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Muhammad Naveed Yousaf

Characterization of the cardiac pacemaker and pathological responses to cardiac diseases in Atlantic salmon (*Salmo salar* L.)

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"*Ignorance is the worst kind of poverty, intellectuality is the greatest of riches, and thought is the best form of worship*".

Hazrat Muhammad (Peace Be Upon Him)

To

My father Muhammad Yousaf (late) and mother Safia Bibi

Parents who sacrificed their entire life in growing up their children and tried to provide them with the best of everything.

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Paper II. **Yousaf MN**, Powell MD. The effects of heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) on serum creatine kinase (CK) and lactate dehydrogenase (LDH) levels in Atlantic salmon (*Salmo salar* L.). The Scientific World Journal 2012, doi:10.1100/2012/741302.

Paper III. **Yousaf MN,** Koppang EO, Skjødt K, Köllner B, Hordvik I, Zou J, Secombes C, Powell, MD. Cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI). Fish Shellfish Immunology 2012, 33(2):305-15.

Paper IV. **Yousaf MN,** Koppang EO, Skjødt K, Köllner B, Hordvik I, Zou J, Secombes C, Powell, MD. Comparative pathological changes of cardiac diseases in Atlantic salmon (*Salmo salar* L.). Veterinary Immunology Immunopathology (under revision).

List of abbreviations

AEC: 3–amino–9–ethyl carbazole,

AMI: acute myocardial infarction,

ANP: atrial natriuretic peptide,

AV: atrio–ventricular,

BNP: B–type natriuretic peptide,

BSA: bovine serum albumin,

Caspase: cysteine–dependent aspartate protease,

CHF: chronic heart failure,

CK: creatine kinase,

CMS: cardiomyopathy syndrome,

CNP: C–type natriuretic peptide,

EGC: eosinophilic granular cells,

DAB: 3, 3'–diaminobenzidine,

dH_2O: distilled water,

H&E: haematoxylin and eosin,

HIF1: hypoxia inducible factor 1,

HRP: horseradish peroxidase,

HSMI: heart and skeletal muscle inflammation,

LDH: lactate dehydrogenase,

LPS: lipopolysacharide,

MAC: membrane attack complex,

MHC: major histocompatibility complex,

NP: natriuretic peptides,

PAMPs: pathogen associated molecular patterns,

PCNA: proliferative cell nuclear antigen,

PD: pancreas disease,

PBS: phosphate buffered saline,

PBST: PBS with 0.1% Tween 20,

PRP/R: pattern recognition proteins or receptors,

PVA: polyvinyl alcohol,

PVDF: polyvinylidene fluoride,

rt–ANP: recombinant rainbow trout atrial natriuretic peptide,

SA: sino–atrial,

SA–HRP: streptavidin–horseradish peroxidase,

sCP: salmon cardiac,

SDS: sodium dodecyl sulfate,

SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis**,**

Tc: cytotoxic T lymphocytes,

Th: helper T lymphocytes,

TLRs: toll–like receptors,

TNF: tumor necrosis factor,

TCR: T cell receptors,

TUNEL: terminal deoxynucleotidyl transferase nick–end labeling,

VNP: ventricular natriuretic peptide.

Abstract

The heart is considered the powerhouse of the cardiovascular system. In this thesis, characterization of cardiac pacemaker and potential biomarkers of cardiac diseases of Atlantic salmon (*Salmo salar* L.) were discussed. The normal performance of the heart requires balance, whether for coronary circulation, the synchrony and effectiveness of myocardial contractions or the influence of its nerves and ganglion cells. Neural control is equally important for the power and complexity of the heart. The pacemaker is the cardiac neural tissue that is responsible for initiation and control of heart beat.

This thesis described the location of cardiac pacemaker of Atlantic salmon at the junction of *sinus venosus* and atrium. Morphologically, the pacemaker tissue was composed of lightly stained plexiform modified cardiomyocytes, wavy appearing nerve bundles with oval, wavy, elongated nuclei with pointed ends and large round to pear–shaped postganglionic nerve cell bodies (ganglion cells). Novel immuno–localization of the natriuretic peptides such as salmon cardiac peptide (sCP) and ventricular natriuretic peptide (VNP) in the postganglionic nerve cell bodies at SA junction was demonstrated, suggesting their neuromodulatory/neurotransmitter roles in teleosts (Atlantic salmon). Besides CD3 as a general T cell marker, novel CD3 immunostaining in the postganglionic nerve cell bodies of cardiac pacemaker of Atlantic salmon was demonstrated, suggesting additional roles of CD3 in teleosts and shared similar patterns to mammals.

Atlantic salmon aquaculture industry bears huge losses due to viral diseases including cardiac viral diseases. Heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD) are viral diseases of marine farmed Atlantic salmon which mainly affect the heart in addition to other vital organs. The main findings of these diseases are necrosis

and mononuclear inflammatory cells infiltrates affecting different regions of the heart. To identify the potential biomarkers of cardiac diseases, blood biochemistry markers were correlated to the CMS– and HSMI–affected Atlantic salmon. Candidate biomarkers included serum enzymes such as creatine kinase (CK) and lactate dehydrogenase (LDH) levels were measured and significantly correlated to the cardiac pathology of HSMI–affected fish, suggesting promising potential biomarkers to predict the disease (HSMI). Non–significant correlations of serum enzymes to CMS–affected fish suggested that serum enzymes could be used to differentiate between the HSMI and CMS.

Further, immunohistochemistry was performed to identify potential markers of cardiac pathological changes of Atlantic salmon affected with similar cardiac diseases (CMS, HSMI and PD). The spectrum of inflammatory cells associated with the cardiac pathology consisted of mainly $CD3\varepsilon^+$ T lymphocytes and lymphocytic response dominated over granulocytes in the CMS–, PD– and HSMI–affected hearts. Macrophage–like and eosinophilic granular cells were identified by rTNFα antiserum in all three investigated diseased hearts, particularly in areas surrounding lesions. MHC class II antiserum identified strong, moderate and low levels of immunopositive cells in diseased hearts in HSMI, CMS and PD respectively. MHC class II immuno–reactivity was mostly confined to inflammatory foci and identified lymphocyte– and dendritic–like cells. Tissue hypoxia was indicated by moderate levels of HIF1α immunoexpression in PD–affected hearts. Increased presence of apoptotic cells in the hearts; particularly in CMS– and PD–affected fish was identified. Coupled with the increased presence of $PCNA^+$, $HIF1\alpha^+$ and apoptotic cells (identified by caspase 3 and TUNEL), these data suggested a high cell turn over where an induction of cell and tissue damage/repair occurred in diseased hearts. The CD3, MHC class II, PCNA, TNFα, caspase 3 and TUNEL staining were mostly confined to lesioned areas in the diseased hearts, pointing to pathological changes and

suggesting the markers appear promising as a supplementary tool in the identification of lesioned areas in the investigated diseased hearts. Interestingly, the apparently similar diseases exhibited differences in the immunopathological responses in Atlantic salmon.

1. Introduction

The heart is considered the powerhouse of the cardiovascular system. Several cardiac abnormalities have been reported in farmed Atlantic salmon in recent years (Poppe and Ferguson, 2006). In Norway, the occurrence of viral cardiac diseases has been increased in farmed Atlantic salmon during last two decades (Bornø et al., 2011; Marta et al., 2012). Viral diseases are a serious threat to aquaculture industry and the specific viral cardiac diseases of farmed Atlantic salmon include cardiomyopathy syndrome (CMS), pancreas disease (PD) and recently identified heart and skeletal muscle inflammation (HSMI) (Poppe and Ferguson, 2006; Silva et al., 2008). Despite many problems in piscine pathology, there has been an increasing focus on heart conditions, and it still lacks the important information regarding the heart anatomy (cardiac conduction system) and immunopathological responses (Poppe and Ferguson, 2006; Kongtorp et al., 2006; Solc, 2007). A better understanding of cardiac conduction system and cardiac pathological responses are required to study the disease pathophysiology and devise the efficient protection/vaccine strategies in farmed Atlantic salmon. In the next sections, the available information on Atlantic salmon heart, cardiac conduction system, blood biochemistry biomarkers, natriuretic peptides, immune system and programmed cell death are summarized following the cardiac diseases, with emphasis on viral cardiac diseases of Atlantic salmon.

1.1 Heart

The fish heart is comparatively smaller than that of vertebrates, comprising of 0.1% of the body mass in most species. Active swimming fish (salmonid) tend to have bigger hearts than more sedentary fish species (Vornanen et al., 2002). The S–shaped Atlantic salmon (*Salmo salar* L.) heart is

located within a pericardial cavity and consists of four serially arranged chambers including: (1) the *sinus venosus*, (2) the atrium, (3) the ventricle and (4) the *bulbus arteriosus* (Fig. 1) (Randall, 1968; Poppe and Ferguson, 2006). The teleost heart is considered a venous heart since it only pumps venous blood. The fish comprises a single circulatory system which ensures unidirectional blood flow (Farrell and Pieperhoff, 2011).

The *sinus venosus* is predominantly composed of connective tissue, and collects and stores venous blood (collecting chamber). The *sinus venosus* is a thin walled chamber with a similar volume to the atrium.

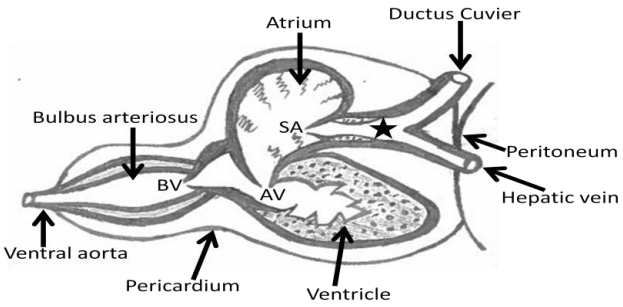

Figure 1. Schematic representation of Atlantic salmon heart. SA: sino–atrial valve, AV: atrio–ventricular valve, BV: Bulbo–ventricular valve, star: SA node (pacemaker area).

The functional role of *sinus venosus* is related to the initiation and control of heart beat, and serves as a site for the cardiac pacemaker tissue in many fishes (Farrell and Jones, 1992; Olson and Farrell, 2006; Poppe and Ferguson, 2006). The *sinus venosus* receives de–oxygenated venous blood from the paired Cuverian ducts, hepatic veins and anterior jugular veins and transfers blood into the next compartment termed the atrium. The one–way ostial valve between atrium and *sinus venosus* is called as the sino–atrial (SA) valve and is composed of connective tissue with sparse and variable arrangement of bundles of cardiac muscle (Farrell and Pieperhoff, 2011). The atrium is filled with blood through

two mechanisms such as *vis–a– fronte* (force from in front) by aspirating effect of ventricular contraction and *vis–a–tergo* (force from behind) by the remaining energy of venous circulation and/or contraction of the *sinus venosus* (Poppe and Ferguson, 2006; Icardo, 2006). The atrium is a thin–walled muscular chamber of trabecular myocardium with an irregular sac–like shape which increases the blood capacity of atrium (Fig. 2). The atrium forms a connection between the *sinus venosus* and the ventricle by the sino–atrial (SA) and atrio–ventricular (AV) valves and through AV valve blood enters into the ventricle (Farrell and Jones, 1992). The salmonid ventricle has a highly variable morphology directly linked to the specific functional needs of the fish.

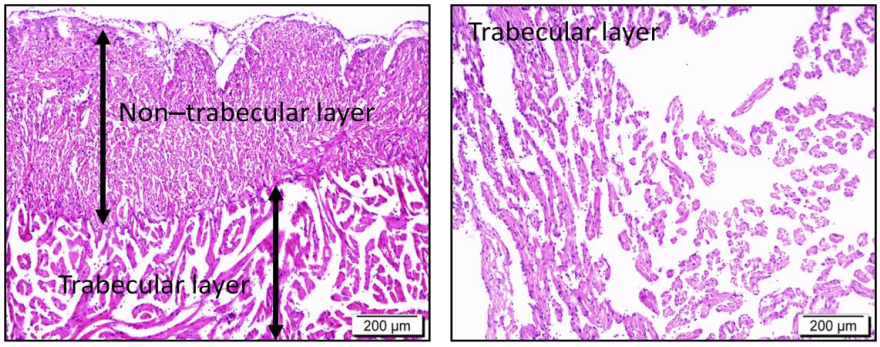

Figure 2. Micrographs showing the ventricle (left) and atrium (right) of Atlantic salmon (H&E stained). Ventricle consists of outer non–trabecular and inner trabecular (spongy) layer while atrium consists of trabecular myocardium.

The Atlantic salmon ventricle is primarily a triangular pyramid in shape due to its active life style with the apex pointing caudo–ventrally in contrast to more sac–like or tubular hearts of other fish groups (Poppe et al., 2003, Farrell and Jones, 1992). The relatively thick–walled, pyramidally shaped ventricle suggests distinct advantages in the blood pressure development with the pyramidal shape providing the most efficient shape for maximal contraction

force per unit mass and volume (Farrell and Jones, 1992; Olsen, 1998). The salmonid ventricle is composed of an inner trabecular myocardium (spongy layer as in the atrium) and an outer non–trabecular (compact) myocardium (Fig. 2). The outer compact layer is present in the hearts of teleost and elasmobranch fishes (Farrell et al., 2010). The outer non–trabecular muscle tissue is organized in a two–layered concentric pattern lying perpendicularly to each other. The thickness of compact layer is usually increased with the age (5% in young to 25% in adult) but also dependant on other factors including life stage, smoltification, sex and physiological demands and health status (Powell et al., 2002). The inner trabecular layer is more loosely arranged to form a sponge–like network (Farrell and Jones, 1992; Poppe and Ferguson, 2006). Recently Pieperhoff et al. (2009) suggested desomosome–like (D) and *fascia adhaerens*–like (FA) adhering junctions between the inner spongy and outer compact myocardium instead of previous understanding of connective tissue layer between spongy and compact layer in teleost heart. The non–trabecular layer is supplied with coronary circulation (oxygenated blood) branching off from hypobranchial artery while trabecular layer relies on the luminal venous blood for oxygen supply (Poppe and Ferguson, 2006; Roberts and Ellis, 2001).

Blood enters from the ventricle to the white colored, thick walled, fibroelastic cylindrical pear–, or onion–shaped compartment termed the *bulbus arteriosus*. In teleosts, this structure provides a passive dampening effect of the peak pulse force through the windkessel effect (Poppe and Taksdal, 2000; Farrell and Jones, 1992) as compared to the equivalent structure in elasmobranchs where the *conus arteriosus* is contractile in nature (Satchell, 1971; Satchell and Jones, 1967). The semilunar valves between the *bulbus arteriosus* and the ventricle prevent the backward flow of blood. The functions of the *bulbus arteriosus* include regulation of blood pressure and depulsator (blood flow to the ventral aorta) by gradual contraction/slow elastic recoil

(Roberts and Ellis, 2001). The *bulbus arteriosus* is connected to the ventral aorta which lies outside of the pericardial cavity and takes blood to the gills (Farrell and Pieperhoff, 2011).

The above mentioned heart chambers are composed of three layers such as (1) pericardium, (2) myocardium (middle) and (3) endocardium (inner) which are discussed as below.

The heart is enclosed in a membranous sac that separates it from rest of the body, termed the pericardium. The pericardium is composed of a layer of mesothelial (epithelial–like) cells, connective and fat tissue. The inner part of the pericardium is called the epicardium that covers the entire surface of heart and is often attached to cardiac chambers. A small amount of pericardial serous fluid bathes the heart and reduces the friction between pericardium and the heart during each heart beat (Farrell and Pieperhoff, 2011; Poppe and Ferguson, 2006; Roberts and Ellis, 2001).

The endocardium comprises a monolayer of endothelial cells and loose connective tissue that covers all of the internal surfaces of the myocardium and is in direct contact with the luminal blood. The endothelial monolayer particularly, in the atrium is phagocytic in some fishes such as Atlantic cod (*Gadus morhua*), platy (*Xiphophorus maculatus*), plaice (*Pleuronectes platessa*) and firemouth cichild (*Thorichthys meeki*), and capable of removing biological macromolecules and waste products (Leknes, 2002, 2011; Seternes et al., 2001, 2007). The endothelium may serve as scavenger cells and is considered a part of immune system in these fishes but other fishes (salmonid) do not have these properties, although scavenger cells have been identified in the head kidney of Atlantic salmon (Froystad et al., 1998; Press and Evensen, 1999).

The myocardium is the thickest and most obvious layer, comprising of cardiac muscle (myocytes). The cardiac muscle (myocardium) is sandwiched

between endocardial and epicardial tissue (Farrell and Pieperhoff, 2011). Trabecular and non–trabecular layers consist of specialized spindle–shaped (long and narrow) cardiomyocytes which are comparatively smaller in diameter in fish (1–12.5 μm) as compared to mammals (10–25 μm) (Farrell and Jones, 1992). The non–trabecular cardiac myocytes are smaller in diameter than trabecular myocytes, providing the shorter diffusion distance from outside to the center of the cell and a high ratio of sarcolemmal area to intracellular volume (Farrell and Jones, 1992). The cross striated cardiac myocytes consist of mostly one centrally located nucleus, numerous mitochondria, variable amount of intracellular myoglobin and no T–tubules (Poppe and Ferguson 2006; Farrell and Pieperhoff, 2011). Fish cardiomyocytes are capable of regeneration and grow by hypertrophy (increase in cell size) as well as hyperplasia (increase in cell number) as compared to post–embryonic mammals where cardiomyocytes mostly grow by hypertrophy (Becker et al., 2011; Borchardt and Braun, 2007; Kikuchi et al., 2010; Major and Poss, 2007; Mommsen, 2001; Poss, 2007; Sun et al., 2009; Vornanen et al., 2002; Poppe and Ferguson, 2006; Soonpaa and Field, 1998).

1.2 Clinical biochemistry markers of cardiac function

Compared with other areas of veterinary medicine, fish blood biochemistry is not common place due to the lack of reference values (physiological and pathological) and in turn there is a very little understanding of disease pathogenesis and pathophysiology (Powell, 2006; Lie and Waagbo, 1988; Chen et al., 2004). However, Lie and Wagboo (1988) and Powell (2006) described the normal blood biochemistry ranges for few important components for Atlantic salmon. Additionally, blood biochemical profile for other fishes such as red lionfish (*Pterois volitans*), great sturgeon (*Huso huso*), cultured sturgeon

hybrids (*Acipenser naccarii* female X *Acipenser baerii* male), black scorpion fish (*Scorpaena porcus* Linneaus, 1758) and Nile tilapia (*Oreochromis niloticus*) have been identified (Almeida et al., 2002; Ishikawa et al., 2007; Celik, 2004; Marco et al., 2011; Rajabipour et al.,, 2009; Anderson et al., 2010). Few studies have been investigated the relationship between blood biochemistry and cardiac diseases of Atlantic salmon (Rodger et al., 1991; Ferguson et al., 1986). In addition, fish heart failures are different from mammals due to the fact that teleost cardiomyocytes can metabolize lactate and local ischemic lesions are rarely identified (Powell, 2006). The creatine kinase (CK) and lactate dehydrogenase (LDH) are biochemical enzymes which catalyze anaerobic or aerobic metabolic reactions in cells, and are well established biomarkers of cardiac disease in humans (Apple et al., 2001; Walker, 2006) and are often used in conjunction with other hormonal biomarkers for myocardial dysfunction diagnosis (Walker, 2006). Both CK and LDH enzymes are released upon cellular degeneration such as necrosis. Lactate dehydrogenase (LDH) is involved in the interconversion of pyruvate and L–lactate during the final reactions of glycolysis and is present in the cytoplasm of all cells (nucleated and non–nucleated cells) (Kemp et al., 2004). Creatine kinase (CK), on the other hand, is predominantly found in the myocyte cytoplasm and in close association with sarcoplasmic reticulum, mitochondria and myofibrils with a half life of about 12 h in humans (Walker, 2006). The creatine kinase levels in blood plasma rise from 4–6 h peaking at 12–36 h and sustained over 3–4 days in humans where cardiac injury has occurred (Kemp et al., 2004). However, raised LDH plasma values are observed from 8–12 h, peaking within 2–3 days and levels are sustained for duration of 7–10 days following cardiac injury (Kemp et al., 2004). In mammals, CK concentrations have been related to the irreversible injury such as myocardial necrosis (Ishikawa et al., 1997). Several piscine studies have been suggested the clinical relevance of blood biochemistry particularly enzymes including CK and LDH

(Congleton and Wagner, 2006; Firat et al., 2011; Rajabipour et al., 2009; Anderson et al., 2010, Powell, 2006). The elevated CK levels have been reported in association with myocytic necrosis seen in pancreas disease (PD) in Atlantic salmon (Rodger et al., 1991; Ferguson et al., 1986) and these results suggested the clinical importance of CK and LDH in cardiac diseases of Atlantic salmon.

1.3 Cardiac conduction system in teleosts

Teleosts such as Atlantic salmon lack a well–formed cardiac conduction system as compared to homeothermic vertebrates. The well–formed cardiac conduction system has been suggested to have evolved later in the homeothermic animals (Solc, 2007). The homeotherm cardiac conduction system is a series of specialized tissues in the heart, consists of three main parts, the sino–atrial node (SA node or 'pacemaker') which generates the pacemaker impulse; the atrio–ventricular node (AV node) which separates the atrial and ventricular contractions by delaying the impulse and allows the atrium to contract first and then the ventricle to ensure blood flow; and the His–Purkinje system (absent in fish) which rapidly conduct the action potential to the ventricle (Boyett, 2009; Mikawa and Hurtado, 2007). The subendocardial ventricular trabaculae are suggested to serve as functional equivalent of the His–Purkinje system in fish (Sedmera et al., 2003). The cardiac conduction system is responsible for the initiation and co–ordination of the heart beat (Boyett, 2009). The action–potential starts in the autonomous pacemaker cells at sino–atrial (SA) junction and propagates impulses to other parts of the heart. The action potential may define as the contraction of each cardiomyocyte by an electric excitation of the cell membrane in the form of a small voltage change (~0.1 V) (Vornanen, 2011). It is suggested that every muscle in the SA area has at least one nerve profile (contact) that confirms the functional role of SA area

as the initiation and controlling of the heart beat (Yamauchi and Burnstock, 1968). Teleost pacemaker is composed of intracardiac postganglionic nerve cell bodies (ganglion cells), specialized or spindle shaped cardiomyocytes and a network of nerve fibers (Laurent et al., 1983; Farrell and Jones, 1992; Vornanen et al., 2002; Boyett, 2009; Zaccone et al., 2011). Electrophysiological studies have identified the specific conduction system–like cells mainly at the junction of the SA area, but also have been reported at the atrio–ventricular (AV) funnel and atrio–ventricular (AV) junction (Saito, 1969; Sedmera et al., 2003; Zaccone et al., 2011).

However, the pacemaker tissue has been identified at the junction of the atrium and the *sinus venosus* in several fish species including rainbow trout (*Oncorhynchus mykiss*), cod (*Gadus morhua*), eel (*Anguilla japonica*), plaice (*Pleuronectes platessa* L), bream (*Ictalurus nebulosus*) and Pacific hagfish (*Eptatretus stouti*) histologically (Yamauchi and Burnstock, 1968; Saito, 1969; Santer, 1972; Laurent et al., 1983; Lukyanov and Sukhova, 1983; Poppe and Ferguson, 2006; Solc, 2007). Teleost pacemaker has been described as a ring of specialized cardiac tissue (nodal tissue) at the sino–atrial (SA) junction. A connective tissue sheet surrounds and infiltrates the nodal tissue to divide it into semicircular smaller areas. The nerves were identified at the periphery and in the nodal tissue at the sino–atrial (SA) junction. The pacemaker tissue has only been identified in histological sections from time to time and one of the reasons was due to its smaller size (Haverinen and Vornanen, 2007), therefore being less obvious in many histological sections of the heart. The pacemaker tissue appears lightly stained by haematoxylin and eosin (H&E) staining with wider muscle fibers and larger nuclei clearly separated by loose connective tissue from the rest of the myocardial cells (Yamauchi and Burnstock, 1968; Sedmera et al., 2003; Haverinen and Vornanen, 2007; Solc, 2007).

