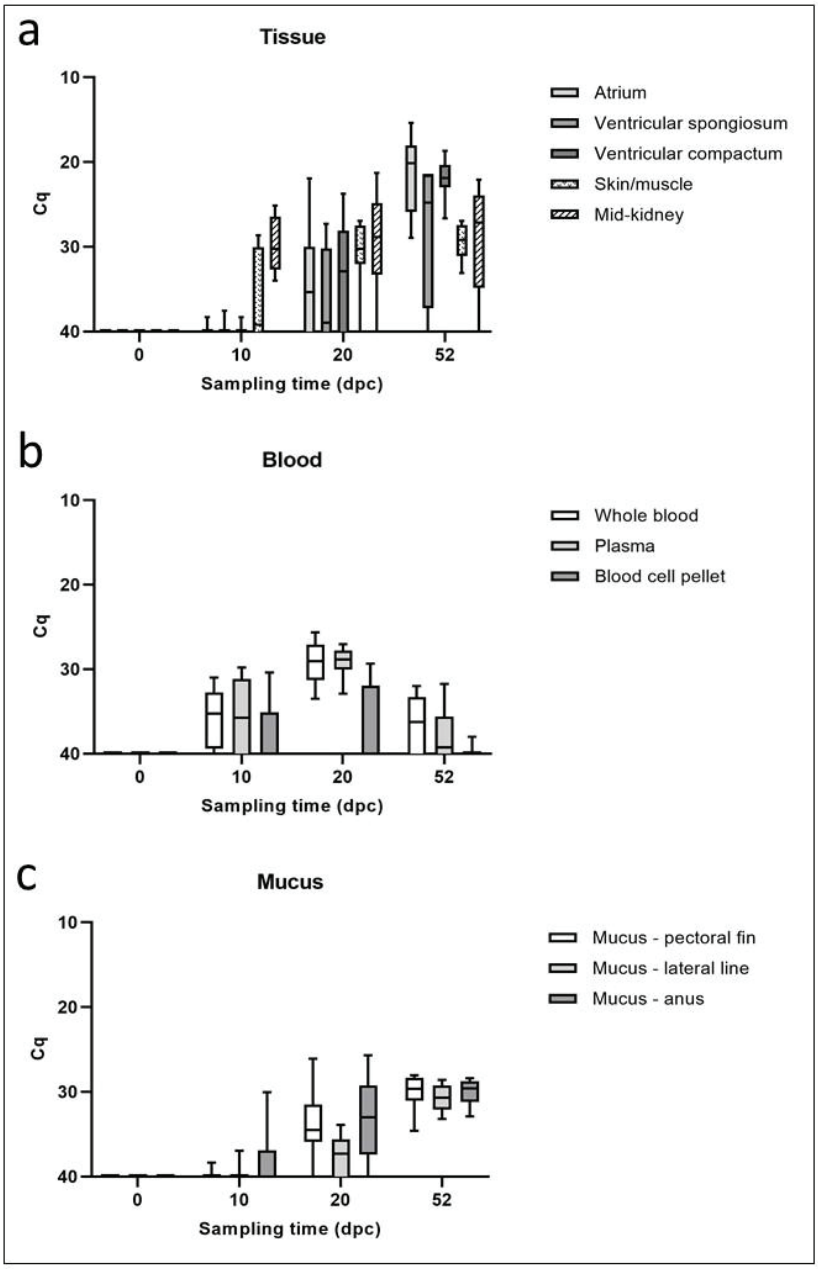


**Figure 3:** Levels of PMCV specific RNA given as Cq values, for all sampling time points, in days post challenge (dpc) in three heart compartments, mid-kidney and skin/muscle and blood components and surface mucus. **a)** Tissue samples of cardiac atrium, ventricular spongiosum, ventricular compactum, mid-kidney and skin/muscle. **b)** Parallel samples of blood: whole blood, plasma and blood cell pellet. **c)** Mucus samples at three different locations of the surface of the fish: Pectoral fins, lateral line and anus. Results are presented as box plot with whiskers representing minimum and maximum values in the individual data set. n=4 at 0 dpc and n=15 at other time points, excluding ventricular spongiosum at 52 dpc where n=4. Note the inverted numbering of the Y-axis.