1.4 The heart as an endocrine organ: Natriuretic peptides

Earlier, it was believed that the heart is a fully differentiated organ which serves only as pump and plays a crucial role in the provision of the circulatory flow of the blood (Takei, 2000). However, de Bold et al. (1981) demonstrated that rat atrial extracts can induce diuresis/natriuresis and hypotension in rats which led to the conclusion of endocrine functions of the heart. The endocrine functions of the heart are mainly served by natriuretic peptides (NPs), a group of hormones synthesized and secreted mainly by the cardiac myocytes. However, the chromaffin cells secreting catecholamines have also been identified in the hearts of hagfish (*Myxine sp.* and *Eptaptreus* sp.) and lampreys (*Petromyzon* sp. and *Lampetra sp.*) as compared to teleosts where chromaffin cells are located within the walls of the posterior cardinal vein (PCV) and in close association with the lymphoid tissue of the kidney. The catecholamines produced by the fish chromaffin cells include adrenaline and noradrenaline (Reid et al., 1998; Perry and Capaldo, 2011). Natriuretic peptides are characterized by 17 amino acid conserved ring structure with N–terminal and C–terminal extensions where later terminal can typically distinguish individual NPs (Fig. 3) (Johnson and Olson, 2008; Inoue et al., 2005). The teleosts NP family members include: (1) atrial NP (ANP), (2) B–type NP (BNP), (3) C–type NP (CNP 1, 2, 3 and 4) and (4) ventricular NP (VNP) (Fig. 3) (Inoue et al., 2005, Takei, 2000; Takei et al., 1994). Salmon cardiac peptide (sCP), which is structurally and functionally similar to mammalian atrial natriuretic peptide (ANP), has been identified in the atrium and ventricle of Atlantic salmon (Tervonen et al., 1998; Arjamaa et al., 2000; Vierimaa et al., 2006). VNP is a unique peptide with a long C–terminal and has been identified so far in the hearts of eel, sturgeon and rainbow trout whereas the VNP gene sequence is highly conserved in these three fish species (Takei, 2000; Inoue et al., 2005).

Natriuretic peptides mediate their biological functions with the help of four natriuretic peptide receptors (NPR). Two receptors such as NPR–A and NPR–B contain membrane–bound guanylyl cyclase (GCs) domains and collectively termed NPR–GC receptors (Toop and Donald, 2004; Takei, 2000). The first NPR–A is suggested as ANP (sCP in Atlantic salmon) and BNP receptor while the NPR–B has a high specificity for CNP.

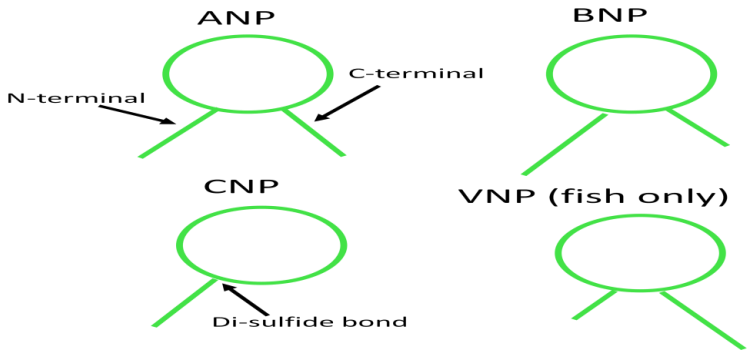

Figure 3. The schematic diagrams showing the basic structure of all piscine members of natriuretic peptides. The 17–amino acid ring structure is conserved among all members. VNP is only identified in teleost fishes.

However, NPR–C and NPR–D lack GC domains and NPR–C has equal affinity to all four NPs which qualifies it as a clearance receptor whereas the NPR–D is a homodimer, structurally similar to the NPR–C and only found in fishes (Toop and Donald, 2004; Loretz and Pollina, 2000; Takei, 2000). VNP has vasoactive and renal effects owing to its high affinity to both NPR–A and NPR–B receptors (Katafuchi et al., 1994; Takei, 2000). Natriuretic peptides play important cardioprotective, endocrine and fluid homeostatic roles (Nishikimi et al., 2006). Atrial natriuretic peptide (ANP) is mainly released by atrial stretch and acts on several organs including the brain, heart, gills, intestine, kidney and interrenal tissue to perform different functions (Fig. 4) (Loretz and Pollina, 2000; Toop and Donald, 2004; Johnson and Olson, 2008; Takei, 2000). Recently,

Johnson and Olson (2009) suggested cardioprotective role of natriuretic peptides in salmonid (rainbow trout).

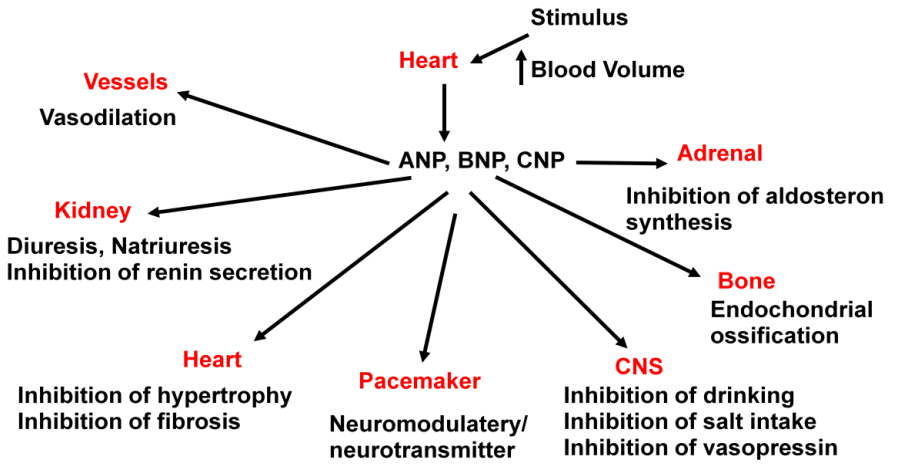

Figure 4. Schematic representation of natriuretic peptides functions in different body systems.

Several piscine studies have suggested the additional neuromodulatory and/or neurotransmitter roles of NPs in addition to cardioprotective/osmoregulatory functions (Donald and Evans, 1992; Donald et al., 1992; Vallarino et al., 1996; Tsukada et al., 2007). Besides cardiomyocytes, natriuretic peptides (ANP) have been identified from the mammalian cardiac conduction system in addition to cardiomyocytes (Hansson, 2002). The ANP has been identified in the intracardial ganglion cells and nerve fiber varicosities in the bovine conduction system (Hansson and Forsgren, 1993, 1995). These findings were further demonstrated in human, cow, sheep, pig and rat (Hansson et al., 1997, 1998). Mammalian nerve fiber varicosities have been reported to contain ANP as demonstrated by immunohistochemistry in the proximity of conduction cells of the AV node and the AV bundle and occasionally in the SA

node, bundle branches, septomarginal trabaculae and false tendons (Hansson and Forsgren, 1993, 1994; Hansson et al., 1998). The mammalian studies have been suggested ANP to act in an autocrine/paracrine fashion on closely found conduction cells to influence the pacemaker velocity (Hansson et al., 1998; Hansson, 2002). ANP has been suggested to be synthesized in the mammalian cardiac conduction system and increased ANP expression has been associated with heart diseases or cardiac sympathectomy in humans (Mochizuki et al., 1991; Hansson et al., 1998).

Elevated plasma levels of NPs in humans are associated with the cardiac diseases which gives yet another role to NPs to serve as biomarkers to predict heart disease (Braunwald, 2008; Minamino et al., 2006). ANP and BNP levels have been used as biomarkers in pathological conditions such as heart failure, acute myocardial infarction (AMI), hypertension, left–ventricular hypertrophy, coronary artery disease, pulmonary hypertension and renal failure (Minamino et al., 2006). Commercial assays are available to measure ANP and BNP in blood plasma and recombinant BNP (nesiritide) is used to treat heart failure (Woodard and Rosado, 2007).

2.1 Immune system

The primary goal of the immune system is to recognize self from non–self and is an inherited prerequisite for the survival of living organisms (Belosevic et al., 2009). The immune system mainly serves to remove invading microorganisms that may cause damage (disease) to the body by the interactions of cells and soluble molecules. In addition, the immune system is involved in the eradication of body's own stressed and damaged cells and protects against tumours (Davies, 2008; Workenhe et al., 2010). It also helps in

maintaining homeostasis during growth and development of the body and following inflammatory reaction or tissue damage (Magnadottir, 2010).

Generally, the immune system is categorized as innate (non–specific) immune system and adaptive or acquired (specific) immune system. Both innate and adaptive systems are mutually dependent on each other and work together. Teleost fishes are the earliest vertebrate class which possess both innate and adaptive immune responses (possession of lymphocytes, major histocompatibility complex (MHC) molecules, immunoglobulin (Ig) and T cell receptor (TCR)) and serve as the evolutionary bridge between the mammals and invertebrates (Flajnik, 1996; Whyte, 2007). Atlantic salmon, as with other teleosts, exhibit both innate and adaptive immune responses (Koppang et al., 2007; Nam et al., 2003; Liu et al., 2008; Moore et al., 2005). As compared to mammals, teleosts lack important lymphoid organs such as lymph nodes, bone marrow and Peyer's patches. However, the anterior or head kidney serves as the major lymphoid organ in teleosts in addition to the thymus, spleen, mucosa/gut–associated lymphoid tissues, and novel intrabranchial lymphoid tissue in salmonid (Fletcher and Secombes, 2010; Koppang et al., 2007, 2010; Press and Evensen, 1999).

2.1.1 Innate immune system

It is well established that the innate immune response is the first line of defence present in all multicellular animals against pathogens/foreign materials until the adaptive immune response is potent and able to take over (Watts et al., 2001; Whyte, 2007). The non–specific immune response gives versatility and is particularly important for fishes due to its temperature independence and slow nature of teleost specific immune response (Watts et al., 2001). Innate immunity does not require prior exposure of the particular pathogen and is

served by a variety of germline–encoded, pattern recognition proteins or receptors (PRP/R). The PRP/R recognize and bind conserved pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysacharides (LPS), peptidoglycans, fungal β 1,3–glucan, mannen–binding lectin (MBL), c–reactive protein (CRP), mannose receptor and double stranded viral RNA and bacterial DNA (Magnadottir, 2006, 2010; Whyte, 2007). The piscine innate immune system detects and reacts quickly against a broad spectrum of pathogens. Toll–like receptors (TLRs) are the transmembrane conserved proteins and important PRPs, and recognize PAMPs from fungi, protozoa, bacteria and viruses, and form an innate immune response. The TLRs have been identified in several fish species (Hynes et al., 2011; Magnadottir, 2010).

Innate immunity may be categorized into three main components such as the physical barriers, the humoral parameters and the cellular components. The physical barriers of fish include mucosal and epithelial surfaces of the gills, alimentary tract and skin with the mucous layer covering the scales which are in direct contact with potentially harmful agents. Antimicrobial peptides, complement factors, lysozome, interferon, c–reactive protein, lectin (haemagglutinin), haemolysin, transferrin and immunoglobulins have been identified from the fish skin mucus which serve as the chemical barriers of the fish. Upon activation of the immune system, both humoral and cellular components are expressed or up–regulated but without memory (Magnadottir, 2006, 2010; Saurabh and Sahoo, 2008).

The humoral parameters comprise special proteins such as complement system and are well developed in fishes. It triggers inflammatory or cytokine response that controls the cellular components (Dixon and Stet, 2001; Magnadottir, 2010). The humoral parameters are expressed either as cell receptors or soluble molecules in the plasma and other body fluids

(Magnadottir, 2010). The humoral components include the well–developed complement system in fishes which consists of the alternative, lectin and classical pathways. All three pathways combine to form the lytic pathway that is well–developed in teleosts and leads to the opsonisation or directly killing of pathogens. All three pathways generate factor C3 which is the central component of the complement system and has been identified in teleosts (Nakao et al., 2003; Boshra et al., 2006). The activation of C3 results in enhanced phagocytosis through opsonisation, immune cells recruitment and promotion of inflammatory response, stimulation of B cell proliferation and activation of membrane attack complex (MAC) (Whyte, 2007). The initial response of the innate immune system includes the actions of neutrophils, macrophages, mast cells, dendritic cells, and natural killer cells. These cells are stimulated by PAMPs linking through receptors or pathogen uptake and in turn participate in the eradication of the pathogens and transmit signals that amplify adaptive immune system (Eltzschig and Carmeliet, 2011; Magnadottir, 2010).

The cellular innate immunity of fish primarily comprises phagocytic cells such as granulocytes (probably neutrophils in fish), monocytes/macrophages, and non–specific cytotoxic such as natural killer cells (tumour attacking non–specific cells in fish) which act on virus infected cells. The increased phagocytosis and neutralization of invading microorganisms are the result of the opsonisation of pathogens. The uptake of pathogens or linking with PAMPs through receptors initiates respiratory or oxidative burst activity (Magnadottir, 2010; Whyte, 2007).

Cytokines consist of the special proteins secreted by the immune cells and are key regulators of the immune system (Tizard, 2004, Secombes et al., 2009). TNFα, as part of innate immune response is the first cytokine released in response to different stimuli including bacteria, virus, parasitic infections,

trauma, and ischemia/reperfusion (I/R). TNFα is released from macrophages, lymphoid cells, mast cells, endothelial cells, fibroblasts and this leads to the downstream expression of chemokines (Kelinbongard et al., 2011; Secombes et al., 2009).

2.2.2 Adaptive immune system

Adaptive immunity may define as the specific host resistance to each individual pathogen or microbial agent with the ability of memory to a prior pathogen exposure that results in an increased response upon repeated exposure (Stevens, 2010). The specific response is achieved by highly specific receptors, either cell–bound (T or few B lymphocytes) or secreted (antibody production by B lymphocytes) for certain regions (epitopes) of the pathogens (Burmester and Pezzutto, 2003). The antigen must be processed inside the cells and the antigen fragments bound to these specific antigen presenting receptors termed the major histocompatibility complex (MHC) molecules (Tizard, 2004). As in mammals, adaptive immunity comprises B and T lymphocytes and antigen presenting cells (macrophages and dendritic cells) in teleosts (Ganassin and Bolc, 1996). Adaptive or acquired immunity composes of the recognition of cell surface MHC–peptide complex by T lymphocytes. This system helps to protect the host from infections and represented mainly by helper T (Th) and cytotoxic T (Tc) lymphocytes defined by the expression of specific CD4 and CD8 markers respectively. The term CD stands for 'cluster of differentiation' and it is referred to define cell–surface molecules on leucocytes that are recognized by a given set of monoclonal antibodies. There are several CDs identified with each given a number (for example: CD3, CD4, CD8 etc.) and each CD may involve in one or more functions (Janeway et al., 2001). These molecules serve as co–receptors and interact specifically with either major histocompatibility class II or I to

determine the discrete stage of T cell development within the thymus. Cytotoxic T lymphocytes bind to MHC class I that presents endogenous antigen via the T cell receptor (TCR) and the CD8 co–receptor, and kills infected cells. MHC molecules interact with either CD4/TCR/CD3 or CD8/TCR/CD3 complexes on antigen presenting cells where CD3 serves as an important trigger of T cell activation (Fig. 5) (Wang et al., 2009; Sun et al., 1995; Salter et al., 1989).

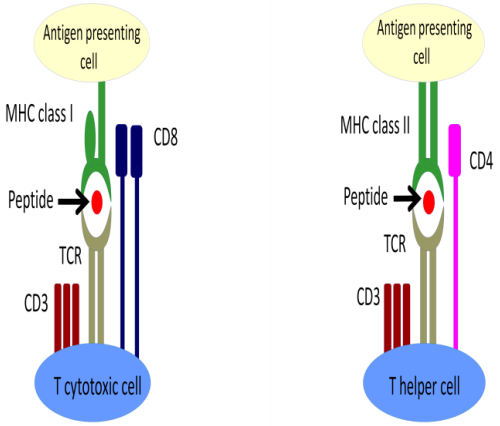

Figure 5. Schematic diagram of TCR–MHC class I–CD8 (left) and TCR–MHC class II–CD4 complex (right). TCR: t cell receptor.

The presence of both cytotoxic and helper T cells in teleosts have been suggested by functional studies and supported by the expression of T cell receptors (TCR), CD8 and CD4 genes in teleosts. The TCR exhibits either α/β or γ/δ isotypes in jawed fishes non–covalently bound to CD3 molecules (Koppang et al., 2007; Nam et al., 2003). CD3 molecules play an important role in signal transduction in the TCR complex and are considered general marker for T lymphocytes (Wang et al., 2009; Koppang et al., 2010). Recently three subunits; CD3ζ, CD3γδ (forerunner of CD3γ and CD3δ in mammals) and CD3ε were cloned

and sequenced in Atlantic salmon (Liu et al., 2008). Besides its role as a T cell marker, the CD3 antigen has also been identified in gastric parietal cells, renal tubular epithelial cells and cerebellar Purkinje cells in several species including humans (Garson et al., 1982; Alroy et al., 2005). Anti–CD3ε antibody has been prepared and validated as a pan T cell marker in various Atlantic salmon tissues including the heart (Koppang et al., 2010), but the additional roles of CD3 antibody, besides that of T cell marker, are still undetermined in teleosts.

2.3 Mechanisms of cardiac cell death in fishes

The term apoptosis was coined by Kerr et al. (1972) to describe a specific morphological pattern of cell death during embryonic development, normal cell turn over in healthy adult tissue and atrophy upon hormone withdrawal. Apoptosis or programmed cell death is an important process to remove damaged or unnecessary cells ensuring normal development of multicellular animals. Caspases (cysteine–dependent aspartate protease) are capable of mediating immune responses (apoptosis, necrosis and inflammation) and are key players in apoptosis (Takle and Andersen, 2007). Caspases comprise pro– and catalytic domains and are synthesized as inactive precursor molecules. A large (p20) and a small (p10) subunit form catalytic domain which assemble into an enzymatically active heterotetrameric complex (p20 + p10) upon proteolytic cleavage of proenzyme (Fig. 6). At least 15 caspases have been identified in mammals. While many caspases have been identified in Atlantic salmon, there are two main subcategories; effectors and initiators that mediate apoptosis by either extrinsic or intrinsic pathways.

Caspase 3 belongs to the effectors group and can be initiated by both extrinsic and intrinsic pathways (Fischer et al., 2005; Gutter, 2000; Takle and Andersen, 2007). The extrinsic pathway is initiated upon binding of specific

cytokine ligands such as FasL and tumor necrosis factor (TNF)–related apoptosis–inducing ligand to the transmembrane death receptors (Fas, DR4 and DR5) initiate the extrinsic pathway (Takle and Andersen, 2007).

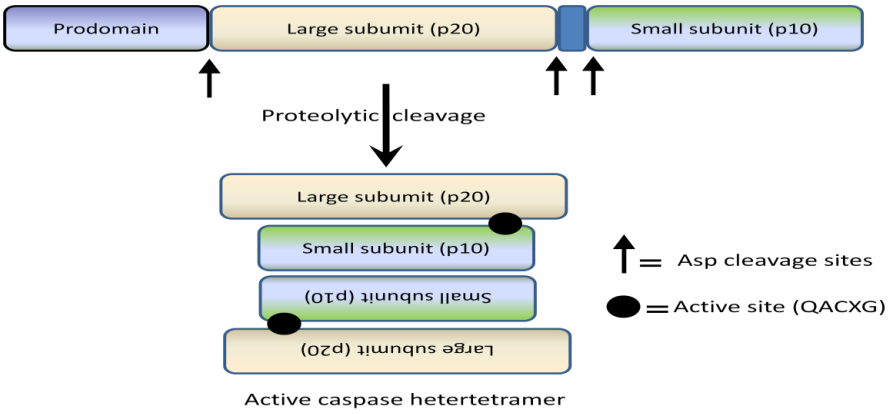

Figure 6. Schematic diagram showing the proteolytic activation of caspases. Caspases are synthesized as single chain inactive precursors consisting of a prodomain and a catalytic domain (large and small subunits). For activation, inactive precursor cleaves at aspartate residue (Asp; arrow) into a larger and a smaller subunit that finally re–associate to form a complex comprising of 2 large and 2 small subunits. Adapted from Takle and Andersen, 2007.

The orthologs of human death receptor ligands have been identified in fishes including TNF (*tnfa and tnfb*), CD95/FasL (*faslg*), Apo2/TRAIL (tnfsf101*, tnfsf102, tnfsf103 and tnfsf104*), and APP (*appa*) by phylogenetic analysis (Eimon and Ashkenazi, 2010). In addition, death receptors are also well conserved in humans and fishes, and orthologs of TNFR1 (*tnfsf1a*), CD95/Fas (fas), and DR6 (*tnfrsf21*) have also been identified in fishes (Eimon and Ashkenazi, 2010). The activation of transmembrane death receptors mediate downstream activity through recruitment of adaptor proteins such as FADD which leads to death inducing signaling complex (DISC) recruitment and drives pro–apoptotic signaling through procaspases–8 and –10. Upon activation, initiator caspases (caspase 8 and 10) activate second group of caspases, known

as effector caspases (caspase–3a, –3b, –6 and –7). Additionally, initiator caspases can also activate intrinsic mitochondrion mediated pathway by activating pro–apoptotic protein Bid to its active form tBid. After cleavage, tBid either activates Bax or enters itself in the mitochondrial membrane and triggers release of various apoptosis–inducing factors. Caspase 3 is suggested as key protease that promotes cleavage of cytoskeletal and nuclear proteins, resulting in apoptosis (Eimon and Ashkenazi, 2010; Takle and Andersen, 2007; Zhao and Vinten–Johansen, 2002).