From 10 to 20 dpc, a significant increase of PMCV RNA load in the cardiac compartments atrium and ventricular compactum (P = 0.01-0.05 and 0.001-0.01, respectively) (Figure 3a) was seen, and a similar increase was also indicated for ventricular spongiousum. The resulting prevalence of detection is 60% of the individuals for each of all the three compartments separately. Still, detection of the virus RNA was not consistently distributed among compartments and individuals, and if any heart compartments is included per individual, the prevalence of detection is 80%. The levels of PMCV specific RNA in mid-kidney samples were almost unchanged, and mid-kidney was still the organ with most individuals with relatively high virus specific RNA levels (Cq around 30), but a wider inter-individual variation in levels were seen, also including a few negative samples. Similar to the heart tissues, viral RNA detection also increased significantly in skin/muscle tissue (P = 0.01-0.05) (Figure 3a) with resulting RNA levels higher, and with less interindividual variation, than each of the heart compartments and also higher prevalence for detection (86%). Both mid-kidney and skin/muscle have significantly higher levels of viral RNA than ventricular spongiosum at this time point (P = 0.01-0.05) (Figure 3a).

At the last sampling point (52 dpc), the distribution of highest PMCV RNA levels changed among the tissues. A significant increase ($P<0.0001$) in the viral RNA levels was seen in atrium and ventricular compactum and probably also in ventricular spongiosum, but due to limited number of spongiosum samples (n=4), statistical analyses for this cardiac compartment was not valid. At this point, all three heart compartments showed significantly higher PMCV RNA levels than mid-kidney and skin/muscle ($P<0.0001$ towards skin/muscle and P = 0.001-0.01 towards mid-kidney, statistics not valid for ventricular spongiosum due to limited number sampled at 52 dpc), while there was no significant change from 20 dpc in viral RNA levels neither in mid-kidney nor skin/muscle samples.

There were no statistically significant differences between the Cq values obtained from the three heart compartments tested at each time point. Still, at individual levels there were variations, with correlation levels ranging from positive to non-existent (Table 3), including strong correlation between atrium and spongiosum at 20dpc. At 52dpc an almost perfect correlation is found ($r=0,98$) between Cq-values of spongiosum and compactum, but this may be related to the inclusion of only fish 1-4 as these are the only available from spongiosum at this time point.

| Tissues | Correlation coefficient (r) |
|---|---|
| Atrium vs ventricular spongiosum 10dpc | -0.02 |
| Atrium vs ventricular spongiosum 20dpc | 0.78 |
| Atrium vs ventricular spongiosum 52dpc F1-4 | 0.16 |
| | |
| Atrium vs ventricular compactum 10dpc | -0.07 |
| Atrium vs ventricular compactum 20dpc | 0.61 |
| Atrium vs ventricular compactum 52dpc | 0.42 |
| | |
| Ventricular spongiosum vs compactum 10dpc | -0.15 |
| Ventricular spongiosum vs compactum 20dpc | 0.70 |
| Ventricular spongiosum vs compactum 52dpc F1-4 | 0.98 |

**Table 3:** Correlation coefficients between Cq values of different cardiac compartments at the samplings 10, 20 and 52 dpc.

An overview of all Cq data revealed that the highest individual sample levels of PMCV specific RNA in both mid-kidney (Cq 21.3) and skin/muscle (Cq 27.0) were detected at 20 dpc, while all sampled cardiac compartments had highest individual RNA levels measured at 52 dpc (atrium Cq 15.4, compactum Cq 18.7 and spongiosum Cq 21.6, respectively). This is consistent with the overall trend observed, where the first significant increase in PMCV specific RNA levels appears in the mid-kidney, and, to some extent in the skin/muscle samples, at 10 dpc and then again at 20 dpc, before the cardiac compartments experienced a similar increase and reached the highest specific viral levels of the experiment at 52 dpc.

**Presence of PMCV specific RNA in blood and mucus**

Real-time RT-PCR detection of PMCV specific RNA was also used on samples of whole blood, plasma, blood cell pellet and mucus to evaluate these sample types as non-lethal virus detection methods, and also to study variation in viral presence in these samples over the time course of the initial phases of a PMCV infection.