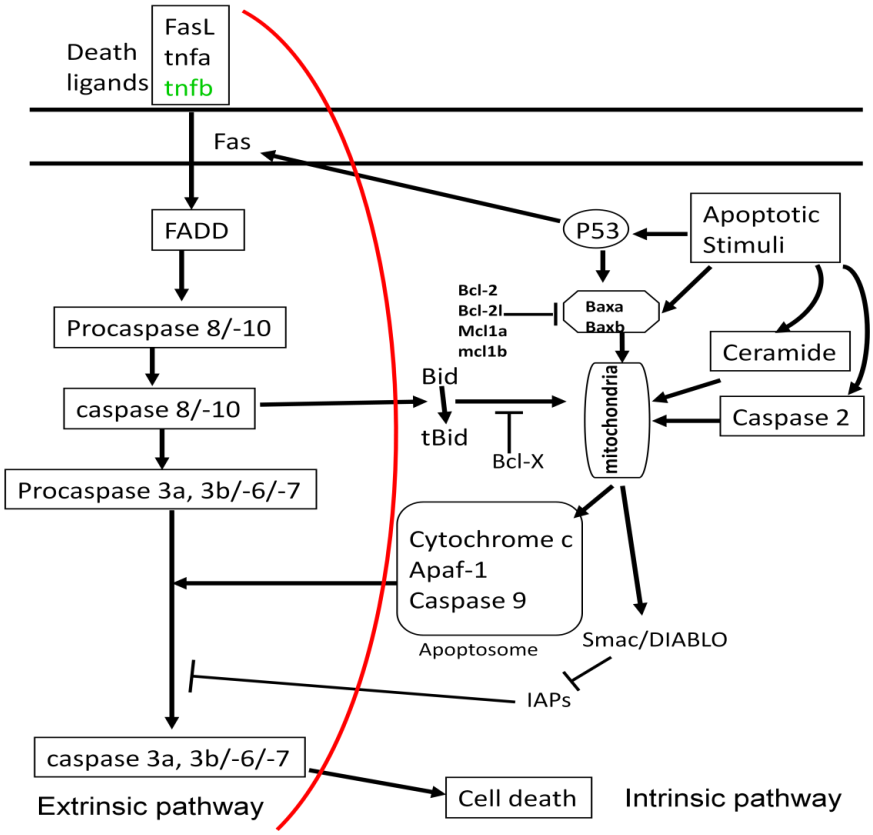

Figure 7. Schematic diagram showing the extrinsic (left) and intrinsic (right) apoptotic pathways. Both pathways converge at the activation of effector caspases (caspase–3a, –3b, –6 and –7). Green color (tnfb) interaction is based on vertebrates and hypothesized in fish. See text for details. Modified from Takle and Andersen, 2007.

Pro–apoptotic Bcl2 family members identified in fish are baxa and baxb while anti–apoptotic members include bcl2, bcl2l, mcl1a, mcl1b (Eimon and Ashkenazi, 2010). The intrinsic pathway is initiated by stressors such as ultraviolet radiation, heat shock, growth factor withdrawal and DNA damage. Upon stimulation, pro–apoptotic Bcl–2 family members induce the permeablizaion of outer mitochondrial membrane and trigger release of pro–apoptotic factors. The important pro–apoptotic factors include Smac/DIABLO, the serine protease Omi/HtrA2, and cytochrome *c* which binds the Adaptor Apaf–1 and the initiator caspase 9 to form a complex called as apoptosome. The mitochondrial factors such as Smac/ DIABLO and the serine protease Omi/HtrA2 indirectly promote apoptosis by inhibiting a family of anti–apoptotic proteins known as inhibitors of apoptosis proteins (IAPs). After assembly, apoptosome activates caspase 9 which in turn activates the effector caspases (caspase–3, –6, –7) through proteolytic processing. These activated effector caspases culminate the apoptotic process through the degradation of key intracellular substrates (Eimon and Ashkenazi, 2010; Takle and Andersen, 2007). Cultured cardiomyocytes exposed to hypoxia and re–oxygenation have been shown to release cytochrome *c* resulting in the cleavage of effector caspases 3 and 9 (Kang et al., 2000). In mammals, apoptosis detected in cardiomyopathy has been associated with intrinsic mitochondrial pathway and higher caspase 3 expression observed (Zhao and Vinten–Johansen, 2002). Caspase 2 has been suggested to be involved in both extrinsic and intrinsic pathways (Takle and Andersen, 2007). Ceramide is a hydrolytic product of sphingomyelin and is generated in response to various stresses such as heat shock, oxidative stress, ultraviolet and γ–irradiation (Yabu et al., 2001; Yamashita, 2003). The ceramide is involved in the activation of effector caspases by intrinsic pathway (Takle and Andersen, 2007). The multifunctional tumor suppressor protein P53 determines how cell respond to DNA damage, hypoxia and oncogenic signaling (Vousden

and Prives, 2009). In response to stressors, P53 stabilizes and activates and perform several functions including activating the intrinsic apoptosis pathway (Eimon and Ashkenazi, 2010). Recently, pro–apoptotic genes such as Bax, Bcl–x, p53 and caspase 6 have been shown to be significantly upregulated in the Atlantic salmon exposed to uranium as compared to control fish (Song et al., 2012).

Tumor necrosis factor is a multifunctional pro–inflammatory cytokine mainly produced by macrophages. TNF can induce apoptosis, necrosis, cell proliferation, differentiation and induction of other cytokines. The receptors for TNF are present in almost all nucleated cells, making the basis for a very complex cytokine network (Idriss and Naismith, 2000; Haugland et al., 2007; Beyaert and Fiers, 1994). Fas (also known as APO–1) belongs to the TNFα receptor family and is expressed on a variety of cells including cardiomyocytes in mammals (Braunwald, 2008). Hypoxia inducible factor–1 (HIF1) is a heterodimer basic helix–loop–helix protein that activates the transcription of hypoxia inducible genes and other important genes for erythropoiesis, iron delivery, angiogenesis, vasomotor tone and ATP metabolism (Chun et al., 2002; Wang et al., 1995). It consists of α–subunit (oxygen sensitive) and β–subunit (oxygen independent) and one of the most important molecules involved in hypoxia (reviewed in Dehne and Brune, 2009; Eltzschig and carmeliet, 2011; Semenza et al., 1997; Gale and Maxwell, 2010). Cytokines and especially TNFα are capable of activating HIF1 that has been suggested to be involved in inflammation, apoptosis and influences adaptive immunity (reviewed in Eltzschig and Carmeliet, 2011; Gale and Maxwell, 2010; Dehne and Brune, 2009). Oxidative stress is one of the major factors causing myocardial damage, and HIF1α is suggested to be important in a number of physiological and pathological conditions (Poppe and Ferguson, 2006; Hopfl et al., 2004; Huang et al., 2004). As in mammals, HIF may play similar roles in fishes including

erythropoiesis, hemoglobin synthesis, angiogenesis, changes in gill surface area, glycolysis, glucose transport and growth suppression (Nikinmaa and Rees, 2005). A link between hypoxia and apoptosis has been proposed in fish where change in gill surface area has been shown after 1 week of hypoxia in crucian carp (*Carassius carassius*) due to possible apoptosis (Solid et al., 2003). The over expression of heart targeted pro–apoptotic protein *Nip3a* resulted in cardiocytes apoptosis which led to abnormal heart development and cardiac dysfunction in zebrafish embryos (Wang et al., 2006). Recently, a link between atrial natriuretic peptide (ANP) and apoptosis in the heart has been proposed where increased ANP expression after being stimulated by heat shock (fish) or hypoxia (mammals) activates caspase–6 or –3 and it may lead to abnormal or deviant heart development (Chen et al., 1997; Takle and Andersen, 2007).

In mammals, two general mechanisms such as apoptosis and necrosis are responsible for cell death during myocardial ischemia and reperfusion, and both mechanisms determine the final degree of lethal myocardial injury (Zhao and Vinten–Johansen, 2002). Since apoptosis is a programmed cell death and energy demanding, the energy deprivation in cardiomyocytes could inhibit the terminal apoptotic events, but leads to calcium release from the sarcoplasmic reticulum (SR). Calcium changes the permeability of the mitochondrial membrane and could lead to programmed cell necrosis (Dorn II, 2009). Viral fish diseases are among the most destructive diseases of fish aquaculture and induce apoptosis (Silva et al., 2008). Fish rhabdoviruses such as spring viremia of carp virus (SVCV), infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) have all been shown to cause apoptosis (Bjorklund et al., 1997). Additionally, betanodaviruses such as greasy grouper nervous necrosis virus (ggNNV) has shown to induce apoptosis in sea bass cell culture via the extrinsic pathway (Guo et al., 2003). Thus, there is accumulating evidence that fish viruses are causing both caspase dependant and independent

apoptosis (Silva et al., 2008). The infectious pancreatic necrosis virus (IPNV) is the most important virus of birnaviridae that is causing a well characterized acute disease in salmonid and a major threat to aquaculture industry (Silva et al., 2008). IPNV has been shown to induce apoptosis in salmonid and zebrafish cell lines (Hong et al., 1999; 2005). Another salmonid virus such as infectious salmonid anemia virus (ISAV) has been shown to trigger *in vitro* cell specific apoptosis in salmonid cell lines such as SHK–1 and CHSE–214 but not in TO cells and may not involve the activation of caspase 3 (Joseph et al., 2004). *In vitro* studies using fish cell lines are proposing that apoptosis induced by betanodavirus and birnavirus is followed by secondary necrosis and it would explain the occurrence of the necrotic histopathology associated with these diseases in fishes (Chen et al., 2006; Hong et al., 1998) and subsequently supported by further studies (Chen et al., 2010; Chiu et al., 2010; Su et al., 2009).

3. Cardiac diseases

Over the last two decades, increasing numbers of cardiac malformations have been identified in marine farmed Atlantic salmon (Poppe and Taksdal, 2000). Farmed Atlantic salmon (*Salmo salar* L.) are prone to numerous cardiac anomalies such as aplasia or hypoplasia of the *septum transversum*, abnormal location and shape of heart (Poppe and Seirstad, 2003), arteriosclerosis (Poppe et al., 2007; Farrell, 2002), ventricular hypoplasia (Poppe and Taksdal, 2000) and specific viral cardiac diseases including cardiomyopathy syndrome (CMS) (Poppe and Ferguson, 2006; Poppe and Seirstad, 2003; Ferguson et al., 1990; Brun et al., 2003; Grotmol et al., 1997), heart and skeletal muscle inflammation (HSMI) (Kongtorp et al., 2004, 2004a, 2006) and pancreas disease (PD) (Christie et al., 1998; Taksdal et al., 2007; Nelson et al., 1995).

3.1 Viral cardiac diseases of Atlantic salmon

Viral fish diseases are among the most destructive diseases of fish aquaculture (Silva et al., 2008). The occurrence of cardiac diseases has increased in recent years in Atlantic salmon aquaculture in Norway. In Norway, CMS causes economic losses in excess of 66 million NOK annually and a single PD outbreak with 500,000 smolts on a fish farm can cause a total loss of 14.4 million NOK (Brun et al., 2003; Aunsmo et al., 2010). The number of HSMI outbreaks has been increased three–fold from 2004 (54 outbreaks) to 2011 (162 outbreaks) while PD has re–emerged from the beginning of the year 2000 (11 outbreaks) to 2011 (89 outbreaks) and CMS remained at uniform pattern with 53 outbreaks in 2010 in Norway (Bornø et al., 2011, Marta et al., 2012).

3.1.1 Heart and skeletal muscle inflammation (HSMI)

Heart and skeletal muscle inflammation (HSMI) is a cardiac disease of marine farmed Atlantic salmon that mainly affects heart and skeletal muscle. HSMI was first reported from mid–Norway in 1999 and has become an increasing problem for salmon industry (Kongtorp et al., 2004). HSMI is characterized as a disease of low mortality (~20%) but with high morbidity (~100%) that commonly affects fish mostly 5 to 9 months after transfer to sea. HSMI has been reported from Norway, Scotland and Chile (Kongtorp et al., 2004a; Ferguson et al., 2005). Macroscopically pale hearts with loose texture, pericardial hemorrhages, ascities and pale/stained liver are observed without haematocrit changes. Cardiac and red skeletal muscles exhibit the most significant histopathological lesions. Lesions first appear and are more frequent in heart than in the skeletal muscle (Kongtorp et al., 2004, 2004a). Presently, HSMI can be diagnosed by histopathology and differentiated from other similar cardiac diseases such as cardiomyopathy syndrome (CMS) and pancreas disease

(PD) (Table 1) (Kongtorp et al., 2006). The diagnosis of HSMI is based upon histopathological changes and presents as epi–, endo–, and myocarditis as well as a pronounced mononuclear cellular infiltration of both trabecular and compact layers of ventricular myocardium accompanied by myocytic necrosis, myositis and necrosis of red skeletal muscle (Table 1). Affected myocytes show signs of degeneration, loss of cardiomyocyte striation and eosinophilia, loss of skeletal muscle striation, vacuolation, centralized nuclei and karyorhexis. Inflammatory changes are more pronounced as compared to necrotic changes in heart and skeletal muscle (Poppe and Ferguson, 2006; Kongtorp et al., 2004, 2006). HSMI appears to be transmissible in laboratory studies by injecting tissue homogenate from diseased fish to healthy fish (Kongtorp et al., 2004). Recently, a viral etiology was suggested where a novel piscine reovirus (PRV) was associated with HSMI (Palacios et al., 2010). As mammalian reoviruses, PRV is also ubiquitous virus and has been identified using unbiased high throughput DNA sequencing (Clarke and Tylor, 2003; Palacios et al., 2010). However, PRV has not been successfully cultured and low quantities of virus were identified in fish with no clinical signs, although at a much lower level than in diseased fish. Low levels of PRV have also been identified in wild Atlantic salmon (Palacios et al., 2010). PRV RNA has also been found in Atlantic salmon brood fish with no clinical signs. However, the absence of PRV RNA from fertilized eggs has been suggested that vertical transmission is not a major route for PRV transmission (Wiik–Nielsen et al., 2012). The fish surviving from HSMI outbreaks are suggested to be lifelong PRV carriers (Wiik–Nielsen et al., 2012). The viral etiology has been supported by challenge trials where infective tissue homogenate was injected intraperitoneally in fish and HSMI–related lesions were identified (Kongtorp and Taksdal, 2009). The association of PRV and HSMI was strengthened by a recent study where PRV immunohistochemical staining

was performed in the heart using specific antibodies against the PRV capsid proteins (Finstad et al., 2012).

3.1.2 Cardiomyopathy syndrome (CMS)

Cardiomyopathy syndrome (CMS) is a cardiac disease of Atlantic salmon that mainly affects the atrium and trabecular ventricle without involvement of the skeletal muscle. It shares similar features with HSMI where both diseases cause myocarditis and pericarditis and has been proposed as a late stage of HSMI (Table 1) (Amin and Trasti, 1988; Ferguson et al., 1990; Kongtorp et al., 2006; Poppe and Ferguson, 2006). It was first reported in late–1980s in the cultured Atlantic salmon in Norway (Amin and Trasti, 1988; Ferguson et al., 1990) and subsequently reported from Faeroe Islands and Scotland (Bruno and Poppe, 1996; Poppe and Sande, 1994; Rodger and Turnbull, 2000). Later, CMS has been identified in wild Atlantic salmon and Chinook salmon (*Oncorhynchus tschawytscha*) in British Columbia, Canada (Brocklebank and Raverty, 2002; Poppe and Seirstad, 2003).

Amin and Trasti (1988) proposed a viral etiology for CMS due to the presence of intranuclear eosinophilic inclusion bodies in unaffected cardiomyocytes situated adjacent to degenerated myocardium. Recently, the experimental transmission of CMS has been shown in unvaccinated Atlantic salmon smolts using intraperitoneal injection of infected tissue homogenate. CMS is a disease of adult, primarily Atlantic salmon; however, post–smolt fish have been identified with same type and severity of CMS lesions but in limited numbers (Fristvold et al., 2009). CMS has also been successfully transmitted in adult salmon (1.1 kg) using Scottish and Norwegian tissue homogenates, leading to the conclusion that similar disease conditions occur in both countries (Bruno and Noguera, 2009). The above mentioned challenge trials supported the viral

etiology of CMS in Atlantic salmon (Bruno and Noguera, 2009; Fristvold et al., 2009).

Recently, a totivirus has been identified in Atlantic salmon by high throughput DNA sequencing (Løvoll et al., 2010). The virus has been identified from several natural CMS outbreaks and from fish where CMS had been induced through experimental transmission. As compared to piscine reovirus (PRV), totivirus is not ubiquitous and suggested to be more closely associated with disease outbreaks (Løvoll et al., 2010). Totivirus is a naked double–stranded RNA virus and the proposed name is piscine myocarditis virus (PMCV) belonging to family *Totiviridae* (Haugland et al., 2011). PMCV is approx. 50 nm in diameter with genome size of 6,688 bp and consists of three open reading frames (ORFs) (Haugland et al., 2011). PMCV RNA has also been identified in healthy Atlantic salmon brood fish and fertilized eggs. It is suggested that PMCV RNA may be transferred from parental fish to progeny but requires further assessment (Wiik–Nielsen et al., 2012). In addition, a distinct strain of PMCV has also been identified in healthy marine Atlantic argentine (*Argentina silus*) which is a pelagic species and lives at the depth of 600 m (Bockerman et al., 2011; Tengs and Bockerman, 2012). Recently, experimental infection by intraperitoneal injection of PMCV has induced CMS specific lesions in Atlantic salmon and viral loads were correlated with histopathological changes (Timmerhaus et al., 2011). Histopathological findings of CMS include necrosis and inflammation of trabecular myocardium of the ventricle and atrium, epicarditis and a cellular infiltrate including mainly lymphocytes and macrophages (Table 1). Rupture of the atrium or *sinus venosus* has been reported at terminal stages of CMS (Ferguson et al., 1990; Poppe and Ferguson, 2006). CMS may occur in adult salmon 12–18 months after sea transfer (Ferguson et al., 1990). Present study identified protein casts in kidney collecting tubules and melanin deposits in the

CMS–affected hearts (unpublished) and in agreement with Fristvold et al. (2009) who reported melanisation in experimentally induced CMS fish.

3.1.3 Pancreas disease (PD)

Pancreas disease is an economically important disease of marine farmed Atlantic salmon in UK, Ireland, Scotland and Norway (Kongtorp et al., 2010; Herath et al., 2012). In Atlantic salmon, pancreas disease (PD) has been known since 1976 and given its name due to the histopathological changes in pancreas (Munro et al., 1984) in addition to skeletal and cardiac muscle (Ferguson et al., 1986). Pancreas disease (PD) was first reported in Norway in 1989 (Poppe et al., 1989) and an associated virus was first isolated from diseased Atlantic salmon in Ireland (Nelson et al., 1995) and subsequently identified from the west coast of Norway in 1998 (Christie et al., 1998). Salmon pancreas disease virus (SPDV) is the causative agent of pancreas disease in Atlantic salmon (McLoughlin and Graham, 2007) and later identified as an alphavirus (Weston et al., 1999). A condition called 'sleeping disease' (SD) has been identified in fresh water farmed rainbow trout (*Oncorhynchus mykiss*) and exhibits similar histopathology to PD (Boucher and Baudin–Laurencin, 1994, 1996). The sleeping disease virus (SDV) has been identified as causative agent of SD in France which is an atypical alphavirus (Castric et al., 1997; Villoing et al., 2000). Both SPDV and SDV viruses belong to the family *Togaviridea* and genus Alphavirus. A name salmonid alphavirus (SAV) has been proposed for these two closely related subtypes of the same virus species (Weston et al., 2002).

To date, at least six subtypes of SAV exist such as SAV1 (Weston et al., 1999), SAV2 (Villoing et al., 2000), SAV3 (Hodneland et al., 2005), SAV4, 5 and 6 (Fringuelli et al., 2008). Except SAV2 which is the causative agent of SD, all other subtypes (SAV1, 3, 4, 5 and 6) are related to PD (Fringuelli et al., 2008). The

subtypes SAV1, 4 and 6 identified in Ireland, SAV1, 2, 4 and 5 found in Scotland and SAV3 only detected in Norway (Hodneland et al., 2005; Hodneland and Endresen, 2006; Fringuelli et al., 2008). However, the Norwegian veterinary institute (NVI) identified SAV2 from two separate outbreaks in Atlantic salmon in Norway (www.vetinst.no). Recently SAV RNA has also been identified by real–time PCR (qPCR) in wild marine non–salmonid flatfish such as common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*) in Scotland (Snow et al., 2010). For all six SAV subtypes, cohabitant challenge models have been successfully established (Graham et al., 2011) and in turn produced long term immunity in fish (Houghton, 1994; Lopez–Doriga et al., 2001). The virus transmission and shedding have been suggested by both faecal and mucosal routes (Graham et al., 2011). SAV is capable of horizontal transmission (Houghton and Ellis, 1996; McLoughlin et al., 1996) and recently Kongtorp et al. (2010) rejected virus transmission from parents to offspring and suggested a minor role of vertical transmission in the spread of SAV3.

During the last decade, PD has re–emerged and has become a major economical and animal welfare issue for farmed Atlantic salmon in Europe (McLoughlin and Graham, 2007). PD has been reported from different regions of Europe including Ireland, Scotland, UK, Spain, Italy and Norway (Ferguson et al., 1990; Christie et al., 1998; Crockford et al., 1999; Graham et al., 2007; McVicar, 1987; Poppe et al., 1989; Rowly et al., 1998; Taksdal et al., 2007). PD has also been reported from USA but without virus isolation and genetic data (Kent and Elston, 1987). Pancreas disease–related mortality ranges from 1 to 42 % in natural outbreaks and an individual outbreak may last for 3–4 months (McLoughlin et al., 2002; Christie et al., 2007). The PD affected fish show inappetence, lethargy, yellow faecal casts as well as an increased mortality. The acute disease phase lasts up until 10 days at 2–14°C with lesions in heart and

pancreas as dominating features. The acute phase is followed by a sub–acute phase 10–21 days after the onset of clinical signs with lesions in pancreas, heart and skeletal muscles, and a chronic phase after 21–42 days with lesions in muscles as dominating feature, and then subsequently a recovery phase (McLoughlin et al., 2002, McLoughlin and Graham, 2007). The important pathological changes involve severe losses of exocrine pancreas, cardiac and skeletal myopathies, epicarditis, focal gliosis of brain stem, white skeletal muscle degeneration and functionally unknown cells in kidney with cytoplasmic eosinophilic granules (Table 1) (Taksdal et al., 2007; Christie et al., 2007).

Table 1. Main tissues affected and histopathological lesions appeared in fish affected with cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD). (compiled from Kongtorp et al. 2004).

Tissue	Lesions description	CMS	HSMI	PD
Heart	Epicarditis	+	+	+
	Compact–myocarditis and degeneration	–	+	+
	Spongy–myocarditis and degeneration	+	+	+
Skeletal muscle	Inflammation and degeneration	–	+	+
Liver	Necrosis of hepatocytes	–	–	+
Pancreas	Necrosis of exocrine tissue.	–	–	+

4. Aims of the study

Viral fish diseases are a serious threat to fish farming industry and presently the number of cardiac diseases outbreaks have increased significantly in Atlantic salmon in Norway compared to the last two decades. In the present study, all of the work was focused on the Atlantic salmon heart.

At the start of the study, there was no peer reviewed data relating to the location and morphology of the cardiac conduction system of Atlantic salmon. The first part of the study focused on the morphology and localization of the cardiac pacemaker tissue of Atlantic salmon due to its functional significance in heart beat and the fact that most cardiac diseases also affect the cardiac pacemaker in humans. Natriuretic peptide and CD3 have been suggesting additional roles in cardiac pacemaker in mammals. Thus, to identify these roles in teleost (Atlantic salmon), immunoexpression of natriuretic peptides (sCP and VNP) and CD3 were also demonstrated in the cardiac pacemaker of Atlantic salmon.

Due to lack of tools for non–lethal diagnosis, the CMS– and HSMI–affected fish were only diagnosed by the increased mortality levels at farm. Second part of the study included the identification of potential biomarkers and associated them with the cardiac pathology of Atlantic salmon affected with cardiac diseases.

Additionally, little was known about the cardiac pathological responses of Atlantic salmon affected with specific viral cardiac diseases such as CMS, PD and the more recently identified HSMI due to lack of specific markers. To address these issues, specific antibodies were used as markers and the third part of this work focused on characterizing and comparing the cardiac pathological changes

of Atlantic salmon affected with similar cardiac diseases such as HSMI, CMS and PD.

The specific aims of the present study could be summarized as follows:

(1) To characterize the localization and morphology of the cardiac pacemaker tissue of Atlantic salmon.