*Blood*

Heparinised whole blood and plasma samples had almost identical PMCV specific RNA levels at all three sampling points with in general low levels of PMCV RNA detected. Remarkably, from 10 to 20 dpc a significant increase in RNA levels was seen ($P<0.0001$) and a 100% prevalence of detection was found, with very low interindividual variation for both sample types and relatively high levels (Cq around or just below 30) (Figure 3b). At 52 dpc, both whole blood and plasma PMCV specific RNA loads were significantly reduced ($P<0.0001$), and the variation in viral RNA levels between individuals were increased, including a higher number of negative fish. The pelleted blood cells showed a similar, but statistically non-significant trend, over time, with lower levels of PMCV RNA and a higher number of negative individual samples per sampling (Figure 3b).

*Mucus*

At 10 dpc the prevalence of individuals with at least one mucus sample with PMCV RNA detection was low (33,3%), and the resulting Cq levels were mainly close to cut off (Figure 3c). The levels increased significantly at 20 dpc ($P <0.0001$, $P=0.001$ - 0.01 and $P=0.01$ - 0.05, respectively, for mucus of pectoral fin, lateral line and anus), where almost all fish had moderate to low amounts of PMCV specific RNA (Figure 3c), but with considerable inter-individual variation for all samples, independent of sampling place. However, at the last sampling, all mucus samples were positive with a very low inter-individual variation and general levels

similar to the mid-kidney samples at this time-point (Figure 3c).

**Immunohistochemistry – IHC**

IHC was performed on 9 cardiac slides selected to represent all time points (Table 1). No specific staining was observed in any of the slides, except for the positive control, where a weak signal was seen in relation to a few atrial lesions, in the atrioventricular valves and in connective tissue at the interphase of ventricular compactum and ventricular spongiosum.

***In situ* hybridisation**

An *in situ* hybridisation procedure including a set of probes specific for PMCV ORF1 was established and optimised for evaluation as a diagnostic method, and to study presence of PMCV specific RNA in relation to cardiac lesions and in mid-kidney tissue over the time course of infection. Selected samples (Table 1) were subjected to the RNAscope *in situ* hybridisation method and subsequently were examined by light microscopy for positive staining resulting from binding of the ORF1-probes.

Slides prepared from heart and mid-kidney showed no positive staining for PMCV at 0 dpc (Figure 4). However, at 10 dpc, two very small foci of specific staining for PMCV were recognised in the atrium of one fish, in addition to very small, pin-point staining of the nuclei of a few myocardial cells. Similarly, in the ventricle of the same fish, a few positive nuclear pin-point signals were observed in apparently normal myocytes in one part of the compactum and the adjacent spongiosum, not related to inflammatory lesions. At the same time point, a few stained foci were observed in the interstitium of the mid-kidney, probably cytoplasmatic, with indications of cytoplasmatic localisation (Figure 4, mid-kidney, 10 dpc).

Ten days later (20 dpc), stronger positive signals were seen in the atrium of both examined fish (Figure 4), including three larger clusters of positive staining and one small cluster, respectively, with some relation to sparse inflammatory lesions. At this time point, pin-point positive nuclear staining of various cells of both atrium and ventricle were also observed in endocardial, myocardial and endothelial cells (see Figure 5a). In the kidney, many distinct foci of positive staining were observed interstitially, mainly cytoplasmatic. In addition, some positive pin-point nuclear staining of tubuli cells, as described for myocardial cells were also observed, in contrast to almost no nuclear staining of interstitial cells at this time point (Figure 4).

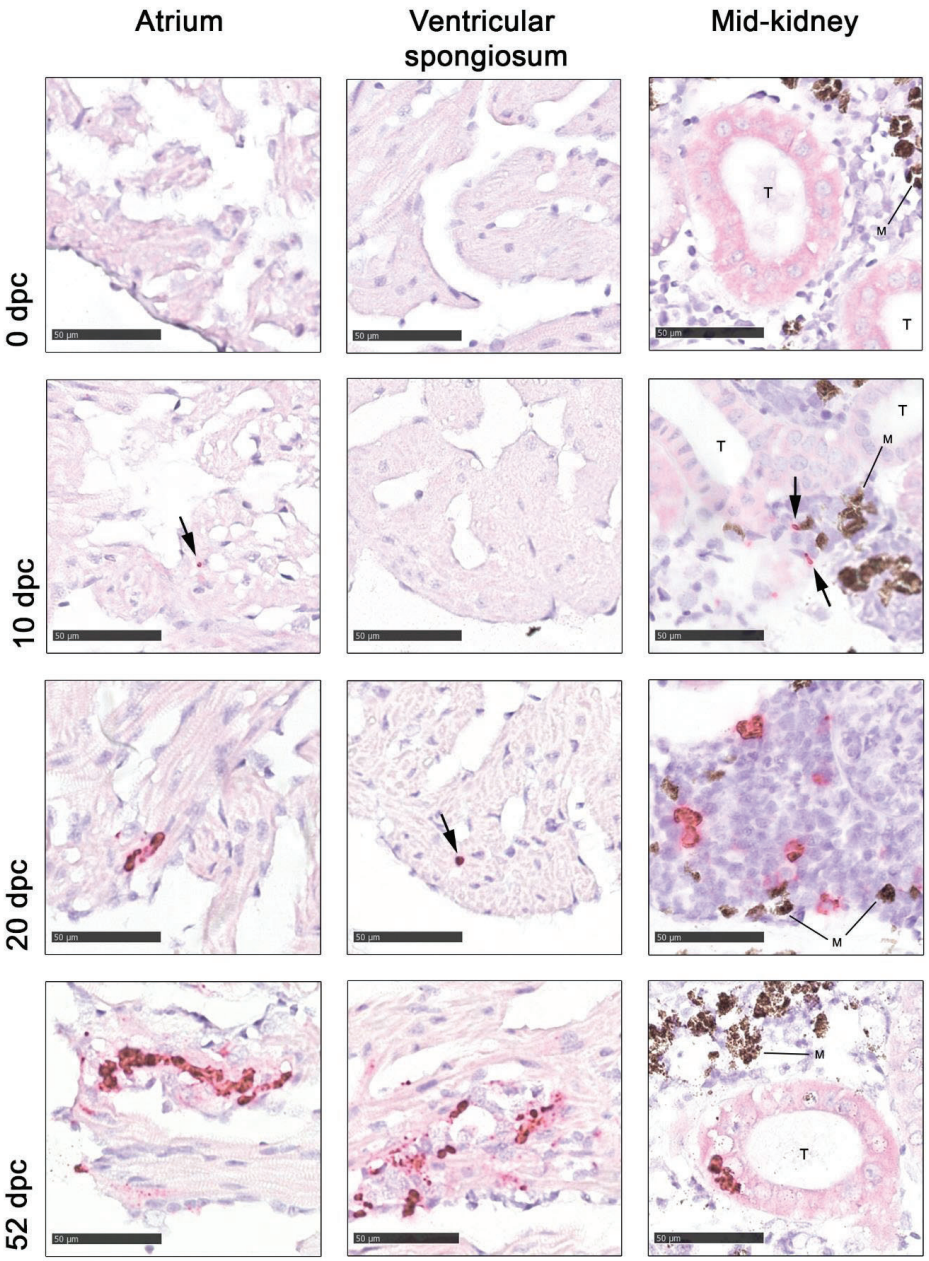


**Figure 4:** Examples from RNAscope in situ hybridisation of cardiac atrium and ventricular spongiosum, represented by hearts of fish F1, F7, F2 and F1 at 0, 10, 20 and 52 dpc, respectively, and mid kidney represented by F3, F3, F2 and F2 at 0, 10, 20 and 52 dpc, respectively. Detection of PMCV specific RNA is visible as dark red staining (arrows in images of sparse staining). S = spongiosum, T = lumen of tubuli, M = melanin deposits and/or melano-macrophages in the interstitium of the kidney. All images 400 x magnification, bar = 50 µm, standard light microscopy.

Towards the end of the experimental period, at 52 dpc, there were abundant positive staining in, and related to, inflammatory foci of both atrium and ventricular spongiosum, with the most intense and widespread staining located in atriae, corresponding with the most severe cardiac lesions (Figure 4 and Figure 1). The staining presented as clustered, strong signals in the middle of inflammatory lesions, more subtle and less intense positive staining of myocardium and surrounding tissue in areas of more sparse or moderate inflammation, in addition to moderate amounts of nuclear pin-point staining in otherwise normal tissue of both compact and spongious layers of the heart. Severe cardiac lesions with strong specific PMCV RNA staining in cytoplasma, showed no staining in myocardial nuclei (see Figure 5b). In this last sampling, most of the positive staining of mid-kidney tissue were seen in tubuli cells, collecting tubuli and their nuclei, rather than in the interstitium as in the earlier samplings (Figure 4), as well as positive pin-point nuclear staining of some glomeruli cells. However, a few positive signals could also be seen interstitially. A transition from strong cellular staining to more sparse nuclear pin point staining was observed as infection progressed.