(2) To identify the additional roles of natriuretic peptides and CD3 in the cardiac pacemaker tissue of Atlantic salmon.

(3) To identify the potential biomarkers of cardiac diseases of Atlantic salmon.

(4) To characterize and compare the specific markers of cardiac pathological changes of Atlantic salmon affected with similar cardiac diseases such as HSMI, CMS and PD.

5. Summary of papers

Paper I: This study describes the location of the primary pacemaker at the sino–atrial (SA) junction and the localization of salmon cardiac peptide (sCP) and ventricular natriuretic peptide (VNP) in Atlantic salmon (*Salmo salar* L.). The pacemaker tissue appeared lightly stained and composed of: (1) wavy nerve bundles with oval elongated wavy appearing nuclei with pointed ends, (2) ganglion cells (12–22 μm) with granular cytoplasm and (3) wide muscle fibers with large nuclei (modified cardiomyocytes) clearly distinguishing them from the other myocardial cells. Pacemaker tissue was further evaluated using immunohistochemical staining. Immunoreactivity of natriuretic peptides (sCP and VNP) antisera showed specific staining in pacemaker ganglion cells in addition to the cardiomyocytes. Positive staining with anti–CD3ε antisera in the pacemaker ganglion cells is a novel finding in teleosts and is consistent with observations in mammals. In conclusion, the Atlantic salmon pacemaker was shown to be located at the SA node and to harbor sCP and VNP peptides, suggesting a possible neuromodulatory and/or neurotransmitter role for these cardiac hormones within the teleost heart.

Paper II: Heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) are putative viral cardiac diseases of Atlantic salmon. This study examined the levels and correlated the serum enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) to the histopathology of clinical outbreaks of HSMI and chronic CMS in farmed Atlantic salmon. A total of 75 fish from 3 different HSMI outbreaks, 30 chronic CMS fish and 68 fish from 3 non–diseased fish groups were used as the study population (N = 173). Serum CK and LDH levels correlated significantly with the total inflammation and total necrosis scores for HSMI fish (P = 0.001). However, no correlation was identified for

enzyme levels and histopathology scores for chronic CMS fish. The significantly increased CK and LDH levels and their positive correlations to histopathology differentiate HSMI from CMS clinically suggesting the potential use of enzymes for screening for HSMI is promising.

Paper III: Heart and skeletal muscle inflammation (HSMI) is a disease of marine farmed Atlantic salmon where the pathological changes associated with the disease involve necrosis and an infiltration of inflammatory cells into different regions of the heart and skeletal muscle. The aim of this work was to characterize cardiac changes and inflammatory cell types associated with a clinical HSMI outbreak in Atlantic salmon using immunohistochemistry. Different immune cells and cardiac tissue responses associated with the disease were identified using different markers. The spectrum of inflammatory cells associated with the cardiac pathology consisted of mainly $CD3\varepsilon^+$ T lymphocytes, moderate numbers of macrophages and eosinophilic granulocytes. Proliferative cell nuclear antigen (PCNA) immuno–reaction identified significantly increased nuclear and cytoplasmic staining as well as identifying hypertrophic nuclei. Strong immunostaining was observed for major histocompatibility complex (MHC) class II in HSMI hearts. Although low in number, a few positive cells in diseased hearts were detected using the mature myeloid cell line granulocytes/monocytes antibody indicating more positive cells in diseased than non–diseased hearts. The recombinant tumor necrosis factor–α (TNFα) antibody identified stained macrophage–like cells and endothelial cells around lesions in addition to eosinophilic granular cells (EGCs). These findings suggested that the inflammatory response in diseased hearts comprised mostly $CD3^+$ T lymphocytes and eosinophilic granular cells and hearts exhibited high cell turn over where DNA damage/repair might be the case (as identified by PCNA,

caspase 3 and terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) reactivity).

Paper IV: Heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD) are diseases of marine farmed Atlantic salmon (*Salmo salar*) which commonly affect the heart in addition to the skeletal muscle, liver and pancreas. The main findings of these diseases are necrosis and inflammatory cells infiltrates affecting different regions of the heart. In order to better characterize the cardiac pathology, study of the inflammatory cell characteristics and cell cycle protein expression was undertaken by immunohistochemistry. Immunohistochemistry was performed on paraffin embedded hearts from confirmed diseased cases applying specific antibodies. The inflammatory cells were predominantly $CD3\varepsilon^{+}$ T lymphocytes while few eosinophilic granulocytes were identified. The PD diseased hearts exhibited moderate hypoxia inducible factor–1α (HIF1α) immuno–reaction that suggested tissue hypoxia while recombinant tumor necrosis factor–α (rTNFα) antibody identified putative macrophages and eosinophilic granular cells (EGCs) in addition to endocardial cells around lesions. There were strong to low levels of major histocompatibility complex (MHC) class II immunostaining in the diseased hearts associated with macrophage–like and lymphocyte–like cells. The diseased hearts expressed strong to low levels of apoptotic cells identified by caspase 3 and terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) staining. The strong signals for proliferative cell nuclear antigen (PCNA) and TUNEL, and moderate levels of caspase 3 immuno–reactivity suggested a high cell turnover where DNA damage/repair might be occurring in the diseased hearts.

6. General discussion

6.1 Cardiac conduction system of Atlantic salmon

The aims of the first study were the localization and detailed morphological description of the cardiac conduction system of Atlantic salmon (*Salmo salar* L.). At the start of the study, there was no conclusive data about the localization and morphology of the cardiac conduction system in Atlantic salmon. The morphological study of the pacemaker tissue was important owing to its functional significance (action–potential). The action–potential initiates in the autonomous pacemaker cells at sino–atrial (SA) junction and propagates impulses to other parts of the heart during contraction in fish (Vornanen et al., 2002; Vornanen, 2011) and suggests the importance of pacemaker tissue of Atlantic salmon. In humans, most cardiac diseases also affect the cardiac conduction system and pathological changes in the conduction system have been observed in several mammalian diseases including myotonic dystrophy, sudden coronary death, coronary atherosclerosis, cardioneuropathy, West Nile virus infection, Parkinson's disease, chagas disease and Aujeszky's disease (Cramer et al., 2011; Fujishiro et al., 2008; Orimo et al., 2007; James, 1983, 1985; Ottaviani et al., 2003; Rassi Jr et al., 2009; Rossi, 1982; Nguyen et al., 1988; Opeskin et al., 2000). Additionally, cardiac arrhythmias have been reported without specialized pacemaker cells in fishes (Farrell and Jones, 1992). These data highlight the importance of localization and detailed morphological study of cardiac pacemaker of Atlantic salmon.

In fish, the identification and localization of the pacemaker tissue was considered ambiguous due to the relatively small size of the heart and the pacemaker center (Haverinen and Vornanen, 2007). Haverinen and Vornanen (2007) identified the pacemaker at the sino–atrial (SA) junction in rainbow trout

(*Oncorhynchus mykiss*) but the study focused mainly on the electrophysiology of the pacemaker without investigating the tissue morphology of the pacemaker. To ensure the presence of the pacemaker tissue in the samples, the heart must be carefully removed from the pericardial cavity, ensuring the inclusion of the *sinus venosus* and atrium in the samples (Paper I). The primary pacemaker tissue was identified as discrete bundles at the sino–atrial (SA) junction (Paper I) and in agreement with other salmonids (rainbow trout and brown trout) and several other fish species (Yamauchi and Burnstock, 1968; Lukyanov and Sukhova, 1983; Haverinen and Vornanen, 2007; Zaccone et al., 2009a, 2009). Haverinen and Vornanen (2007) have shown the circular distribution of pacemaker in rainbow trout and supports our observation of circular distribution of pacemaker tissue at the SA node in Atlantic salmon (Figure 8A and B).

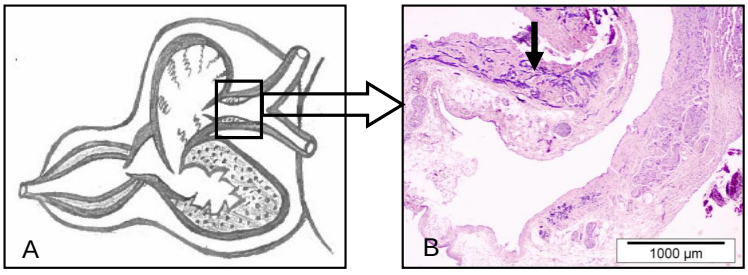

Figure 8. A, A schematic diagram of Atlantic salmon heart and pacemaker area (within the box) was shown on both sides forming a ring at SA node. B, Low power view of sino–atrial junction showing a circular distribution of nodal tissue (pacemaker). Arrow: elastic tissue.

The loose connective tissue separated the neural tissue from surrounding cardiomyocytes and infiltrated into the nodal tissue, dividing it in several small semicircular areas (Paper I) and in agreement with other salmonid (Haverinen and Vornanen, 2007). The highly innervated modified myocardiocytes were identified in the atrial region close to the junction of the *sinus venosus* and the atrium (Paper I) and other piscine studies have suggested that every muscle in

the SA area has at least one nerve profile (contact) (Yamauchi and Burnstock, 1968; Zaccone et al., 2011). The subendocardial pacemaker tissue was composed of three cell types: (1) plexiform modified myocardiocytes that appeared lightly stained than the rest of atrial myocardiocytes; (2) wavy appearing nerve bundles with oval, wavy, elongated nuclei with pointed ends; (3) large round to pear–shaped postganglionic nerve cell bodies (ganglion cells) with granular cytoplasm (Nissle bodies) and prominent nucleoli in the nuclei. The size of ganglion cells was in range of 12–22 µm. The pacemaker tissue (ganglion cells) was identified by H&E and cresyl violet staining at the SA junction. Other stain such as Gomori's methenamine silver stain was also used to identify the nerve fibers and ganglion cells at the SA junction (Paper I). The nerve bundles or postganglionic nerve cell bodies were not identified in the ventricle of Atlantic salmon (Paper I) (Solc, 2007). Through the identification and detailed morphological description of the cardiac pacemaker, the first aim of the study was fulfilled.

Multifunctional natriuretic peptides (NPs) are involved in key physiological functions including cardioprotection in salmonid (Johnson and Olson, 2009). The accumulating evidence from mammalian and piscine studies has proposed additional neuromodulatory and/or neurotransmitter roles of NPs (Donald and Evans, 1992; Donald et al., 1992; Hansson, 2002; Vallarino et al., 1996; Tsukada et al., 2007). Natriuretic peptides (ANP) have been identified in the intracardial ganglion cells and nerve fiber varicosities in the bovine conduction system (Hansson and Forsgren, 1993). Similar findings were further demonstrated in cardiac conduction system of other mammals such as human, sheep, pig and rat (Hansson et al., 1997, 1998). In light of these studies, ANP was proposed to act in an autocrine/paracrine fashion on conduction cells to influence the pacemaker velocity in mammals (Hansson et al., 1998; Hansson, 2002). ANP synthesis has been shown in the mammalian cardiac conduction

system and increased ANP expression has been associated with heart diseases or cardiac sympathectomy in humans (Mochizuki et al., 1991; Hansson et al., 1998). Present study performed immunohistochemistry by using natriuretic peptides (sCP and VNP) antisera and identified specific staining in the intracardiac ganglion cells in Atlantic salmon (Paper I). Previously, the immunohistochemical localization of natriuretic peptides has been demonstrated in the neural tissue (brain) and heart of several fishes such as Atlantic hagfish (*Myxine glutinosa*) and gulf toadfish (*Opsanus beta*), African lungfish (*Protopterus annectens)* and cartilaginous elasmobranch fish *(Scyliorhinus canicula)* (Donald and Evans, 1992; Donald et al., 1992; Vallarino et al., 1990, 1996) and in agreement with the immunolocalization of natriuretic peptides in the present study (Paper I). The specific NPs binding sites have been shown in the neural tissue of Atlantic hagfish (*Myxine glutinosa*) and eel (*Anguilla japonica*) (Donald et al., 1999; Tsukada et al., 2007), supporting the existence of NPs binding sites and receptors in the cardiac neural tissue (pacemaker). Besides cardioprotective functions, additional neuromodulatory and/or neurotransmitter roles of NPs have been suggested in fishes (Donald and Evans, 1992; Donald et al., 1992; Vallarino et al., 1996; Tsukada et al., 2007). Recently, Zaccone et al. (2009a, 2009) have shown the specific binding sites for neurotransmitters such as substance P and galanin (GA) in the cardiac pacemaker tissue at the SA junction in ray–finned fish (*Polypterus bichir bichir*) and teleosts such as rainbow trout (*Oncorhynchus mykiss*), mullet (*Mugil cephalus*) and Nile catfish (*Synodontis nigriventris*), supporting the existence of NPs neural tissue binding sites and possible effects on the cardiac pacemaker tissue. In view of above–mentioned studies, the localization of sCP and VNP in the cardiac pacemaker tissue suggested their additional neurotransmitter and/or neuromodulatory roles particularly relative to the cardiac pacemaker in Atlantic salmon (Paper I) and fulfilled the second aim of the study.

6.2 Clinical biochemistry biomarkers of Atlantic salmon

After achieving the first two aims, the work was focused to identify potential biomarkers of cardiac diseases in Atlantic salmon and correlated them with histopathology. According to Biomarkers Definitions Working Group (2001), '*a biomarker is defined as a characteristic that is measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic process to a therapeutic intervention*'.

In paper II, the clinical biochemistry biomarkers were identified and correlated with cardiac diseases of Atlantic salmon. The diseases CMS and HSMI are presently diagnosed by histopathology which is a lethal method. Fish must be killed to perform the standard paraffin wax protocol and H&E staining on heart and other vital organs. The H&E stained slides are examined under the microscope for pathological changes (necrosis and inflammation) characteristic of CMS and HSMI. Both CMS and HSMI are noticed on farms with increased mortality rates. CMS causes huge losses due to the mortality in pre–harvest adult fish while HSMI and PD significantly decreases the fish filet quality due to lesions in the skeletal muscle (Larsson et al., 2012; Poppe and Ferguson, 2006; Kongtorp, 2009). Recently, the piscine reovirus (PRV) and piscine myocarditis virus (PMCV) are associated with the HSMI and CMS respectively but Koch's postulates are unfulfilled to date (Løvoll et al., 2010; Palacios et al., 2010). The piscine reovirus is ubiquitous and identified in both diseased and non–diseased fish. In addition, both PRV and PMCV have been identified from healthy Atlantic salmon broodfish and PMCV was also identified in marine Atlantic argentine (Bockerman et al., 2011; Wiik–Nielsen et al., 2012), and in turn may limit the potential diagnosis of these diseases by virus isolation or identification by PCR.

The current situation demands proper tools to diagnose or predict the disease as early as possible using suitable biomarkers. Biomarkers include

enzymes, hormones, biological substances, markers of cardiac stress and malfunction, and myocyte injury (Braunwald, 2008). The biomarkers of inflammation (C–reactive protein (CRP)), myocyte stress (natriuretic peptides) and myocyte injury (troponin I, creatine kinase and lactate dehydrogenase) were tested in Atlantic salmon to fulfill the third aim of the study. CRP is an acute–phase reactant protein synthesized by liver in response to inflammation. The troponin (Tn) complex comprises 3 proteins such as TnC (calcium binding protein), TnI (inhibits actin from binding to myosin heads) and TnT (binds tropomyosin) (Jaffe et al., 2006; Tibbits and Moyes, 1992). Troponin complex helps regulate excitation–contraction coupling in the cardiomyocytes. Cardiac injury results in detachment of cTnI from actin and leakage into the general circulation. Therefore, the higher levels of cTnI in serum/plasma are considered to be a highly specific and sensitive biomarker of myocardial injury and necrosis (Reynolds and Oyama, 2008).

Present study used human CRP and natriuretic peptide (ANP, BNP) assays but did not identify any correlation to the HSMI–affected fish serum while cTnI assay could not be measured in the samples. The reason(s) may include the lack of cross–reactivity among human and Atlantic salmon assays. Additionally, Jaffe et al. (2006) have reported the fluctuating levels of CRP in response to acute illness including myocardial injury in humans.

Other biomarkers of myocyte injury such as creatine kinase (CK) and lactate dehydrogenase (LDH) were used to correlate with histopathology (inflammation and necrosis) of HSMI– and chronic CMS–affected Atlantic salmon (Paper II). The term chronic–CMS was used to refer CMS–affected fish that were sampled approx. 6 months after peak mortality phase of disease. Several piscine studies have measured the blood biochemical parameters particularly enzymes including CK and LDH (Rajabipour et al., 2009; Anderson et

al., 2010, Powell, 2006). CK and LDH are biochemical enzymes that are present in almost all cell types and are released upon cellular degeneration such as necrosis (Kemp et al., 2004; Walker, 2006). CK and LDH levels in blood plasma sustain over 3–4 days to 7–10 days in humans following cardiac injury respectively (Kemp et al., 2004) and provided an opportunity to extrapolate from human studies and use them as biomarkers in teleosts (Atlantic salmon) due to their prolong presence in the blood. In big game fish (*Chrysophrys auratus*), plasma CK and LDH exhibit the physiological stress responses after capture, probably because of muscle damage that release the enzymes in plasma (Wells et al., 1986). Boucher (1990) has shown the serum enzymes CK and LDH levels respond to temperature variations in rainbow trout. Furthermore, CK concentrations have been associated with irreversible injury such as myocardial necrosis in mammals (Ishikawa et al., 1997). In Atlantic salmon, PD–affected fish have shown the significantly increased CK levels (Rodger et al., 1991; Ferguson et al., 1986) and above mentioned studies led to the hypothesis of clinical importance of CK and LDH in cardiac diseases of Atlantic salmon.

To the best of our knowledge, this was the first study which performed extensive correlations among enzyme levels and different anatomical regions of heart and skeletal muscle of CMS– and HSMI–affected fish. The significantly increased CK and LDH levels were identified in HSMI–affected fish and in agreement with previous studies where significantly increased CK levels were identified in PD–affected fish (Rodger et al., 1991; Ferguson et al., 1986). The significant serum enzyme correlations were identified in different HSMI disease phases (start, peak and late phases) that were based on the mortality levels at farms (Paper II). There were significant correlations between CK levels and individual anatomical regions for HSMI–affected fish. Most of the individual parameters identified significant correlations to LDH levels for HSMI–affected

fish. Taken together, CK and LDH levels identified significant correlations to total inflammation and total necrosis scores. However, there were no significant correlations identified for chronic CMS–affected fish and one of reasons may include the delayed sampling (6 months after disease outbreak) and enzymes levels drop down to normal ranges in that period due to the fact the enzymes levels peak for 10–12 days in humans (Paper II). The ranges for CK and LDH enzymes levels were identified for diseased (chronic CMS– and HSMI–affected fish) and non–diseased fish (Paper II). HSMI is suggested as a disease with more inflammatory lesions than necrosis (Kongtorp et al., 2004; Kongtorp, 2009) and current study concurs with the previous studies (Paper II). Histopathological scores (for inflammation and necrosis) of HSMI– and CMS–affected fish were significantly different and comparatively higher than non–diseased fish except necrosis scores of CMS–affected fish which were not significant. These significantly higher enzymes levels and their significant correlations to histopathology supported the potential use of serum enzymes for screening of HSMI–affected fish is promising (Paper II).

6.3 Cardiac pathological responses of Atlantic salmon affected with cardiac diseases

The investigated cardiac diseases (CMS, HSMI and PD) have been associated with inflammation and necrosis in the heart, however, an overall evaluation of the cardiac pathological responses in Atlantic salmon was unexplored (Poppe and Ferguson, 2006). To determine the cardiac pathological responses, broader investigations were performed to identify the specific biomarkers in Atlantic salmon. In papers III and IV, markers (antibodies) were used to identify and compare the cardiac pathological responses in Atlantic salmon heart affected with HSMI, PD and CMS. Through immunohistochemistry,

specific antibodies were tested on HSMI–, CMS– and PD–affected paraffin embedded hearts to fulfill the third and fourth aims of the study. Presently histopathological findings are used to diagnose HSMI and CMS and immunohistochemistry has been suggested as a useful addition in the diagnostic tools for these diseases (Finstad et al., 2012). Immunohistochemistry is not a quantitative assay but recent studies showed a linear correlation between the results of measuring quantitative RNA expression by real time PCR and semiquantitative protein expression by immunohistochemistry (Barton and Levine, 2008; O'Connor et al., 2010).

6.3.1 Nature of inflammatory cells

The involvement of mononuclear lymphocyte–like cells in all three investigated diseases is a well–known fact (Ferguson et al., 1990; Poppe and Ferguson, 2006; Kongtorp et al., 2004, 2006; Taksdal et al., 2007; Christie et al., 2007). Inflammation is suggested as a protective mechanism to tissue damage, irrespective of the cause, and if the tissue injury did not completely damage the effected tissue (Roberts and Rodger, 2001). Lymphocyte like–cells have been reported in trabecular (PD, HSMI and CMS) and non–trabecular (HSMI and PD) layers of the heart in the investigated diseases (Paper II) (Poppe and Ferguson, 2006; Kongtorp, 2009). Inflammatory cells have not been characterized in Atlantic salmon particularly affected with cardiac diseases (Kongtorp, 2009). Until recently, it was not possible to characterize these inflammatory cells due to non–availability of specific markers (antibodies), but nowadays several salmonid–specific antibodies are available and validated, including CD3ε (Koppang et al., 2010; Boardman et al., 2012), CD8α (Hetland et al., 2010, 2011; Olsen et al., 2011) and recombinant tumor necrosis factor–α (rTNFα) (Zou et al., 2003). There were moderate levels of $CD3\varepsilon^{+}$ cells in atria as compared to strong levels in the HSMI–affected ventricles, specifically in the affected areas with

inflammatory foci and degenerative changes (Paper III). This observation was supported by moderate severe lesions reported in atria as compared to ventricles in HSMI–affected fish (Kongtorp et al., 2006) and subsequently by moderate levels of CD3ε immunostaining identified in the early stages of soybean enteropathy in Atlantic salmon (Lilleeng et al., 2009). The CD3ε immunostaining of HSMI–affected hearts was significantly different from non–diseased fish where only few $CD3\varepsilon^{+}$ cells were identified (Paper III) and in agreement with Koppang et al. (2010) where few $CD3\varepsilon^{+}$ cells were identified in non–diseased heart. Interestingly, there were differences in CD3ε immunoexpression among three investigated diseases where HSMI–affected hearts presented with strong levels of CD3ε immunopositive cells as compared to moderate levels in CMS– and PD–affected hearts (Paper IV). Besides a general T cell marker, CD3 has been suggested to have additional roles and the CD3 immunolocalization has also been identified in gastric parietal cells, renal tubular epithelial cells and cerebellar Purkinje cells in several species such as human, mouse, rat, quail and guinea pig (Garson et al., 1982; Alroy et al., 2005). Recently, Xu et al. (2010) suggested the involvement of CD3 in the normal development of retinal ganglion cells in mice. To test these additional roles of CD3 in teleosts (Atlantic salmon), cardiac pacemaker tissue was stained with CD3ε antiserum. It identified specific positive immunostaining in postganglionic nerve cell bodies, suggesting additional roles of CD3 in Atlantic salmon (Paper I). The novel CD3ε immuno–reaction in Atlantic salmon cardiac neural tissue shares similar pattern to that of mammals but requires further studies to determine its functional significance.