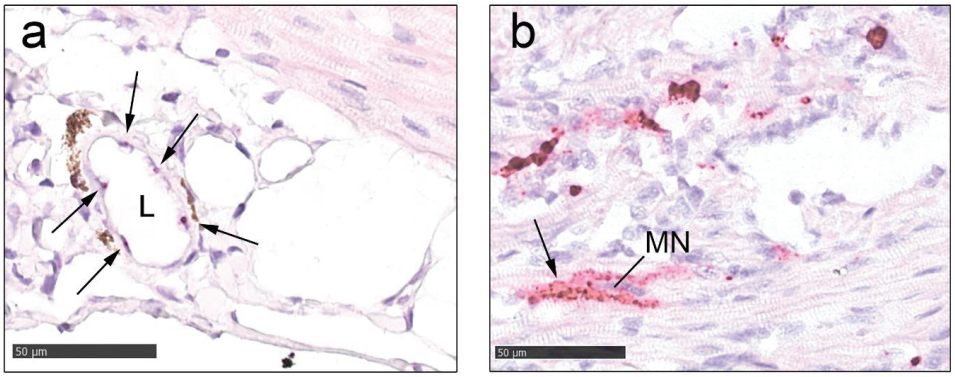


**Figure 5:** Details of RNAscope *in situ* hybridisation results. Detection of PMCV specific RNA are visible as dark red staining. **a)** Dotted staining in endothelial nuclei in a coronary vessel (arrows). L = lumen of coronary vessel. **b)** Staining (arrow) in atrial myocardial cells. Note the lack of staining in the myocardial nucleus (MN). Both images, 400 x magnification, bar = 50 µm, standard light microscopy.

## Discussion

Cardiomyopathy syndrome has since the first description in 1985 become a regular and relatively widespread disease in Atlantic salmon, in particular for the Norwegian, Scottish and Irish farming industry. In Norway, it has been ranked as the most important viral disease regarding both mortality and economic losses for the last years in Norway (FHR 2019, 2020). Still, a decade after the viral agent was found (Haugland et al, Lovoll), detailed knowledge on infection and pathogenesis, in particular in early phases, and a wide set of tools for diagnostic and research studies, are still lacking. Similar, there is also a need for a wider set of tools for diagnostic and research studies. Here we have compared detection of PMCV and CMS related tissue lesions using previously used and well known diagnostic methods, i.e. histopathology and real-time RT-PCR, to immunohistochemistry, an existing, but less used method, and the not previous published method RNAscope *in situ*

hybridisation. The methods were also used to study virus tropism by comparing PMCV specific RNA levels in a wider organ sample set, including non-tissue sample types of various blood components and mucus.

Low or no mortality and almost complete absence of clinical signs typical for CMS, is a common finding in CMS challenge trials of similar duration to this study (Haugland et al., 2011; Timmerhaus et al., 2011). Here, the only clinical sign observed was an enlarged atrium observed at autopsy 52 dpc. This was probably related to CMS, as histopathological cardiac lesions were sparse to moderate in all cardiac compartments, in combination with high levels of PMCV specific RNA load (Cq atrium 18.3). In general in this challenge, histopathological examinations confirmed CMS lesions characteristic for early phase infection, described as multiple, sparse foci of few inflammatory cells subendocardially in the atrium (Fritsvold et al., 2009; Haugland et al., 2011; Timmerhaus et al., 2011), and that these atrial lesions were recognised earlier than lesions in the spongiosum of the ventricle. Also, a typical development pattern of CMS lesions was seen over time in ventricular spongiosum, subsequent to the increased number and severity of atrial lesions. The compact layer had few and sparse lesions, in accordance with earlier descriptions (D. W. Bruno et al., 2013; Ferguson et al., 2006; Fritsvold et al., 2009; Haugland et al., 2011).