The CD8 is considered the main marker of cytotoxic T lymphocytes and part of the T cell receptor (TCR). Cell mediated cytotoxicity has been suggested by $CD8\alpha^{+}$ lymphocytes in rainbow trout (Takizawa et al., 2011). Besides low levels of CD8α positive staining, CMS–affected hearts expressed more $CD8\alpha^{+}$

cells as compared to other diseased (HSMI– and PD–affected hearts) and non–diseased hearts (Paper IV). The CD8 immunostaining was confined to lymphocyte–like cells in diseased and non–diseased hearts (Papers III and IV). Timmerhaus et al. (2011) have shown the correlation between histopathology, viral loads and $CD8^+$ T cells, suggesting the involvement of $CD8^+$ T lymphocytes in clearing the experimental PMCV infection in Atlantic salmon. The low levels of $CD8\alpha^+$ cells have been suggested to have protective roles in Atlantic salmon (Hetland et al., 2011). Few $CD8\alpha^+$ cells have been identified in parasitic infection of rainbow trout and infectious salmon anaemia (ISA) infection of Atlantic salmon (Hetland et al., 2011; Olsen et al., 2011). Present study suggested that mononuclear inflammatory cells were predominantly $CD3\varepsilon^+$ in all investigated diseases (Papers III and IV).

However, moderate levels of immunostaining were observed using rTNFα antiserum where immunopositive cells included macrophage–like and eosinophilic granular cells (EGCs) in all three investigated diseases. $rTNF\alpha^+$ cells were located in the areas surrounding lesions (Papers III and IV). Due to central role of TNFα in inflammation and immunity, it has been suggested as inflammatory biomarkers in salmonids (Haugland, 2008). Macrophages are the main antigen presenting cells (APC), although dendritic cells have also been identified in fish, and shown to present antigen to T lymphocytes and to secrete cytokines (TNFα) (Lovy et al., 2008; Magnadottir, 2010; Tizard, 2004). Eosinophilic granular cells were also identified in *bulbus arteriosus* in diseased and non–diseased fish (Papers III and IV) and supported by Amin and Trasti, (1989) who reported abundant EGCs in *bulbus arteriosus* and at borders between *bulbus arteriosus* and ventricle of Atlantic salmon. Qin et al. (2001) have shown the production of TNFα by macrophages in rainbow trout and histopathological observations have identified macrophage–like cells in CMS– and HSMI–affected hearts that support our observation of immunopositive

macrophage–like cells in Atlantic salmon by rTNFα antiserum in the present study (Papers III and IV) (Ferguson et al., 1990; Kongtorp, 2009). The rTNFα immunostaining for HSMI–affected hearts was significantly different from non–diseased hearts (Paper III). EGCs as functional analogue of the mammalian mast cells are also recruited at the site of inflammation in teleosts (Reite and Evensen, 2006) and are likely to be involved in enhancing T cell activation by presenting antigens and release of TNFα similar to that seen in mammalian mast cells (Hogan et al., 2008; Rothenberg and Hogan, 2006). It has been previously suggested that the final stages of EGCs maturation take place locally in teleosts (Reite and Evensen, 2006) and rainbow trout gill explants showed a significant increase in EGCs as compared to controls upon stimulation with LPS and TNFα (Holland and Rowley, 1998). In mammals, the antigen presentation ability of mast cells has been demonstrated by *in vitro* stimulation of mast cells with LPS and IFN–γ which induced the MHC class II expression, and subsequently by showing *in vivo* increased presence of MHC class II^+ mast cells recorded by inflammatory stimuli (*Leishmania major*) *in situ* (Kambayashi et al., 2009). The increased presence of EGCs has been reported in several salmonid conditions including acanthocephalan infection, ectoparasites (*Ichthyobodo* sp.) infection and ulcerative dermal necrosis of nasal epithelium, suggesting the recruitment of EGCs in persistent inflammatory conditions of salmonid (reviewed in Reite and Evensen, 2006). The above mentioned studies were in agreement with the present findings of moderate levels of EGCs in diseased hearts which were likely to be either recruited at the lesioned sites (heart) or possibly involved in T cell activation by performing antigen presenting roles but it requires further assessment to confirm (Papers III and IV).

Another monoclonal rainbow trout anti–granulocytes/monocytes antibody was used which identified few granulocyte/monocytes–like cells in diseased and non–diseased hearts (Papers III and IV). These results suggested

that lymphocytic response dominant over granulocytes in the investigated diseased hearts and spectrum of inflammatory cells associated with the cardiac pathology consisted of mainly CD3ε^{+} T lymphocytes in CMS–, PD– and HSMI–affected hearts (Papers III and IV).

6.3.2 MHC class I and II antigen presentation

Strong levels of MHC class I immuno–reaction were identified in diseased and non–diseased fish hearts because MHC class I molecules are abundantly expressed in nearly all known cell types (Randelli et al., 2008). MHC class I presents peptides derived from cytosolic proteins and the pathway termed cytosolic and endogenous pathway. Viruses infect the cells by entering the cytoplasm and endogenous pathway presents these virus infected cells to T lymphocytes. MHC class I molecules exclusively interact with CD8^{+} cytotoxic T lymphocytes (CTL). Generally apoptosis results as the outcome of the virus–infected cells by CTL to prevent infection of the neighboring cells (Randelli et al., 2008). In the present study, macrophage–like cells were observed in diseased hearts by MHC class I immunostain which were suggested important for clearing of tissue of infected or necrosed cells whereby performing similar functions in piscine as in mammals (Boshra et al., 2006; Ferguson et al., 1990; Magnadottir, 2006; Tizard, 2004). MHC class I immuno–reaction identified the perinuclear myocardial and endocardial staining in the diseased (HSMI–, CMS– and PD–affected) hearts. However, non–diseased hearts were identified predominantly with endocardial MHC class I immunostaining (Papers III and IV) (Dijkstra et al., 2003; Hetland et al., 2010).

MHC class II molecules, on the other hand, bind peptides for presentation to the CD4^{+} T helper cells by the exogenous pathway (Randelli et al., 2008). MHC class II molecules are expressed on antigen presenting cells such as dendritic

cells, macrophages, B and T lymphocytes, and possibly epithelial cells and is considered important molecule for initiation of adaptive immune responses (Glimcher and Kara, 1992; Koppang et al., 2004; Toda et al., 2011). MHC class II *β* chain antiserum used in the present study has already been used in several studies to identify T lymphocytes, epithelial cells, multinucleated giant cells (MGC), macrophages and dendritic–like cells in Atlantic salmon (Koppang et al., 2003, 2003a, 2004; Kongtorp, 2009; Morrison et al., 2006). MHC class II immuno–reactivity was mostly confined to inflammatory foci and identified lymphocyte– and dendritic–like cells in the diseased hearts (Papers III and IV). This observation was in line with a previous study where Lovy et al. (2008) identified increased numbers of dendritic–like cells in the gills of rainbow trout affected with microsporidial gill disease (MGD) as compared to non–diseased gills. The increased MHC class II immunoexpression in HSMI–affected hearts was significantly different from non–diseased hearts (Paper III). Previously, the same MHC class II antiserum has been used to immunostain HSMI–affected hearts and identified moderate levels of staining (Kongtorp, 2009) and supports our observation of increased presence of MHC class II^+ cells in HSMI–affected hearts (Paper III). Interestingly, MHC class II immunopositive cells frequency were different among three diseases with strong, moderate and low levels to no staining in HSMI–, CMS– and PD–affected hearts respectively (Paper IV). Atlantic salmon infected with amoebic gill disease has been identified with increased number of MHC class II^+ cells in the gills and MHC class II positive cells were suggested as indictor of immune cell trafficking and possible contribution in the antigen presentation (Morrison et al., 2006). Additionally, the increased presence of MHC class II^+ cells have also been described in granulomatous uveitis associated with vaccination in Atlantic salmon (Koppang et al., 2004). Low levels of MHC class II immunostaining in PD hearts may be explained by a transcriptional study where induction of MHC class I but not II has shown in

different organs including heart in Atlantic salmon infected with infectious salmon anaemia virus (ISAV) (Jorgensen et al., 2007). In Atlantic salmon, MHC class I and II expression experiments have shown transcriptional regulatory mechanisms similar to those in mammals (Koppang et al., 1998, 1999). The inflammatory spectrum of diseased hearts was predominantly comprised CD3Ɛ$^{+}$ and MHC class II^{+} cells, suggested the inflammatory cells as activated T helper cells; however, this assumption requires CD4 antibodies to identify the immunopositive inflammatory cells in the diseased hearts (Papers III and IV).

6.3.3 Programmed cell death

The myocytic necrosis is a common finding in all three investigated diseased hearts (Poppe and Ferguson, 2006). Together apoptosis and necrosis determine the final degree of lethal myocardial injury (Zhao and Vinten–Johansen, 2002). In Paper IV, caspase 3 and TUNEL staining identified strong to moderate levels of apoptotic cells in the investigated diseased hearts and in agreement with significantly increased caspase 3 immunostaining identified in the teleost gills (*Thalassoma pavo* L.) exposed to cadmium (Brunelli et al., 2011). Recently, caspase–3–mediated apoptosis has been shown in the regenerating spinal cord in teleost (*Apteronotus leptorhynchus*), suggesting its role in tissue replacement after injury (Sirbulescu and Zupanc, 2009). Additionally, pro–apoptotic genes such as Bax and Bcl–x have been shown significantly upregulated in Atlantic salmon exposed to uranium (U) as compared to controls (Song et al., 2012). The increased presence of caspase 3^{+} cells has also been noted in the adult brain of brown ghost knifefish (*Apteronotus leptorhynchus*) affected with aneuploidy (Rajendran et al., 2008) and significantly increased caspase 3 staining identified in the intestine of soybean meal (SBM)–induced enteritis in Atlantic salmon (Bakke–McKellep et al., 2007). The above mentioned

studies support the increased presence of apoptotic cells (identified by caspase 3 and TUNEL) in the diseased hearts in the current study (Papers III and IV). The increased number of apoptotic cells (in CMS and PD–affected hearts) could be explained by the marked tissue regeneration (heart) capacity in Atlantic salmon affected with the investigated diseases (Ferguson et al., 1990; Kongtorp, 2009; McLoughlin et al., 2002; Taksdal et al., 2007). As apoptosis is a highly ordered and energy demanding process, energy deprivation in cardiomyocytes could inhibit the terminal apoptotic events, and could lead to the programmed cell necrosis (Dorn II, 2009). CMS and PD were the cardiac diseases identified with more cardiac necrotic changes as compared to HSMI where mononuclear cells infiltration predominated (Papers II and IV) (Kongtorp et al., 2004, 2004a; Grammes et al., 2012). This was reflected by the moderate to low levels of TUNEL and caspase 3^{+} cells in HSMI–affected hearts respectively (Paper III) and corroborated by low levels of caspase 3 immunostaining in HSMI–affected hearts (Grammes et al., 2012). Recently, a transcriptomic study of PMCV injected fish has shown the correlation of CMS–related lesions and upregulation of T cells and apoptotic genes at peak cardiac pathology/viral load (8 weeks post infection) in the hearts (Timmerhaus et al., 2011). This was in agreement with the increased presence of apoptotic cells in the CMS and PD–affected hearts (Paper IV).

As in mammals, fish viruses are also capable of inducing apoptosis in the hosts (Hay and Kannourakis, 2002; Silva et al., 2008). Phylogenetic analysis of piscine reovirus (PRV) associated with HSMI disease in Atlantic salmon revealed that PRV lies taxonomically in between the orthoreovirus and aquareovirus. PRV comprises 10 dsRNA genome segments and in line with orthoreoviruses as compared to aquareoviruses consisted of 11 segments (Palacios et al., 2010). Although, the structural and functional properties of PRV are undetermined to date but sequence homologies suggested PRV closer to mammalian

orthoreoviruses (Finstad et al., 2012; Palacios et al., 2010). In mammals, reoviruses including orthoreoviruses induce apoptosis in a wide variety of cultured cells (*in vitro*) and in target tissues (*in vivo*) including the heart and CNS (Clarke et al., 2005). Reoviruses induce apoptosis by regulating several important genes (TNF ligand, Bid, Smac) related to extrinsic and intrinsic apoptosis pathways and apoptosis considered critical mechanism by which disease is triggered in the host (Clarke and Tyler, 2003; Clarke et al., 2005). As suggested by Kongtorp (2009), the HSMI–associated virus possesses the immunoregulatory properties. The cytopathic effect of chum salmon reovirus (CSV) has shown the apoptosis and syncytia (large multinucleated giant cells formed by the fusion of neighboring cells) formation in salmonid cell lines including epithelial–like CHSE–214, fibroblast–like RTG–2 and monocyte/macrophage–like RTS11 cell lines. The hemotypic aggregation was observed in RTS11 instead of syncytia formation that suggested the potential for CSV to modulate macrophage functions (DeWitte–Orr and Bols, 2007). Apart from giant cells, the above mentioned studies support the present observation of increased numbers of apoptotic cells in HSMI–affected hearts and suggest the similar cellular responses to those found in other orthoreoviruses (Paper III). Mammalian alphaviruses such as Sindbis virus (SIN) and Semliki forestvirus (SFV) have shown to induce apoptosis in both cell cultures and target organs (Kiver, 2009), and further syncytia and apoptosis have also been shown in CHSE–214 cell culture by Norwegian salmonid alphavirs (SAV3) (Skotheim, 2009; Yousaf, 2008). Infectious pancreatic necrosis virus (IPNV) has been shown to regulate apoptosis and necrosis death pathways through the upregulation of TNFα in zebrafish cell culture (Wang et al., 2011) and it is likely that other fish RNA viruses including PRV, SAV or PMCV follow the similar patterns of cell death but it requires further assessment. Besides necrotic changes, apoptosis has also been reported in heart and pancreas tissues of Atlantic salmon in natural and

experimentally induced SAV3 infection by histopathology (Taksdal et al., 2007). Recently, *in vitro* apoptosis has been shown in the chum salmon heart–1 cells (CHH–1) and CHSE–214 cells infected with SAV1 (Herath, 2010). The presence of apoptosis in heart cell line (CHH–1) supports the apoptosis identified in the hearts of the investigated diseases particularly PD. IPNV and viral nervous necrosis virus (VNNV) have been shown to induce apoptosis followed by secondary necrosis in cell cultures (Chen et al., 2006; Chen et al., 2010; Chiu et al., 2010; Hong et al., 1998; Su et al., 2009) and support the current findings of strong levels of both apoptosis (identified by caspase 3 and TUNEL) and necrosis (identified by H&E staining) cells in PD– and CMS–diseased hearts (Paper IV). However, further evaluation is required to determine if apoptosis follows necrosis in the investigated diseases, although, apoptosis and necrosis were observed in the present study (Papers III and IV). Comparatively TUNEL identified more positive cells than caspase 3 immunostaining (Papers III and IV) due to the reason that TUNEL positivity might also indicate necrosis as suggested by Bianciardi et al. (2006).

6.3.4 Tissue hypoxia

PD–affected fish have shown significant reductions in antioxidant status including blood plasma and liver vitamin E levels as compared to healthy fish (Ferguson et al., 1986) and further confirmed by Rodger et al. (1991). At that time, the marked decreased levels of vitamin E were suggested to be as a consequence of the disease instead the cause of the disease (Ferguson et al., 1986). Presently it is known that the anti–oxidant shift (oxidative stress) is a well–established effect of infectious diseases and feature of cell death (Hay and Kannourakis, 2002), and suggested as major source of myocardial damage in salmonid viral diseases instead of viruses itself (Poppe and Ferguson, 2006). It is

suggested that the increase in reactive oxygen species (ROS) during inflammation contributes to HIF1α accumulation and activation (Dehne and Brune, 2009). HIF1α is a transcriptional factor that is upregulated under hypoxia and is considered essential for normal heart physiology and particularly plays important roles in cardiac conditions such as ischemia and pressure overload (Hopfl et al., 2004; Gale and Maxwell., 2010; Dehne and Brune, 2009). HIF1α immunostaining showed moderate levels of staining in PD–affected hearts; while low levels were identified in CMS– and HSMI–affected hearts as compared to low to no staining in non–diseased hearts (Papers III and IV). Being a transcriptional factor, HIF1α is upregulated under hypoxic conditions and moderate levels of HIF1α immunostaining in PD–affected hearts suggested possible myocardial hypoxia (Hopfl et al., 2004; Gale and Maxwell, 2010; Dehne and Brune, 2009). Caretti et al. (2007) have shown *in vivo* overexpression of HIF1α in hypoxic myocardium in mammals. Immunohistochemical studies identified HIF1α^{+} cells in several tissues including heart under normoxia in mammals, although increased expression was observed under hypoxia (Stroka et al., 2001). The marked increase in HIF1α expression has been observed in salmonid cell cultures upon hypoxia, although, low levels of HIF1α levels were also expressed under normoxia (Soitamo et al., 2001). HIF1α protein stabilization and degradation have been suggested similar mechanisms in mammals and teleosts, although in salmonids (rainbow trout and chinook salmon) stabilization occurred at much higher oxygen levels than in mammals, suggesting HIF1α roles in piscine physiology (Stroka et al., 2001; Soitamo et al., 2001). It supports our observation of low levels of HIF1α immunostaining in normoxic non–diseased hearts (Papers III and IV). Recently, Terova et al. (2008) have shown the significantly increased HIF1α expression by real time PCR in response to acute and chronic hypoxia in teleost (seabass, *Dicentrarchus labrax*). During development stages of Baltic salmon (*Salmo salar*), HIF1α

protein expression increased with age as identified by Northern and Western blot analysis (Vuori et al., 2004), and HIF1α protein has been shown to be expressed in tissues derived from salmonid liver, gonad, embryonic tissues (Soitamo et al., 2001), and in cardiac and skeletal muscle in the present study (Papers III and IV). The disturbances in HIF1α functions have been associated with Baltic salmon yolk–sac fry mortality (M74–syndrome) and increased HIF1α expression has been identified in Atlantic salmon affected with vertebral column deformity (Sanchez et al., 2011; Vuori et al., 2004). In humans, HIF1α expression may lead to cardiac degeneration, dysfunction and leading to heart failure (Bekeredjian et al., 2010; Lei et al., 2008). Moreover, TNFα which is rapidly released after infection has been suggested to activate HIF1 in mammals (Dehne and Brune, 2009) and may strengthen the increased TNFα and HIF1α immunoexpression in response to cardiac diseases of Atlantic salmon in the present study (Papers III and IV).

6.3.5 Mitotic activity

Proliferative cell nuclear antigen (PCNA) is a well conserved protein identified in all eukaryotic species based on sequence, structural and functional similarities (Strzalka and Ziemienowicz, 2011). PCNA was first identified in the serum of some patients with systemic lupus erythematosus (Miyachi et al., 1978). PCNA serves as probably the most commonly used marker for cellular proliferation and surrogate marker for DNA synthesis, cell cycle control, DNA repair and apoptosis (Soonpaa and Field, 1998; Eldridge and Goldsworthy, 1996; Chapman and Wolgemuth, 1994; Panday and Wang, 1995; Strzalka and Ziemienowicz, 2011). It has been suggested as marker of cell proliferation in myocardial hypertrophy, myocarditis, valvular heart disease, ischemic heart

disease and cardiomyopathy in humans (Matturri et al., 1997, 2002; Arbustini et al., 1993).

Moderate levels of PCNA immuno–reaction were observed in HSMI–affected diseased hearts and were significantly different from a low number of $PCNA^+$ cells in non–diseased hearts (Paper III) (Grammes et al., 2012). PCNA staining was localized to the nuclei and cytoplasm as well as hypertrophic nuclei (Papers III and IV). Additionally, strong to moderate levels of immuno–reactivity resulting from PCNA were observed in CMS– and PD–affected hearts respectively while focal low levels of immuno–reactivity were identified in non–diseased hearts (Paper IV) and supported by other piscine studies (Zenker et al., 1987; Ortego et al., 1995; Borucinska et al., 2008). The significant increase in the number of $PCNA^+$ cells has been suggested as an indicator of high cell turn over and recruitment in Atlantic salmon affected with amoebic gill disease (Morrison et al., 2006; Adams and Nowak, 2003) and subsequently in the intestine of Atlantic salmon infected with soybean meal (SBM)–induced enteritis (Bakke–McKellep et al., 2007). The intense and high levels of immunostaining cardiomyocytes with anti–PCNA antibodies suggested active myocardial hyperplasia in diseased hearts (Papers III and IV). Heart regeneration has been shown in CMS–, PD– and HSMI–affected fish (Ferguson et al., 1990; Kongtorp, 2009; McLoughlin et al., 2002; Taksdal et al., 2007). Furthermore, Kongtorp (2009) has described complete heart regeneration in post–clinical phase of HSMI–affected fish despite disease severity. Many cells have been lost in HSMI–affected heart, however, surviving endocardial and epicardial cells in HSMI–affected fish were suggested to be involved in the regenerating process. Additionally, it was hypothesized that scar formation was not important and fibrotic tissue cleared in healing phase following HSMI outbreak (Kongtorp, 2009). Fish retain the ability of heart remodeling after development as compared to mammals where heart regeneration capacity is not completely lost

but severely limited in postnatal and adult hearts (Becker et al., 2011; Kikuchi et al., 2010; Major and Poss, 2007; Mommsen, 2001; Poss, 2007; Sun et al., 2009; Vornanen et al., 2002; Poppe and Ferguson, 2006; Soonpaa and Field, 1998). For example, Jopling et al. (2010) have shown zebrafish heart regeneration by cardiomyocyte dedifferentiation and proliferation without involvement of stem or progenitor cells. Cardiac remodeling has also been described by showing the thickening of compact ventricle layer of Atlantic salmon infected with amoebic gill disease (AGD) (Powell et al., 2002) and supported the increased presence of $PCNA^+$ cells in diseased hearts as identified in Papers III and IV. PCNA immunostaining suggested high levels of cell division activity in diseased hearts and identified hypertrophic nuclei which have been suggested representing compensatory hypertrophy in CMS–affected hearts in Atlantic salmon (Papers III and IV) (Ferguson et al., 1990).

Taken together, the increased presence of $PCNA^+$ cells and strong to moderate levels of apoptosis and hypoxia suggested a high cell turn over where an induction of cell and tissue damage/repair occurring in diseased hearts (Papers III and IV).

7.1 Conclusions

The current study successfully identified and characterized the cardiac pacemaker tissue in Atlantic salmon at SA junction and subsequently demonstrated immunoexpression of natriuretic peptides (sCP and VNP) and CD3 in the cardiac pacemaker, suggesting their additional roles in cardiac neural tissue. Based on the observations in Paper I, to identify the cardiac pacemaker in fish (Atlantic salmon), the heart must be carefully removed from the pericardial cavity, ensuring the inclusion of the *sinus venosus* and atrium in the samples. Furthermore, biomarkers of clinical biochemistry and cardiac pathological changes were identified and validated in the cardiac diseases of Atlantic salmon (Papers II and III). Presently, HSMI and CMS are mostly diagnosed by histopathology and immunohistochemistry would be beneficial as a supplementary tool for these cardiac diseases. In end, the identified specific markers (antibodies) were used to characterize and compare the cardiac pathological responses in Atlantic salmon affected with HSMI, CMS and PD (Papers III and IV). It is noteworthy these apparently similar cardiac diseases of Atlantic salmon exhibited differences in cardiac immunopathological responses in the heart.

In summary:

- Location and detailed morphology of Atlantic salmon pacemaker at SA node were described.
- Novel neuromodulatory/neurotransmitter roles of natriuretic peptides (sCP and VNP) and additional CD3 roles were suggested in teleosts (Atlantic salmon).

- Inflammatory cells comprised mainly CD3ε^{+} T lymphocytes in all investigated diseases and lymphocytic cell population dominated over granulocytes.
- Moderate levels of eosinophilic granular cells (EGCs), and macrophage–like were identified in all three investigated diseases. MHC class II^{+} cells included antigen presenting cells including lymphocyte– and dendritic–like cells.
- Strong to moderate levels of apoptotic cells were identified besides necrosis in all investigated diseases.
- The active myocardial hyperplasia was indicated in diseased hearts due to pronounced and extensive PCNA immunostaining of cardiomyocytes and suggested the induction of cell and tissue damage/repair occurring in the diseased hearts.
- PD–affected hearts appeared comparatively more hypoxic than CMS– and HSMI–affected hearts due to moderate levels of HIF1α immunoexpression.
- The CD3, MHC class II, PCNA, TNFα, caspase 3 and TUNEL staining were confined to the lesioned areas in the diseased hearts, pointing to the pathological changes and appeared promising in the identification of lesioned areas in the investigated diseased hearts.

7.2 Future prospects

The present study performed the characterization of cardiac pacemaker of Atlantic salmon and the detailed evaluation of pathological changes of cardiac pacemaker tissue in response to cardiac diseases. Mononuclear cells infiltrations (lymphocyte–like cells) were observed in the pacemaker tissue besides other heart compartments such as atrium and ventricle in the investigated diseases. Ganglionitis and neuritis (cardioneuropathy) were identified in the cardiac pacemaker at SA node of CMS–, PD– and HSMI–affected fish (unpublished). Cardioneuropathies have been observed with atrial arrhythmia such as sustained sinus tachycardia and sinus arrhythmia, atrial fibrillation, sino–atrial block and sinus sick syndrome in humans (Rossi, 1985). Recently, autonomic dysfunction due to reduced heart rate has been identified in hamsters infected with West Nile virus, pointing the reduced heart rate may lead to sudden cardiac death (Wang et al., 2011a). Additionally, subtle cardiac conduction system pathology has been associated with sudden unexpected death in epilepsy patients due to cardiac arrhythmia (Opeskin et al., 2000). The above mentioned data led to the hypothesis that Atlantic salmon heart with cardioneuropathy may exhibit cardiac arrhythmia due to the pathological changes in cardiac neural tissue. For example, despite severe degenerative heart lesions, CMS–affected fish remain alive and die mostly at harvesting stage, suggesting similar mechanisms of cardiac arrhythmias in teleosts as in mammals (Ferguson et al., 1990; Opeskin et al., 2000; Poppe and Ferguson, 2006; Wang et al., 2011a). No mortality has been identified in PMCV injected CMS challenge trials, pointing the lack of additional stressors which were required for mortality (Timmerhaus et al., 2011). HSMI–affected dead fish exhibit more severe inflammation as compared to moribund or healthy fish, suggesting more intense immune reaction blown out of proportion (Kongtorp, 2009). Taken together, these observations highlight the importance of cardioneuropathy in fish and

suggest carrying out studies to characterize the pathological changes of cardiac pacemaker of Atlantic salmon, their functional significance on the heart performance and potential association with mortality are necessary. Novel sCP and VNP immunostaining should be supported by the identification of receptor binding sites of [^{125}I] sCP and VNP in the cardiac neural tissue of Atlantic salmon. Furthermore, immunolocalization of NP receptors including NPR–A, NPR–B and NPR–C will be useful. It will strengthen the neurotransmitter/neuromodulatory roles of natriuretic peptides and further explores cardiac autonomic nervous system of heart in Atlantic salmon. The serum enzymes (CK and LDH) levels were measured and correlated to histopathology in diseased and non–diseased fish in the present work. The identification and evaluation of serum enzymes in Paper II enabled use CK and LDH as biomarkers, which may become relatively cheap and easily available assays to the local farmers. Longitudinal studies should be performed at the farms and enzymes levels measured throughout the production cycle. It will establish normal and diseased enzymes ranges, and in turn contributes in limited piscine clinical biochemistry. Moreover, Atlantic salmon–specific clinical biochemical assays should be developed. It will provide us non–lethal diagnostic tools to diagnose heart diseases that are causing huge economic losses to Atlantic salmon aquaculture. Immunohistochemical markers are supplementary tools to identify protein expression at tissue levels and there is a need for more antibodies. As the apparently similar cardiac diseases exhibited differences in immunopathological responses in Atlantic salmon, so the care should be taken to devise protection/vaccination strategies regarding these diseases separately. The research should be continued to produce more Atlantic salmon–specific polyclonal and monoclonal antibodies particularly CD4 antibodies to confirm the presence of T helper cells suggested by the present study.

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Paper I

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Localization of natriuretic peptides in the cardiac pacemaker of Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

This study describes the location of the primary pacemaker at the sino-atrial (SA) junction and the localization of salmon cardiac peptide (sCP) and ventricular natriuretic peptide (VNP) in Atlantic salmon (*Salmo salar* L.). The pacemaker tissue appeared lightly stained and composed of: (1) wavy nerve bundles with oval elongated wavy appearing nuclei with pointed ends, (2) ganglion cells (12–22 μm) with granular cytoplasm and (3) wide muscle fibers with large nuclei (modified cardiomyocytes) clearly distinguishing them from the other myocardial cells. Pacemaker tissue was further evaluated using immunohistochemical staining. Immunoreactivity of natriuretic peptides (sCP and VNP) antisera showed specific staining in pacemaker ganglion cells in addition to the cardiomyocytes. Positive staining with anti-CD3ε antisera in the pacemaker ganglion cells is a novel finding in teleosts and is consistent with observations in mammals. In conclusion, the Atlantic salmon pacemaker was shown to be located at the SA node and to harbor sCP and VNP peptides, suggesting a possible neuromodulatory and/or neurotransmitter role for these cardiac hormones within the teleost heart.

Introduction

The S-shaped teleost fish heart consists of four serially arranged chambers that include: (1) the *sinus venosus*, (2) the atrium, (3) the ventricle and (4) the *bulbus arteriosus* (Randall, 1968). The heart shape, in particular the ventricle in fish, is highly variable depending upon specific functional needs. The ventricle is composed of an inner trabecular layer (spongy layer as in the atrium) and an outer non-trabecular (compact) layer. Compared to the ventricle, the atrium is a thin trabecular chamber with an irregular sac-like shape. The atrium forms a connection between the *sinus venosus* and the ventricle by the atrio-ventricular (AV) valve that ensures a unidirectional flow of blood. The *sinus venosus* is predominantly composed of connective tissue. It is related to the initiation and control of heartbeat and serves as a site for the cardiac pacemaker in many fishes (Farrell and Jones, 1992; Jobling, 1995; Olson and Farrell, 2006; Poppe and Ferguson, 2006).

Ectotherms lack a well-formed cardiac conduction system in contrast to endotherms. It has been suggested that the cardiac conduction system evolved later in endothermic animals (Sedmera et al., 2003; Solc, 2007). The mammalian cardiac conduction system consists of three main parts, the sino-atrial node (SA node or 'pacemaker'), the atrio-ventricular node (AV node) and the His-Purkinje system (which is absent in fish). The action-potential starts in the autonomous pacemaker cells and propagates impulses to other parts of the heart. Irregular and slow contraction rates have been reported in the absence of specialized pacemaker cells. The pacemaker is composed of intracardiac postganglionic nerve cell bodies (ganglion cells) and a network of nerve fibers and is responsible for the initiation of the heart beat (Laurent et al., 1983; Farrell and Jones, 1992; Voranen et al., 2002; Boyett, 2009; Zaccone et al., 2011). Specific conduction system-like cells have been identified mainly at the junction of the SA area, but also have been reported at the atrio-ventricular (AV) funnel and atrio-ventricular (AV) junction (Saito, 1969; Zaccone et al., 2009a,b, 2011).

Abbreviations: AEC, 3-amino-9-ethyl carbazole; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; BSA, bovine serum albumin; CNP, C-type natriuretic peptide; DAB, 3,3′-diaminobenzidine; dH_2O, distilled water; AV, atrio-ventricular; H&E, hematoxylin and eosin; NP, natriuretic peptides; PBS, phosphate buffered saline; PBST, PBS with 0.1% Tween 20; PVA, polyvinyl alcohol; PVDF, polyvinylidene fluoride; rt-ANP, recombinant rainbow trout atrial natriuretic peptide; SA, sino-atrial; sCP, salmon cardiac peptide; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCR, T cell receptors; VNP, ventricular natriuretic peptide.

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Histologically, the pacemaker tissue has been identified at the junction of the atrium and the *sinus venosus* in several fish species such as rainbow trout, cod, eel, plaice and bream (Yamauchi and Burnstock, 1968; Saito, 1969; Santer, 1972; Laurent et al., 1983; Lukyanov and Sukhova, 1983; Poppe and Ferguson, 2006; Solc, 2007). Haverinen and Vornanen (2007) described the pacemaker as a ring of specialized cardiac tissue (nodal tissue) at the sino-atrial (SA) junction in rainbow trout (*Oncorhynchus mykiss*). This study mainly focused on the electrophysiology of the pacemaker without investigating the tissue morphology of the pacemaker. A connective tissue sheet surrounded and infiltrated the nodal tissue to divide it into smaller areas. Nerves were identified at the periphery and in the nodal tissue at the sino-atrial junction. The identification and localization of pacemaker cells is ambiguous and one of the reasons was based on their smaller size (~0.2 mm in width, diameter and ~3 mm in length) in a 200–300 g rainbow trout at the sino-atrial junction (Haverinen and Vornanen, 2007). Pacemaker tissue appears lightly stained by hematoxylin and eosin (H&E) staining with wider muscle fibers and larger nuclei clearly separated by loose connective tissue from the rest of the myocardial cells (Yamauchi and Burnstock, 1968; Sedmera et al., 2003; Haverinen and Vornanen, 2007; Solc, 2007).

Natriuretic peptides (NP) are a group of hormones originally described by de Bold et al. (1981), synthesized and secreted mainly by the heart. The four NP members identified in vertebrates include: (1) atrial NP (ANP), (2) B-type NP (BNP), (3) C-type NP (CNP) and (4) ventricular NP (VNP). VNP has been identified so far in the heart of teleosts (atrium and ventricle) such as eel, sturgeon and salmonid (rainbow trout) with the VNP gene sequence highly conserved in these three fishes (Takei, 2000; Inoue et al., 2005). VNP is a unique peptide with a long COOH-terminal tail sequence and has vasoactive and renal effects owing to its high affinity to both NPR-A and NPR-B receptors (Katafuchi et al., 1994; Takei, 2000). The specific antibodies against synthetic salmon cardiac peptide (sCP), which is structurally and functionally similar to mammalian atrial natriuretic peptide (ANP), have been used to localize and quantify the sCP in the atrium and ventricle of Atlantic salmon (Tervonen et al., 1998; Arjamaa et al., 2000; Vierimaa et al., 2006). Immunohistochemical localization of ANP has been identified in the intra-cardial ganglion cells and nerve fiber varicosities in the bovine conduction system (Hansson and Forsgren, 1993). These findings were further demonstrated in human, cow, sheep, pig and rat. Mammalian nerve fiber varicosities have been reported to contain ANP as demonstrated by immunohistochemistry in the proximity of conduction cells of the AV node and the AV bundle and occasionally in the SA node, bundle branches, septomarginal trabeculae and false tendons (Hansson and Forsgren, 1993, 1994; Hansson et al., 1998; Hansson, 2002). ANP has been suggested to be synthesized in the mammalian cardiac conduction system and increased ANP expression has been reported associated with heart diseases or cardiac sympathectomy (Mochizuki et al., 1991; Hansson et al., 1998). Immunolocalization of salmon cardiac peptide (sCP) and ventricular NP (VNP) has been established in atrial and ventricular myocytes (Arjamaa et al., 2000; Loretz et al., 1997), however, the localization in the cardiac conduction system (pacemaker) of Atlantic salmon has not been described.

The CD3 molecule plays an important role in signal transduction in T cell receptors (TCR) complex and is a specific marker for T lymphocytes (Wang et al., 2009; Koppang et al., 2010). Besides its role as a T lymphocyte marker, CD3 antigen has also been identified in gastric parietal cells, renal tubular epithelial cells and cerebellar Purkinje cells in several species including humans (Garson et al., 1982; Alroy et al., 2005). Anti-CD3ε antibody has been prepared and validated as a pan T cell marker in various Atlantic salmon tissues including the heart (Koppang et al., 2010), but the additional roles of CD3 antibody, besides that of T cell marker, are still undetermined in teleosts. To answer this question, we used the salmon CD3ε antibody to identify the cardiac pacemaker localization of Atlantic salmon by immunohistochemistry. Recently cloned genes and antibodies against these genes (sCP, VNP and CD3ε) in Atlantic salmon have provided us with tools to identify their localization in relation to the cardiac pacemaker of Atlantic salmon.

In this study, the morphology and location of the cardiac pacemaker tissue and the immunohistochemical distributions of sCP, VNP and CD3ε were demonstrated using specific antibodies against sCP, VNP and CD3ε respectively in the pacemaker of Atlantic salmon (Tervonen et al., 1998; Koppang et al., 2010).

Materials and methods

Histology

The hearts from naive Atlantic salmon weighing between 0.5 and 3 kg were collected from two sea farms ($n = 20 + 30$) Wenberg Fiskeoppdrett, Fauske and Gildeskål Forskningsstasjon AS (GIFAS), Gildeskål, respectively. Fish were maintained from smolts for approximately 1–3 years with an average weight range (0.7–2.5 kg) at the Mørkvedbukta research station, Bodø, Norway in 2 m^3 tanks supplied with fresh ambient sea water (range 7–8 °C) and fed 0.7% of body weight with a commercial pelleted diet (Spirit, Skretting, Stavanger, Norway) three times weekly ($n = 20$). All fish were sacrificed by a blow to the head. Fish were carefully dissected and the heart removed from the pericardial cavity, ensuring that the *sinus venosus* along with the atrium were included in the samples. Hearts were fixed in 10% neutral phosphate buffered formalin solution (Sigma–Aldrich Norway AS, Oslo, Norway) and processed by standard protocols for histological procedures (dehydration, embedding in paraffin wax, sectioning 3 μm thick, and staining). Additional staining procedures such as Gomori's methenamine silver stain (Sigma–Aldrich, Norway) and cresyl violet (Sigma–Aldrich, Norway) as described elsewhere (Downing, 1992) were performed to demonstrate nodal tissue (nerve fibers and ganglion cells).

Immunostaining

Immunostaining was performed according to Haugarvoll et al. (2008) with slight modifications. Briefly, 3 μm cardiac sections ($n = 3–7$) were mounted on poly-L-lysine coated slides (Sigma–Aldrich, Norway) and dried at 50 °C for 30 min. Sections were deparaffinized with xylene followed by a graded series of ethanol. Antigen unmasking was undertaken by autoclaving the slides at 121 °C for 21 min in 10 mM citrate buffer, pH 6.0 containing 0.1% Tween 20 (Sigma–Aldrich, Norway). Slides were kept at room temperature for 20 min and then washed twice with distilled water (dH_2O) for 2 min. All incubations were performed in a closed-lid humidity chamber. To inhibit endogenous peroxidase activity, the slides were incubated with 3% H_2O_2 (Sigma–Aldrich, Norway) in methanol for 10 min at room temperature. Then slides were washed twice with phosphate buffered saline (PBS). The sections were incubated in 5% bovine serum albumin (BSA) (Sigma–Aldrich, Norway) in PBS for 1 h. After BSA removal, slides were incubated with primary antibodies such as polyclonal rabbit anti-salmon CD3ε antibody (Koppang et al., 2010), polyclonal goat anti-salmon cardiac peptide (sCP) serum (Pelle-210497) (Tervonen et al., 1998; Arjamaa et al., 2000) and polyclonal goat anti-vendace ventricular natriuretic peptide (VNP) serum (F978-250701) overnight at 4 °C. Following incubation, slides were washed three times with PBS with 0.1% Tween 20 (PBST) (Sigma–Aldrich, Norway), pH 7.4 for 5 min each. Slides were incubated with secondary antibodies (sc-2020, donkey anti-goat IgG, Santa Cruz Biotechnology, Santa Cruz,

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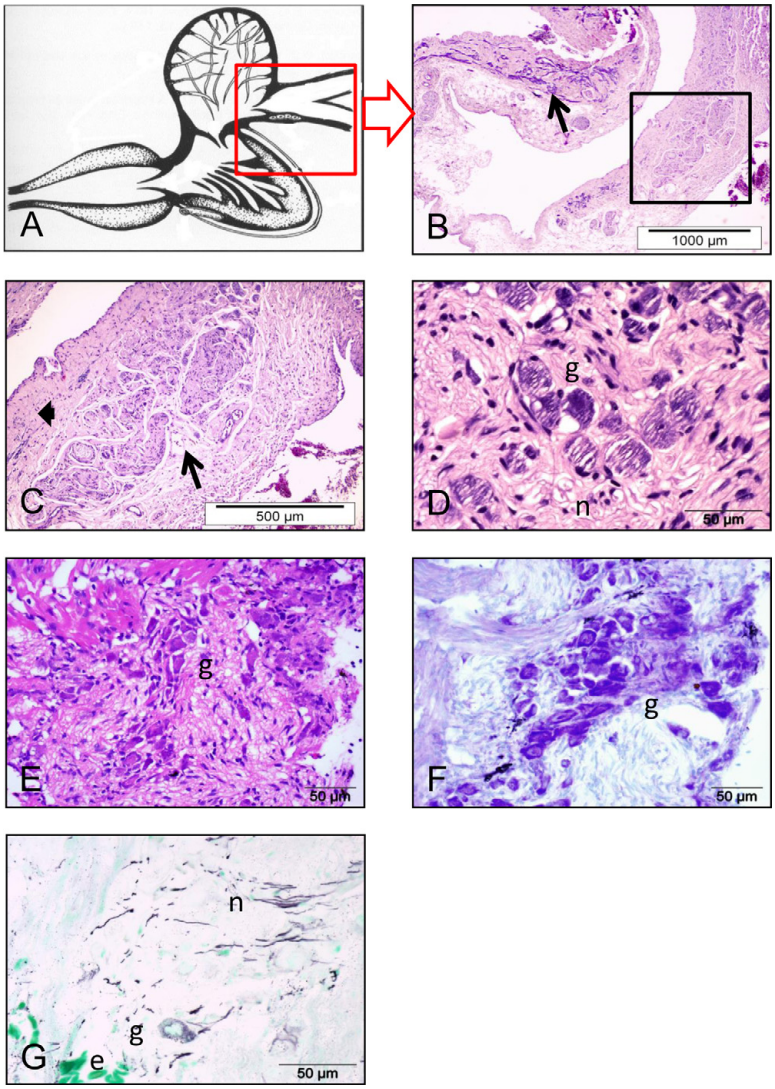

Fig. 1. Diagram and micrographs of the cardiac pacemaker area at the sino-atrial junction. (A) A schematic diagram of Atlantic salmon heart and area for histological and immunological investigation (within the red box). (B) Low power view of sino-atrial (SA) junction and area within black box is shown in (C). Arrow: elastic tissue. (C) Enlarged image of selected part (black box) of pacemaker area from (B). Arrow head: cardiomyocytes, arrow: modified cardiomyocytes. (D) Distribution of ganglion cells (12–22 µm in size) and nerves in pacemaker tissue. n, nerve; g, ganglion cells. (E and F) Cluster of ganglion cells identified by H&E and cresyl violet staining respectively. g, ganglion cells. (G) Nerve fibers and ganglion cells were identified by Gomori's methenamine silver staining. e, erythrocytes; g, ganglion cells; n, nerve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CA, USA or goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) in 1.5% BSA in PBS for 30 min at room temperature. Slides were washed three times with PBST at room temperature for 5 min each. Slides were incubated with 3,3′-diaminobenzidine (DAB; Sigma–Aldrich, Norway) or 3-amino-9-ethyl carbazole (AEC; Sigma–Aldrich, Norway) for 5 min and then washed with distilled water (dH_2O) for 5 min. Slides were dipped in hematoxylin for 10 sec for counterstaining and then dehydrated in a graded series of ethanol for 10 sec each followed by xylene and mounted with polyvinyl alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway) or ImmunoHistoMount (Sigma–Aldrich, Norway). Negative controls included primary antibodies replaced with 1.5% BSA in PBS while specificity was confirmed using pre-immune serum (CD3), and pre-adsorption controls in which primary antibody (sCP antiserum) was incubated with sCP antigen and/or recombinant rainbow trout atrial natriuretic peptide (rt-ANP) (10^{-4} mol L^{-1} and

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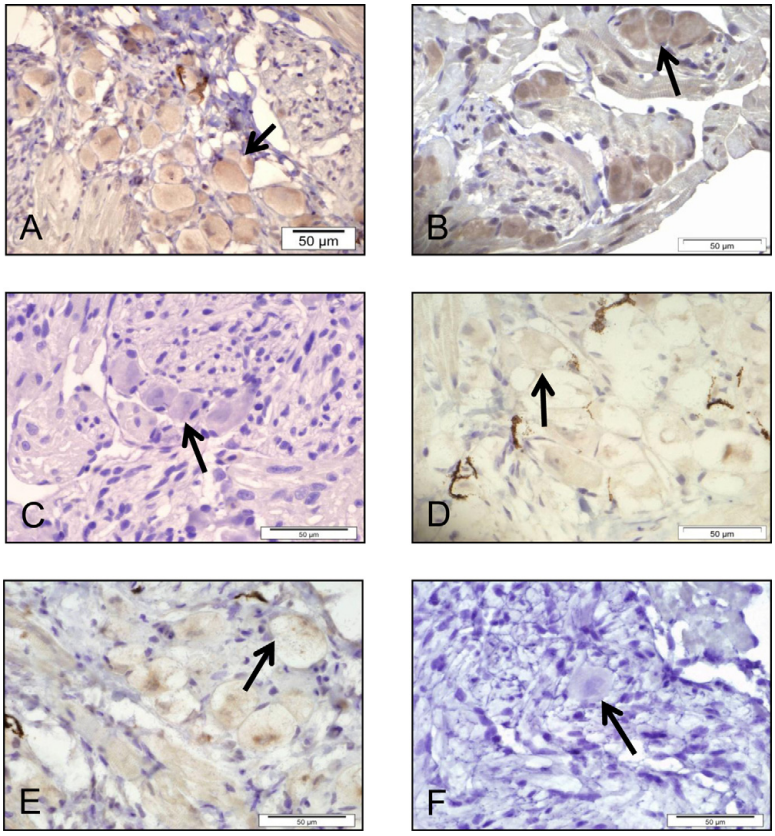

Fig. 2. Natriuretic peptides immunostaining in the cardiac pacemaker tissue of Atlantic salmon. (A) Anti-salmon cardiac peptide (sCP) and (B) anti-vendace VNP immunostaining of ganglion cells in pacemaker. Arrow: ganglion cells. (C) Negative control by replacing primary antibody with 1.5% BSA in PBS. (D and E) Pre-adsorption test using 10^{-6} mol L^{-1} rt-ANP and sCP antigen identified weak staining in ganglion cell. (F) No was staining identified at 10^{-4} mol L^{-1} (rt-ANP). Arrow: ganglion cells.

10^{-6} mol L^{-1}) at 4 °C overnight. Slides were evaluated by light microscopy using an Olympus microscope BX51 equipped with Cell^{B} software (Olympus Corporation, Tokyo, Japan).