Compared to PD and HSMI challenge trials (Christie et al., 2007; Kongtorp & Taksdal, 2009; Lund et al., 2016; Taksdal et al., 2015; Wessel et al., 2020), experimental CMS seems to have a slightly slower onset of infection (Fritsvold et al., 2009; Haugland et al., 2011; Timmerhaus et al., 2011), which probably also reflects the more protracted development seen in the field for CMS than in cases of e.g. PD (Graham et al., 2010; Kongtorp, Taksdal, & Lyngoy, 2004; McLoughlin & Graham, 2007; Taksdal et al., 2007). At the first sampling post challenge, 10 dpc, PMCV specific RNA was detected in highest concentrations in the mid-kidney, while heart tissues, considered as the target organ of PMCV (Haugland et al., 2011; Timmerhaus et al., 2011), only showed detections close to cut-off limit in a few samples. This might indicate that PMCV replicates and increases in concentration in kidney before it is transferred to the target organ, the heart. An early phase of viremia or primary viremia, where virus spreads from its initial infection site, is also supported by the increasing levels of virus RNA in blood samples, especially the ones including plasma.

The viremic phase seemed to continue towards the sampling ten days later, as the presence of viral RNA was increasing in all organs tested and in mucus and blood. The levels of PMCV RNA detected in plasma at this time point was remarkably high and consistent among the individuals, compared to levels and consistency among individuals in the other tissues tested, and gives a strong support to an ongoing viremia at this stage of infection, although the exact time of the viremic peak cannot be decided as the sampling points included are too few. Cell damage is thought to trigger inflammation (Van Vleet & Ferrans, 1995), and increasing damage to infected cardiac cells was seen concurrently with increasing levels of PMCV RNA in the heart. These sparse early phase histopathological atrial lesions can be observed together with initial, sparse lesions in the spongy ventricle. The continuous high blood flow passing through the kidneys and the nature of basic renal functions, especially filtration, may explain the moderate to high levels of specific PMCV RNA in kidney samples during a viremia, as have been described previously in challenge trials (Timmerhaus et al., 2012).

At the last sampling point (52 dpc), the real-time RT-PCR results indicate that the viremia seen at 10 and 20 dpc was over. The relatively high and consistent levels of

PMCV specific RNA in blood was reduced, including several samples where no virus RNA could be detected. At this time point, high levels of PMCV RNA in all cardiac tissues suggests that the virus probably has reached its target cells of the myocardium, where more efficient replication may take place, simultaneously inducing a phase of increasing inflammatory responses in the myocardium. Also interesting, concurrently with moderate virus RNA levels and high consistency among the individuals in skin/muscle at this time point, the mucus samples from both pectoral fin, lateral line and anus, all had their maximum levels of PMCV RNA in this sample set, also including very low interindividual variation. This could indicate that this stage of infection represents a phase where viral shedding from the fish through mucus is high. Shedding and horizontal transfer of the virus is supported by previous challenges including injection challenged fish transferring virus to cohabitants (Haugland et al., 2011). The time frame for such shedding to occur is not possible to define due to the limited sampling time points in this study and especially low frequency at this late time point. Similar studies on SAV have shown that the virus was present in mucus from 2-3 wpc and the presence continued for further 1-3 weeks, depending on virus subtype, and demonstrated that shedding and transmission of virus may occur through mucosal routes (Graham et al., 2011).

In addition to the expected strong positive staining shown in cardiac lesions at 52 dpc using the RNAscope *in situ* hybridisation, a strong positive staining was detected in a few foci in interstitium of mid-kidney at 10 dpc, corresponding well with the increased levels of PMCV RNA shown in mid-kidney at this early time point. The change to staining in kidney tubuli cells at 20 and 52 dpc, may indicate a possible progression of the PMCV infection in the mid-kidney: from interstitial cells with hematopoietic function between sinusoids at the peak of the viremia, to cells of the excretory part of the kidney, most often in tubuli, but to some extent also in glomeruli, in the proposed post-viremic phase. The RNAscope *in situ* hybridisation was performed on 1 to 3 selected samples from each time point, representing the individuals with highest amounts of PMCV specific RNA in heart tissues, and does not necessarily represent the full sample set at each time point.

In some tissues and phases of the PMCV infection, very small, pin-point positive staining was observed in the nuclei of apparently normal cells, in general not in relation to inflammatory or other pathological lesions. The pin-point staining was found in nuclei of myocardial cells in cardiac spongiosum and compactum, in endothelium of coronary vessels, in mid-kidney interstitial cells and tubuli cells. Replication of PMCV is described as cytoplasmatical (Haugland et al., 2011), and similar findings of strong specific PMCV RNA staining in cytoplasma and no staining in myocardial nuclei in relation to severe cardiac lesions found in our study, supports this. Also, presence of a RNA virus, or viral genomic RNA fragments, in the nuclei of such cells is unexpected and should be investigated further to conclude the significance of presence of PMCV RNA in the nuclei.