Western blot

Approximately 30 mg of three Atlantic salmon frozen hearts were minced on ice and transferred to tubes containing RIPA buffer (150 mmol L^{-1} NaCl, 50 mmol L^{-1} Tris–HCl at pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) and sonicated until the tissue was dissolved at 4 °C. The suspension was collected, centrifuged at 13,000 × *g* for 30 min at 4 °C and the supernatant collected. 50 μg supernatant was used and separated by SDS-PAGE 12% separating gels with 4% stacking gels using a Mini Protean Tetra Cell (BioRad, Hercules, CA, USA) at 100 V for 100 min by following protocol described by Laemmli (1970). Samples were blotted on polyvinylidene fluoride (PVDF) membrane and incubated with vendace VNP antiserum (1:1000 or 1:500) overnight at 4 °C and visualized with 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, Norway). Negative control included primary antibody replaced with 1% BSA in PBS with 0.5% Tween 20 (PBST) (Sigma–Aldrich, Norway).

Results

Histology

Pacemaker (nodal tissue) seen as discrete bundles was identified at the sino-atrial (SA) junction with evident *sinus venosus* elastic tissue (Fig. 1A and B). Nodal tissue was separated from surrounding tissue by loose connective tissue, which also infiltrated the nodal tissue, dividing it in several small semicircular areas. The densely innervated myocardial cells were identified in the atrial region close to the junction of the *sinus venosus* and the atrium (Fig. 1C). The pacemaker was located sub-endocardially and was composed of three cell types: (1) plexiform modified muscle cells that appeared more lightly stained than the atrial myocardial cells; (2) wavy appearing nerve bundles of lightly stained fibers with oval, wavy, elongated nuclei with pointed ends; (3) large round to pear-shaped postganglionic nerve cell bodies (ganglion cells) (12–22 μm) with a granular cytoplasm and nuclei with prominent nucleoli were also identified in the pacemaker tissue (Fig. 1C and D). The clusters of ganglion cells were identified by H&E and cresyl violet staining at the SA junction (Fig. 1E and F). The nerve fibers and ganglion cells were also identified with Gomori's methenamine silver stain at the SA junction (Fig. 1G).

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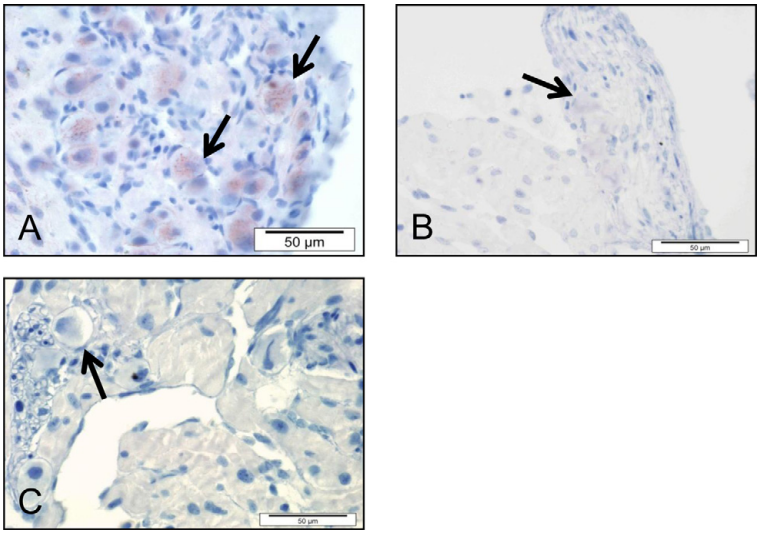

Fig. 3. Anti-CD3ε immunostaining in the cardiac pacemaker tissue of Atlantic salmon. (A) Ganglion cells of pacemaker showed granular cytoplasmic CD3ε staining. (B and C) No staining was observed using either pre-immune serum or primary antibody replaced with 1.5% BSA in PBS, respectively. Arrow: ganglion cells.

Immunostaining

Natriuretic peptides immunostaining using salmon cardiac peptide (sCP) and vendace ventricular natriuretic peptide (VNP) antisera showed specific staining in the intracardiac ganglion cells (Fig. 2A and B). Negative controls in which the primary antibodies were replaced with BSA did not stain cardiomyocytes, ganglion cells or nerve fibers (Fig. 2C). Pre-adsorption controls showed the staining in a dose-dependent manner where the sCP antiserum blocked with sCP antigen and 10^{-6} mol L^{-1} recombinant rainbow trout ANP identified weak staining in the ganglion cells respectively (Fig. 2D and E). However, the 10^{-4} mol L^{-1} recombinant rainbow trout ANP did not identify any staining in the pacemaker tissue (Fig. 2F). Anti-CD3ε immunostaining was evident (granular staining) in the ganglion cells (Fig. 3A). Pre-immune CD3ε serum or BSA did not identify any positive staining in the pacemaker tissue respectively (Fig. 3B and C).

Western blot

The specificity of polyclonal goat anti-vendace VNP antibody was tested on the heart tissue of Atlantic salmon to confirm the cross reactivity. The VNP antibody identified a single band of approximately 14 kDa in the heart. No visible band could be identified in the negative control (Fig. 4).

Discussion

The present study identified the cardiac pacemaker of Atlantic salmon at the sino-atrial junction, in agreement with other closely related salmonids such as brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) as well as in Atlantic cod (*Gadus morhua*), catfish, mullet (*Mugil cephalus*), Nile catfish (*Synodontis nigriventris*) and bichir (*Polypterus bichir bichir* Geoffory St. Hillaire, 1802) (Yamauchi and Burnstock, 1968; Lukyanov and Sukhova, 1983; Haverinen and Vornanen, 2007; Zaccone et al., 2009a,b). Tissue identified as the pacemaker was visible only in the hearts where the sino-atrial junction was included in the sections. Pacemaker tissue was presented as several nerve bundles surrounded by loose connective tissue that also infiltrated the tissue, dividing it into smaller semicircular areas. Pacemaker tissue was composed of ganglion cells, innervated with network nerve fibers and modified cardiomyocytes identified within and around the nodal tissue and was in agreement with the rainbow trout pacemaker (Haverinen and Vornanen, 2007). The cluster of ganglion cells and nerves were also identified by Gomori's methenamine and cresyl violet stains at the junction of the *sinus venosus* and atrium. The morphological study of the pacemaker tissue was important owing to its functional significance. The pacemaker is the area where excitation is initiated and leads to the activation of all cells of the heart during contraction (Voranen et al., 2002). No nerve bundles or intracardiac ganglion cells were identified in the ventricle of Atlantic salmon and this is in line with other studies where conducting tissue bundles have not been identified by histology in the ventricle of the fish (Solc, 2007).

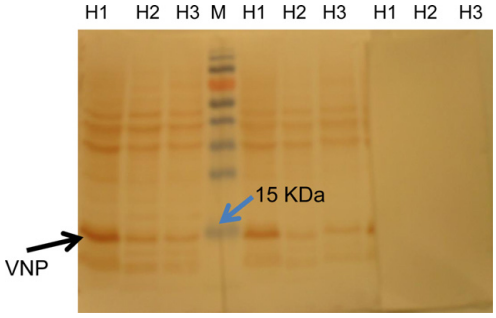

Fig. 4. Western blot analysis of heart tissues of Atlantic salmon ($n = 3$) by using polyclonal goat anti-vendace VNP antibody (1:1000 and 1:500). Negative control was performed by replacing primary antibody with 1% BSA in PBST. Lanes 1–3 were incubated with polyclonal goat anti-vendace VNP (1:500), lanes 4–6 incubated with polyclonal goat anti-vendace VNP antibody (1:1000) and lanes 8–10 incubated with 1% BSA in PBST. H1: heart 1, H2: heart 2, H3: heart 3, M: PageRuler™ prestained protein ladder (SM0671, Fermentas GmbH, St. Leon-Rot, Germany).

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Recently, CD3ε antiserum was prepared and validated in the different tissues of Atlantic salmon including the heart (Koppang et al., 2010). The CD3 antibody normally recognizes all mature T cells. Immunostaining using the above described CD3ε antiserum identified specific staining in the ganglion cells of Atlantic salmon pacemaker. To the best of our knowledge, novel anti-CD3ε$^+$ immunostaining in the cardiac pacemaker tissue has not been reported previously in teleosts. Such immunostaining in the ganglion cells was in agreement with the previous studies where normal Purkinje cells of the cerebellum showed anti-CD3$^+$ immunostaining in several mammals such as human, mouse, rat, quail and guinea pig (Garson et al., 1982). The CD3 antigen was formerly considered specific for T lymphocytes, but it has also been identified in gastric parietal cells and renal tubular epithelial cells of several species (Alroy et al., 2005) indicating additional roles of CD3 besides T cell identification. Anti-CD3ε immunostaining in teleost ganglion cells shares a similar pattern to that of mammals and requires further studies to determine its functional significance.

Natriuretic peptides (NP) play important cardioprotective and fluid homeostatic roles. ANP is mainly released by atrial stretch and acts on several organs including the brain, heart, gills, intestine, kidney and interrenal tissue to perform different functions (Loretz and Pollina, 2000; Toop and Donald, 2004; Johnson and Olson, 2008). Salmon cardiac peptide (sCP) has been isolated from atrial and ventricular myocytes (Tervonen et al., 1998) and its localization and specificity have been demonstrated in Atlantic salmon cardiomyocytes (Arjamaa et al., 2000). The present study used the same antiserum (sCP) and identified the localization of sCP in the cardiac pacemaker tissue of Atlantic salmon where anti-sCP immunostaining was evident. However, the functional significance of sCP and VNP in the pacemaker is still undetermined. However, vendace VNP antiserum also identified positive immunostaining in the ganglion cells of the Atlantic salmon pacemaker. The specific cross-reactivity of anti-vendace VNP antibody to Atlantic salmon heart tissue was confirmed by Western blot and identified approximately 14 kDa band and is in line with eel proVNP (Takei et al., 1994). The non-specific bands were faintly visible at higher primary antibody dilutions (1:1000) as compared to lower dilutions (1:500) and can be reduced by further titration of primary antibody. The recombinant rainbow trout ANP (rt-ANP) was used for pre-adsorption controls due to its structural and functional similarities to sCP (Tervonen et al., 1998; Vierimaa et al., 2006) in addition to sCP antigen which blocked the sCP antiserum in a dose-dependent manner and identified no staining or weak staining from higher to lower doses of rt-ANP respectively.

Immunohistochemical localization of natriuretic peptides has been established in the brain and heart of Atlantic hagfish (*Myxine glutinosa*) and gulf toadfish (*Opsanus beta*). Anti-sCP and VNP sera identified neural tissue (ganglion cells) and were in agreement with the previous studies which identified natriuretic peptide immunoreactive perikarya in the different regions of the brains of several fishes such as Atlantic hagfish (*Myxine glutinosa*), spiny dogfish (*Squalus acanthias*), gulf toadfish (*Opsanus beta*), African lungfish (*Protopterus annectens*) and cartilaginous elasmobranch fish (*Scyliorhinus canicula*) (Donald and Evans, 1992; Donald et al., 1992; Vallarino et al., 1990, 1996), supporting the localization of natriuretic peptides (sCP and VNP) in the heart neural tissue (pacemaker). The specific neural tissue binding sites using ^{125}I-rat ANP and NPR-A receptors have been shown in the different regions of the brain of Atlantic hagfish and eel (*Anguilla japonica*) (Donald et al., 1999; Tsukada et al., 2007) suggesting the existence of NPs binding sites and receptors in the cardiac pacemaker (ganglion cells). Several piscine studies have suggested the neuromodulator and/or neurotransmitter roles of NPs in addition to cardioprotective/osmoregulatory functions (Donald and Evans, 1992; Donald et al., 1992; Vallarino et al., 1996; Tsukada et al., 2007). Similarly, the mammalian studies have shown the localization of NPs in the cardiac conduction system (Hansson, 2002).

Mammalian ganglion cells and nerve fiber varicosities have been shown to exhibit specific immunostaining for ANP at the SA node area (Hansson and Forsgren, 1993; Hansson et al., 1997). ANP was also shown to be synthesized in the cardiac conduction system of normal rats and the increased ANP levels were recorded after cardiac sympathectomy (Hansson et al., 1998). Although, the effects of NPs (sCP and VNP) were not investigated on pacemaker tissue in the current study, preliminary human studies have proposed that ANP may act in an autocrine/paracrine fashion on closely found conduction cells to influence the pacemaker velocity (Hansson et al., 1998). Active receptors were suggested in Purkinje fibers to fulfill the functions (Hansson et al., 1998; Hansson, 2002).

Recently the specific binding sites for neurotransmitters such as substance P and galanin (GA) have been shown in the pacemaker tissue at the SA junction in ray-finned fish (bichir) and teleosts (mullet and Nile catfish) (Zaccone et al., 2009a,b), supporting the existence of NPs neural tissue binding sites and possible effects on the cardiac pacemaker tissue. The identification of NPs and their binding sites in piscine neural tissue (brain) supported the localization of sCP and VNP in the cardiac pacemaker tissue and suggested additional neurotransmitter and/or neuromodulator role(s) of piscine NPs (sCP and VNP) particularly in relation to the cardiac pacemaker. Immunohistochemical localization of CD3ε and natriuretic peptides (sCP and VNP) in teleost (Atlantic salmon) shared a similar fashion to mammalian counterparts and requires further studies to find its significance in teleosts (Hansson, 2002; Tsukada et al., 2007).

This study provides a detailed morphological description of cardiac pacemaker in Atlantic salmon and demonstrated the novel localization of piscine CD3ε and natriuretic peptides (sCP and VNP) in the pacemaker tissue by immunohistochemistry. The presence of these peptides in the pacemaker tissue suggests their potential neurotransmitter and/or neuromodulatory role for sCP and VNP in the heart conduction system (Hansson and Forsgren, 1994; Hansson, 2002).

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Paper II

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Research Article

The Effects of Heart and Skeletal Muscle Inflammation and Cardiomyopathy Syndrome on Creatine Kinase and Lactate Dehydrogenase Levels in Atlantic Salmon (*Salmo salar* L.)

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Heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) are putative viral cardiac diseases of Atlantic salmon. This study examined the levels and correlated the serum enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) to the histopathology of clinical outbreaks of HSMI and chronic CMS in farmed Atlantic salmon. A total of 75 fish from 3 different HSMI outbreaks, 30 chronic CMS fish, and 68 fish from 3 nondiseased fish groups were used as the study population ($N = 173$). Serum CK and LDH levels correlated significantly with the total inflammation and total necrosis scores for HSMI fish ($P = 0.001$). However, no correlation was identified for enzyme levels and histopathology scores for chronic CMS fish. The significantly increased CK and LDH levels and their positive correlations to histopathology differentiate HSMI from CMS clinically suggesting the potential use of enzymes for screening for HSMI is promising.

1. Introduction

The marine farmed Atlantic salmon (*Salmo salar* L.) exhibits a variety of cardiac diseases, and the reason for this likely includes low activity in relatively confined spaces, continuous food supply, low oxygen level, crowding, stress in handling, and temperature [1]. The cardiac anomalies and defects of Atlantic salmon include aplasia or hypoplasia of the septum transversum, abnormal location and shape of heart [1], arteriosclerosis [2, 3], and ventricular hypoplasia [4], but specific diseases include cardiomyopathy syndrome (CMS) [5–7], pancreas disease (PD) [8–10], and heart and skeletal muscle inflammation (HSMI) [11, 12]. Annual economical losses due to cardiomyopathy syndrome (CMS) alone have been estimated up to € 4.5–8.8 millions [7].

Heart and skeletal muscle inflammation (HSMI) is a disease of marine farmed Atlantic salmon reported from Norway, Scotland and Chile. HSMI is a disease which mainly affects heart and red skeletal muscle. It is typically a disease of moderate mortality (~20%) but high morbidity (~100%) that affects fish 5 to 9 months after transfer to sea. Presently, HSMI can be diagnosed by histopathology and presents as epi- and endocarditis as well as mononuclear cellular infiltration of both trabecular and compact layers of ventricle myocardium accompanied by myocytic necrosis [11–14]. HSMI is transmissible in laboratory studies by injecting tissue homogenate from diseased fish to healthy fish [11, 15], and recently piscine reovirus (PRV) has been suggested to be associated with HSMI infection [16, 17]. Lesions first appear and are more frequent in heart than red skeletal muscle. Affected myocytes show signs of degeneration, loss of cardiomyocytes striation and eosinophilia, loss of skeletal muscle striation, vacuolation, centralized nuclei, and karyorrhexis. There are more inflammatory changes as compared to necrotic changes in heart and red skeletal muscle [1, 12, 13]. HSMI has become more significant where outbreaks have increased from 54 in 2004 [18] to 162 cases reported in 2011 [19].

Cardiomyopathy syndrome (CMS) is a cardiac disease of Atlantic salmon with a suggested totiviral etiology [20] that mainly affects atrium and trabecular ventricle myocardium without involvement of skeletal muscle. It shares similar

features with HSMI where both cause myocarditis [1]. Histopathological changes include necrosis and inflammation of trabecular layer of ventricle and atrium, epicarditis, cellular infiltrates of mainly mononuclear lymphocytes and macrophages, and rupture of atrium or sinus venosus macroscopically [1, 5]. CMS affects adult salmon after 12–18 months of sea transfer, and recently a totivirus (piscine myocarditis virus (PMCV)) is proposed as causative agent for cardiomyopathy [5, 17, 20, 21]. The piscine myocarditis virus is a double-stranded RNA virus with diameter of 50 nm and 6688 bp genome size [21]. The haematological tests and serum analysis for fish, compared with other areas of veterinary medicine, are not common place compared to higher vertebrates due to the lack of reference values for clinical chemistry (physiological and pathological) and understanding of disease pathogenesis and pathophysiology [22–24]. There is a strong need for blood biochemistry ranges especially where the etiology is unclear (HSMI and CMS) since the associated viruses can be present asymptomatically [17, 25]. Biochemical enzymes such as creatine kinase (CK) and lactate dehydrogenase (LDH) are well-established biomarkers of cardiac disease in humans [26] and are often used in conjunction with other hormonal biomarkers for a myocardial dysfunction diagnosis [27]. Both enzymes are released upon cellular degeneration such as necrosis. Lactate dehydrogenase (LDH) is involved in the interconversion of pyruvate and L lactate during the final reactions of glycolysis and is present in the cytoplasm of all cells (nucleated and nonnucleated cells). In humans, raised LDH plasma values are observed from 8 to 12 h, peaking within 2–3 days, and levels are sustained for duration of 7–10 days following cardiac injury [28]. Creatine kinase (CK), on the other hand, is found in the myocyte cytoplasm, sarcoplasmic reticulum, mitochondria, and myofibrils with a half-life of about 12 h in humans. Creatine kinase levels in blood plasma rise from 4 to 6 h peaking at 12–36 h and sustained over 3-4 days in humans where a cardiac injury has occurred [28]. The creatine kinase concentrations are related to the irreversible injury associated with myocardial necrosis in mammals (dogs) [29]. The elevated CK levels have been reported in association with myocytic necrosis seen in pancreas disease (PD) in Atlantic salmon [30, 31], and these results suggested that CK could be a useful candidate indicator of cardiac diseases in Atlantic salmon.

The aims of the present study were to measure the serum CK and LDH levels and examining their relationship to the cardiac diseases (HSMI and CMS) of Atlantic salmon.

2. Material and Methods

2.1. Fish Sampling. Seven groups of Atlantic salmon (*Salmo salar* L.) were involved in this study. All samples from diseased fish were taken from the farms diagnosed with both diseases by National Veterinary Institute, Oslo, Norway (NVI) and further histopathology was performed to confirm the disease diagnosis during the study. Group 1 ($n = 28$) consisted of fish (S1) collected from a confirmed HSMI outbreak at a sea site during the peak mortality of the HSMI episode. Group 2 ($n = 16$) comprised fish (S0) collected from a confirmed HSMI sea cage outbreak two months after peak mortality period. Group 3 ($n = 31$) included fish (S0) from a sea cage site collected during the early onset of a HSMI outbreak. The clinical phase of the disease has been defined as the time period with increased mortality at farm due to HSMI [32]. The disease phase was determined from the peak mortality time at the farm [16]. All diseased groups were in the sea phase of salmon production and opportunistic samples collected from diseased cages on each farm. All three diseased farms were widely distant from each other in Nordland county, Norway. Group 4 ($n = 30$) included chronic CMS infected fish (S2) and had confirmed CMS outbreak in the past, and sampling was performed 6 months after CMS outbreak. Diseased groups included fish with average weight range (600–1000 g) for HSMI and (6000–7000 g) for CMS fish.

Group 5 ($n = 28$) was nondiseased fish which were taken from a study where Atlantic salmon (S0) had been made anaemic using phenylhydrazine, and cardiac hypertrophy had been characterized [33]. Group 6 ($n = 20$) consisted of nondiseased, apparently healthy fish (S0 + S1) kept in a laboratory facility (University of Nordland, Mørkvedbukta Research Station, Bodo, Norway) in 2 m^3 tanks with fresh ambient sea water (temperature range 7-8°C) and fed 0.7% commercial feed (Spirit, Skretting, Stavanger, Norway) of their body weight three times weekly. Group 7 ($n = 20$) comprised of apparently healthy, nondiseased Atlantic salmon (S1) from the sea cages. Nondiseased groups included fish with average weight range (400–2000 g).

2.2. Blood Collection. All fish were killed by a blow to the head or overdose of tricaine methanesulfonate (MS222) (100 mg mL^{-1}). Blood was collected immediately from the caudal vein with a 5 mL syringe using 23 G needle, allowed to clot in Eppendorf tubes for 2–4 h, centrifuged at 8,000 g for 5 min, and the serum collected except group 4 where heparinised blood plasma was collected and frozen at −20°C.

2.3. Serum Analysis. All samples were frozen and sent on dry ice to Norwegian School of Veterinary Sciences, Oslo Central Laboratory and to the Nordland Hospital, Department of Medicine Biochemistry, Bodo for creatine kinase (CK) and lactate dehydrogenase (LDH) analysis. Creatine kinase (CK) and lactate dehydrogenase (LDH) were measured by using ADIVA 1650 (Siemens Medical Solution Diagnostics Inc., Tarrytown, NY, USA) at Norwegian school of veterinary sciences, Oslo central laboratory and ADVIA 1650/1800 (Bayer Diagnostics, Tarrytown, NY, USA) at Nordland Hospital, Department of Medicine Biochemistry, Bodo on the basis of their enzyme activity and measured by increase in absorbance at 340/410 nm. Both laboratories used the same methods. Briefly the principle of the procedures for LDH and CK is as follows: LD catalyzes the conversion of L lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD). The enzyme activity of LD is proportional to the rate of production of NADH (reduced NAD). Creatine kinase reacts with creatine phosphate and

ADP to form ATP which is coupled to the hexokinase-G6PD reaction, generating NADPH. The concentrations of NADH and NADPH were measured by the increase in absorbance at 340/410 nm for LDH and CK, respectively.