We have previously studied detection of PMCV specific RNA in the three heart tissues atrium, ventricular spongiosum and ventricular compactum, in field samples from an ongoing CMS outbreak with a very high viral load. These results indicated that sampling from any of the three examined cardiac compartments would result in similar sensitivity of virus detection among the three heart compartments in similar late stages of CMS (Fritsvold et al., 2021). In this study, we include similar heart tissue compartment samples from individuals in early infection phases and with a lower viral load. Similar to the field study, there were no statistical significant difference between cardiac compartments

over the samples set in PMCV RNA levels at any time points. Still, the results here show that at individual level, the correlation of levels of PMCV RNA detected between the three included compartments were varying.

In this study, samples taken by non-lethal methods were also included for comparison with a standard tissue sampling. Whole blood, plasma and pelleted blood cells from all individuals were tested for presence of PMCV specific RNA. The results showed that moderate levels of PMCV RNA may be found in plasma, while the viral load in pelleted blood cells was low or non-detectable at all time points. Also, similar levels were found in whole blood, but since the levels are comparable to plasma and pelleted cells almost negative, the presence found in whole blood is probably due to the plasma part and not the cells present. Mucus collected from pectoral fin, lateral line and anus reached similar detection levels as plasma samples, but in contrast, at late phase of infection.

For diagnostic purposes, heart have been the standard tissue for detection of PMCV as virus specific RNA (Fritsvold & Jensen, 2021) and previous studies (Fritsvold et al., 2021; Timmerhaus et al., 2011), and present results confirm the heart as the organ of choice in active phases of PMCV infection with manifested cardiac inflammation and disease. Still, if the purpose is to screen for PMCV in early phases of an infection, the present results point towards a higher sensitivity of virus detection when kidney is chosen or included as sampled tissue. Also interesting, at 20 dpc in the present challenge, a comparable high sensitivity and low inter-individual variation to the mid-kidney were also found in plasma samples. However, compared to these results, mucus is probably not ideal for early phase screening purposes. Using non-lethal blood sampling is beneficial to reduce loss for the farmer if screening is required for surveillance, and is also beneficial from an ethics perspective. More research is needed to confirm and optimise the high sensitivity using blood plasma samples to detect early PMCV presence in field cases.

As a consequence of an increasing number of field cases with double or even triple infections with PMCV, PRV-1, ISAV or SAV, all causing heart pathology, assigning a diagnosis of CMS based on histopathology alone have become more challenging. As our results show, the real-time RT-PCR detects specific PMCV RNA from a very early infection stage, especially in kidney and plasma, and with high sensitivity throughout the infection. However, detection of PMCV specific RNA by this method may precede any specific CMS pathology and disease by weeks or even months (Svendsen et al., 2019)(pers.comm. H. Sindre). Although immunohistochemistry has been used to detect PMCV antigen in heart tissue with pathological changes (Fritsvold et al., 2021), our present results show that of immunohistochemistry is dependent on a reliable primary antibody resulting in repeatable, sensitive and robust results to be suitable for diagnostic purposes.

In contrast, the RNAscope *in situ* hybridisation method specifically detects PMCV specific RNA in connection with observed heart pathology, showing increased sensitivity and robustness compared to the immunohistochemistry. The RNAscope ISH method may therefore, be a valuable supplement to histopathology to provide reliable CMS diagnoses in situations where the pathological changes are uncharacteristic or mixed.

In conclusion, this study adds knowledge on how PMCV infection progress in individual fish in the early phases of CMS. Our results indicate a viremic phase of PMCV at 10 to 20 dpc, preceding the cardiac lesions. In this early phase, virus could also be detected in relatively high amount in mid-kidney. Plasma and/or mid-kidney samples may therefore be candidates to screen for

early phase PMCV infection. Secondly, our results supports the heart as the organ of choice for both viral detection and histopathological diagnosis when cardiac lesions have emerged. In addition, the RNA scope *in situ* method has the potential to become a very useful diagnostic tool, both in pathogenesis studies and for routine diagnostics.


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