2.4. Histopathology and Scoring Method. To correlate biochemical enzymes (CK and LDH), histopathology was method of choice for diagnosis of HSMI. Hearts, skeletal red and white muscle from below the dorsal fin and above the lateral line, and other vital organs, were collected and fixed in 10% neutral phosphate-buffered formalin solution. External and internal visual examination was performed in addition to histological observation of other vital organs for other abnormalities or signs of overt disease in the fish. Tissues were processed by a standard paraffin wax protocol (dehydrated, embedded in paraffin, 3 μm thick sectioned and H and E stained) and examined for changes (necrosis and inflammation) characteristic of HSMI [11]. The case definition for HSMI includes inflammation and necrosis of trabecular and compact ventricle myocardium, epicarditis, endocarditis, mononuclear inflammatory cell infiltration, and a higher level of inflammation compared with necrosis while supportive signs may also include inflammation and necrosis of red skeletal muscle, atrium, and absence of pancreatic lesions [32]. A semiquantitative assessment of each slide was adapted from McLoughlin et al. [34] for scoring histopathological findings in heart and muscle tissues obtained from seven groups of fishes used in this study. It has been used and established in PD, a similar cardiac disease to HSMI and CMS (Table 1).

2.5. Different Anatomical Regions. The scoring method was used for the detailed study of the tissue in anatomically distinct areas of the heart and skeletal muscle: (1) atrial trabecular myocardial inflammation, (2) and necrosis, (3) atrial epicarditis, (4) ventricle compact myocardial inflammation, (5) and necrosis, (6) ventricle trabecular myocardial inflammation, (7) and necrosis, (8) ventricle epicarditis, (9) skeletal muscle inflammation, (10) and necrosis. A total inflammation score was determined from the summed scores of all parameters (atrial and ventricular trabecular inflammation, atrial and ventricular epicarditis, ventricle compact inflammation, and skeletal muscle inflammation) and total necrosis score determined from the summed scores of all parameters (atrial and ventricular trabecular necrosis, ventricle compact necrosis, and skeletal muscle necrosis). The sum scores of inflammation and necrosis in heart and skeletal muscle were correlated with the biochemical enzymes (CK and LDH) levels. Total inflammation and total necrosis scores were correlated to the HSMI plus nondiseased fish and the CMS fish plus nondiseased fish to differentiate the enzymatic effects in each disease separately.

2.6. Slides Evaluation. Slides were evaluated blindly by two persons, histopathological results compared between the groups and correlated with respective biochemical enzymes values for each sample. Sometimes the conflict for slide score was around 0.5–1 between two persons, and then they agreed after discussing case definition and scoring system on most suitable score for the slide.

Table 1: Semiquantitative lesion scoring system adapted from McLoughlin et al. [34]. System covers heart and skeletal muscle lesions separately. Lesions starting from 0 (healthy tissue) to 3 (severe changes). (a) Heart lesion classification. (b) Skeletal muscle classification.

Score	Description
(a)	
0	Normal appearance
1	Focal myocytic necrosis ± inflammation (<50 fibers affected)
2	Multifocal myocytic necrosis ± inflammation (50–100 fibers affected)
3	Severe diffuse myocytic necrosis ± inflammation (>100 fibers affected)
(b)	
0	Normal appearance
1	Focal myocytic necrosis ± inflammation
2	Multifocal myocytic necrosis ± inflammation
3	Severe diffuse myocytic necrosis ± inflammation

2.7. Statistical Analysis. Spearman rank coefficient correlations were performed using SigmaPlot (10.0) and were considered statistically significant at P values ≤ 0.05. The mean histopathology (total inflammation and total necrosis scores) and enzyme (CK and LDH) values were analyzed using Kruskal-Wallis One Way Analysis of Variance on ranks with differences isolated using Dunn's post hoc analysis. Data were presented as mean ± SD.

3. Results

The highest and lowest mean CK values were identified in HSMI fish group 1 = 16479.25 ± 1844.49 IU.L^{-1} and nondiseased fish group 5 = 1581.71 ± 425.33 IU.L^{-1} respectively. However, the highest and lowest mean LDH values were identified in HSMI fish group 2 = 1838.25 ± 957.47 IU.L^{-1} and nondiseased fish group 5 = 235.39 ± 27.43 IU.L^{-1}, respectively. The mean CK and LDH values for CMS fish were (5207.93 ± 967.81 IU.L^{-1}) and (426.2 ± 60.68 IU.L^{-1}), respectively (Table 4).

Diseased group 1 (HSMI fish) had significantly the highest levels for CK activity as compared to other HSMI groups 2 and 3 (Kruskal-Wallis One-Way Analysis: H = 65.217, d.f. = 6; ($P \leq 0.001$) (Figure 2(a)). However, LDH activity levels were higher in HSMI fish (group 3) as compared to other HSMI fish (groups 1 and 2) (Kruskal-Wallis One-Way Analysis: H = 73.838, d.f. = 6; $P \leq 0.001$) (Figure 2(b)). The CMS (group 4) CK and LDH values were significantly different from nondiseased fish (groups 5, 6, and 7) (Figures 2(a) and 2(b)).

The scoring grades which were used to score the inflammatory changes represented by micrographs (Figure 1) and reflected the semiquantitative scoring system which was

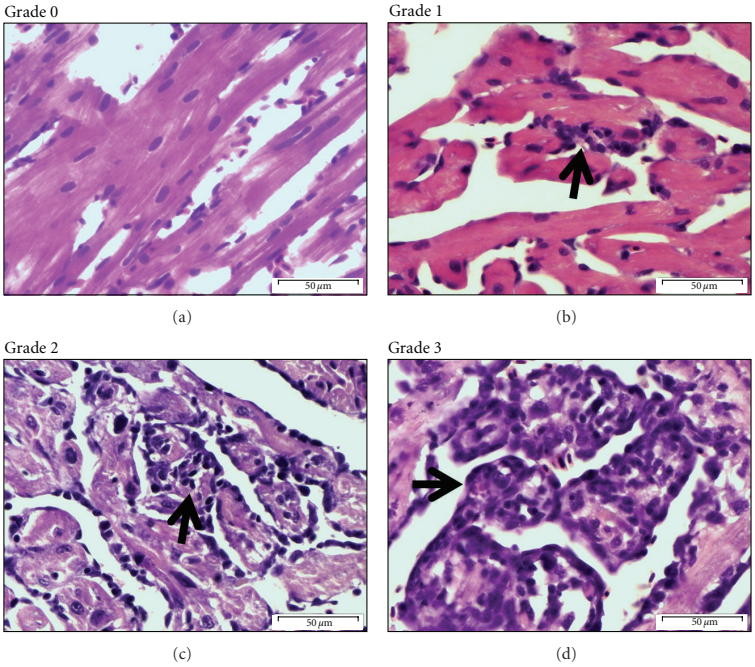

Figure 1: The representative micrographs of the semiquantitative scoring system described in Table 1, represented the heart histopathology. Grade 0: no pathological changes. Grade 1: minor inflammatory lesions comprises of focal subendocardial mononuclear leukocytes. Grade 2: several distinct lesions with moderately increased number of mononuclear leukocytes. Grade 3: severe lesions where almost all myofibres have been replaced by inflammatory cells, predominantly by mononuclear lymphocyte-like cells. Arrow: inflammation. Scale bars = 50 μm.

Table 2: Total mean (± SD) scores of inflammation and necrosis for HSMI (groups 1, 2, and 3), CMS (group 4), and nondiseased fish (groups 5, 6, and 7) in heart and heart with skeletal muscle. Different letters represent significant differences between groups ($P < 0.05$).

Parameter	Nondiseased	HSMI	CMS
Inflammation			
Heart	0.83 ± 0.30	5.30 ± 1.16	2.44 ± 1.39
Heart + muscle	0.99 ± 0.16[a]	5.59 ± 1.03[b]	2.44 ± 1.39[c]
Necrosis			
Heart	0.35 ± 0.36	2.01 ± 1.34	0.1 ± 0.40
Heart + muscle	0.62 ± 0.45[a,c]	2.92 ± 1.15[b]	0.38 ± 0.68[c]

applied to each anatomical region of the heart (atrium, compact and trabecular ventricular myocardium, pericardium) and the red skeletal muscle (Table 1).

Seven fish groups were compared on the basis of inflammation and necrosis scores. Inflammatory mononuclear cells were more frequent as compared to focal areas of necrosis in heart tissue, but the opposite was apparent in the red skeletal muscle where necrosis predominated. The mean total inflammation (Kruskal-Wallis One-Way Analysis: H = 111.216, d.f. = 2; ($P \leq 0.001$) and total necrosis (Kruskal-Wallis One-Way Analysis: H = 90.484, d.f. = 2; ($P \leq 0.001$) scores of HSMI and CMS fish were significantly different from nondiseased fish with the exception of total necrosis scores of CMS fish which were not significantly different from nondiseased fish (Table 2). More lesions were present in the heart as compared to the skeletal muscle (Table 2). Total inflammation scores ranged from 1 to 11 in diseased groups while 0 to 3.5 in nondiseased groups. Total necrosis scores ranged from 0 to 7 for diseased groups while from 0 to 3 for nondiseased groups. Total necrosis (Kruskal-Wallis One-Way Analysis: H = 118.135, d.f. = 6; ($P \leq 0.001$) (Figure 3(b)) and total inflammation (Kruskal-Wallis One-Way Analysis: H = 119.558, d.f. = 6; ($P \leq 0.001$) (Figure 3(a)) scores were compared for all seven groups and identified higher scores in HSMI group 1 while being significant and lower in the group 2 and 3 as compared

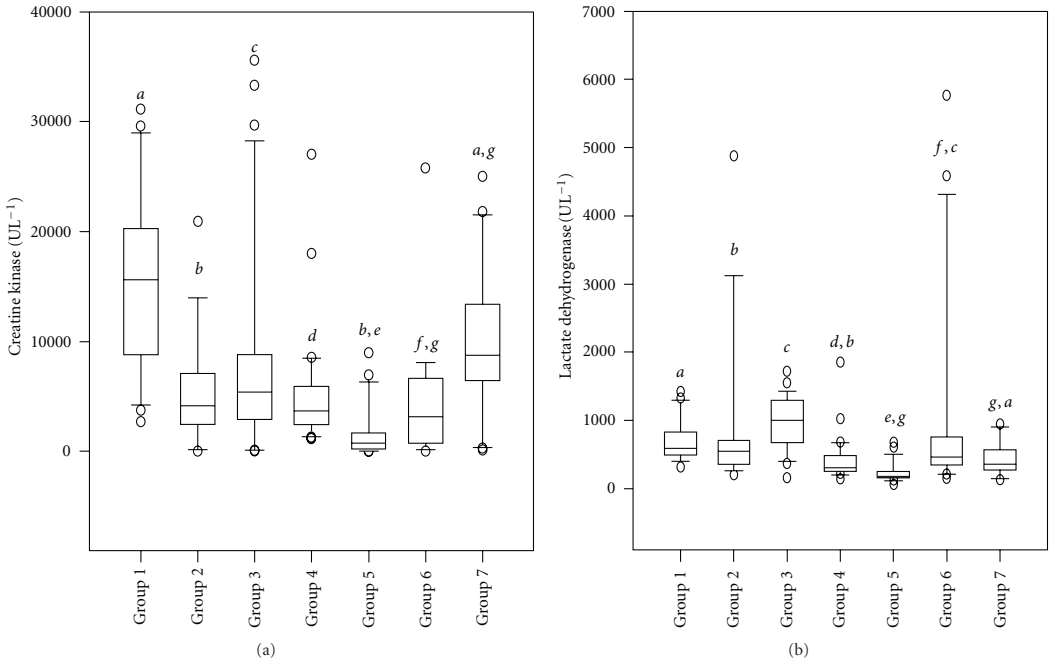

Figure 2: The box plots of (a) creatine kinase (CK) and (b) lactate dehydrogenase (LDH) enzymes activity values in the serum/plasma of Atlantic salmon for all seven groups ($N = 173$). Group 1 ($n = 28$) and group 2 ($n = 16$) represented fish from an acute and late phase of a HSMI outbreak, respectively, while group 3 ($n = 31$) represented values from fish from an early phase of HSMI. Group 4 ($n = 30$) included chronic CMS fish. Group 5 ($n = 28$), group 6 ($n = 20$), and group 7 ($n = 20$) represented values from nondiseased fish. Bars with different letters represented significant differences between groups ($P < 0.05$). (∘) denotes outliers.

to none to low level of changes seen in nondiseased fish (groups 5, 6, and 7) (Figures 3(a) and 3(b)). In general, muscle necrosis and inflammation (cardiac and skeletal) were negligible to mild in nondiseased fish (groups 5, 6, and 7) as compared to CMS and HSMI fish (groups 1, 2, 3, and 4). Total inflammation and total necrosis results of all HSMI-diseased fish (groups 1, 2, and 3) were significantly different from nondiseased groups (groups 5, 6, and 7) with the exceptions of total necrosis scores of CMS fish (group 4) which was not significantly different from nondiseased fish (group 6) (Figures 3(a) and 3(b)).

The histopathology scores (sum score of heart and muscle necrosis and inflammation) were correlated with serum/plasma CK and LDH levels. The correlations which were made among CK enzyme levels, and different anatomical parameters of all fish groups excluding CMS fish (group 4) gave significant relationships ($P \leq 0.001$) (Table 3). The significant correlations of CK levels to individual parameters included atrial inflammation (S coeff. = 0.451, $P < 0.001$), atrial necrosis (S coeff. = 0.252, $P = 0.002$), atrial epicarditis (S coeff. = 0.314, $P < 0.001$), ventricle compact layer inflammation (S coeff. = 0.440, $P < 0.001$), ventricle compact layer necrosis (S coeff. = 0.249, $P = 0.002$), ventricle trabecular inflammation (S coeff. = 0.526, $P < 0.001$), ventricle trabecular necrosis (S coeff. = 0.283, $P < 0.001$), ventricle epicarditis (S coeff. = 0.333, $P < 0.001$), skeletal muscle necrosis (S coeff. = 0.206, $P = 0.035$), and skeletal muscle inflammation (S coeff. = 0.169, $P = 0.084$). The CK enzyme levels significantly and positively correlated with the both total inflammation (S coeff. = 0.552, $P < 0.001$) and total necrosis (S coeff. = 0.526, $P < 0.001$) scores (Table 3). The LDH levels were also correlated in the same manner as above for CK which showed significant relationships ($P = 0.05$) (Table 3). The significant correlations for LDH levels to different parameters were atrial inflammation (S coeff. = 0.254, $P = 0.002$), ventricle compact layer inflammation (S coeff. = 0.297, $P = 0.001$), ventricle trabecular inflammation (S coeff. = 0.166, $P = 0.049$), skeletal muscle inflammation (S coeff. = 0.373, $P = 0.001$), ventricle epicarditis (S coeff. = 0.20, $P = 0.016$), and skeletal muscle necrosis (S coeff. = 0.414, $P = 0.001$) (Table 3). However, few non-significant relationships were identified for LDH and anatomical parameters such as ventricle compact layer necrosis (S coeff. = 0.080, $P = 0.341$), ventricle trabecular necrosis (S coeff. = 0.052, $P = 0.534$), atrial necrosis (S coeff. = 0.034, $P = 0.688$), and atrial epicarditis (S coeff. = −0.049, $P = 0.558$). There were significant positive correlations between LDH levels and the total inflammation (S coeff. = 0.266, $P < 0.001$)

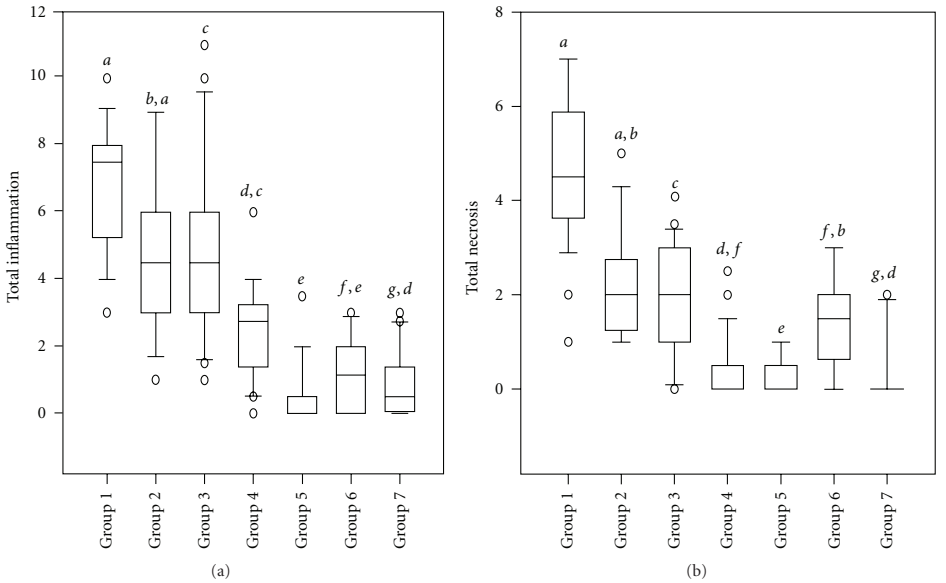

Figure 3: The box plots of all seven groups of Atlantic salmon on the basis of (a) total inflammation and (b) total necrosis in the heart and skeletal muscle ($N = 173$). Group 1 ($n = 28$) and group 2 ($n = 16$) represented fish from an acute and late phase of a HSMI outbreak, respectively, while group 3 ($n = 31$) represented values from fish from an early phase of HSMI, and group 4 ($n = 30$) included chronic CMS fish. Group 5 ($n = 28$), group 6 ($n = 20$), and group 7 ($n = 20$) represented values from nondiseased (non-HSMI) fish. Higher scores of inflammation and necrosis were found in diseased (groups 1, 2, 3, and 4) fish while low levels of scores in nondiseased (groups 5, 6, and 7) fish were present. Bars with different letters represented significant differences between groups ($P < 0.05$). (∘) denotes outliers.

Table 3: The Spearman correlation coefficient for anatomically distinct regions of the HSMI and CMS infected fish to creatine kinase (CK) and lactate dehydrogenase (LDH). P values given in parentheses.

Parameter	HSMI		CMS	
	CK	LDH	CK	LDH
Ventricle compact necrosis	0.249 (0.002)	0.080 (0.341)	—	—
Ventricle trabecular necrosis	0.283 (<0.001)	0.052 (0.534)	0.198 (0.291)	0.122 (0.516)
Atrium necrosis	0.252 (0.002)	0.034 (0.688)	0.327 (0.077)	0.242 (0.195)
Skeletal muscle necrosis	0.206 (0.035)	0.414 (0.001)	0.068 (0.719)	−0.024 (0.899)
Ventricle compact inflammation	0.440 (<0.001)	0.297 (0.001)	—	—
Ventricle trabecular inflammation	0.526 (<0.001)	0.166 (0.049)	0.157 (0.405)	−0.043 (0.822)
Atrial inflammation	0.451 (<0.001)	0.254 (0.002)	0.108 (0.580)	0.052 (0.790)
Ventricle epicarditis	0.333 (<0.001)	0.20 (0.016)	0.340 (0.065)	0.309 (0.096)
Atrium epicarditis	0.314 (<0.001)	−0.049 (0.558)	—	—
Muscle inflammation	0.169 (0.084)	0.373 (0.001)	—	—
Total inflammation	0.552 (<0.001)	0.266 (<0.001)	0.089 (0.635)	−0.075 (0.691)
Total necrosis	0.526 (<0.001)	0.247 (0.003)	0.355 (0.075)	0.240 (0.209)

and total necrosis (S coeff. = 0.247, $P < 0.003$) scores (Table 3). The correlations between CK enzyme levels and different anatomical parameters of all fish groups excluding HSMI fish (groups 1, 2, and 3) identified non-significant relationships ($P = 0.05$) (Table 3). The correlations made between CK levels and different anatomical regions were atrial inflammation (S coeff. = 0.108, $P = 0.580$), atrial necrosis (S coeff. = 0.327, $P = 0.077$), ventricle trabecular inflammation (S coeff. = 0.157, $P = 0.405$), ventricle trabecular necrosis (S coeff. = 0.198, $P = 0.291$), ventricle epicarditis (S coeff. = 0.340, $P = 0.065$), and skeletal muscle necrosis (S coeff. = 0.068, $P = 0.719$). The LDH levels

Table 4: Blood serum enzymes in different fish groups (Mean ± SE).

	Creatine kinase ($IU.L^{-1}$)	Lactate dehydrogenase ($IU.L^{-1}$)
Group 1	16479.25 ± 1844.49	697.43 ± 56.61
Group 2	10280 ± 5246.18	1838.25 ± 957.47
Group 3	8333.34 ± 1709.08	966 ± 71.94
Group 4	5207.93 ± 967.81	426.2 ± 60.68
Group 5	1581.71 ± 425.33	235.39 ± 27.43
Group 6	7098.35 ± 2916.95	1027.9 ± 334.04
Group 7	10297.15 ± 1531.11	423.3 ± 53.06

were also correlated in the same manner as above for CK and included atrial inflammation (S coeff. = 0.052, P = 0.790), atrial necrosis (S coeff. = 0.242, P = 0.195), ventricle trabecular inflammation (S coeff. = −0.043, P = 0.822), ventricle trabecular necrosis (S coeff. = 0.122, P = 0.516), ventricle epicarditis (S coeff. = 0.309, P = 0.096), and skeletal muscle necrosis (S coeff. = −0.024, P = 0.899). The combined CMS and nondiseased group's correlations with CK levels were also made in the same manner as described above and identified correlations for total inflammation (S coeff. = 0.089, P = 0.635) and total necrosis (S coeff. = 0.355, P = 0.075) scores (Table 3). There were non-significant negative and positive correlations between LDH levels and total inflammation and total necrosis for CMS group, respectively (Table 3).

4. Discussion

The CK and LDH values of all seven fish groups were compared and diseased fish (HSMI) identified with significantly higher enzymes levels as compared to nondiseased fish. The significantly higher and lower mean enzymes levels in diseased and nondiseased fish, respectively, were consistent with the CK enzyme ranges already reported in farmed Atlantic salmon affected with a similar pancreas disease (PD) [30, 31]. The highest mean LDH levels were identified at the earlier phase of HSMI disease while highest CK levels were present in acute phase of the disease. Previous *in vivo* studies identified the increased CK and LDH activities in Atlantic salmon and Nile tilapia (*Oreochromis niloticus*) treated with tributyltin (TBT) and cadmium, respectively [35, 36].

Histopathology was used as a method of choice to diagnose the diseases (HSMI and CMS). This study described the histopathology in the heart and skeletal muscle by using a semiquantitative scoring system that addressed the pathological changes in both tissues (cardiac and somatic muscle). The diseased fish showed the histopathological changes in the heart and skeletal muscle similar to HSMI and in hearts for CMS fish [5, 11, 13]. The histological changes were identified in both atrium and ventricle (compact and trabecular) of HSMI fish while mostly ventricular trabecular layer was involved in CMS fish. The histopathological changes were compared for all seven fish groups, and hearts were identified with most tissue damage and suggested to be the contributing source of enzymes (CK and LDH) which released upon cellular damage and in line with Rodger et al. [30] that suggested the significantly higher CK levels due to myopathy in PD-affected Atlantic salmon.

The mean CK levels and histopathology scores for acute phase HSMI fish (group 1) were doubled than early or late phase of HSMI fish (groups 2 and 3), and mean CK levels (group 1) were up to four times greater than nondiseased fish (groups 5 and 6). These higher CK levels and total inflammation scores were suggested to be the disease (HSMI) outcome and supported the notion that fish included in group 1 were in the acute phase of disease whereas groups 2 and 3 were not in clinical phase of a HSMI outbreak. The acute phase of the disease (HSMI) corresponded to higher mortality rates on the farm and creatine kinase levels in blood sustained over 3-4 days in humans where a cardiac injury has occurred [28].

The HSMI-infected fish showed significantly higher histopathological scores as compared to the nondiseased fish. The higher histopathological scores were consistent with the higher CK levels in diseased fish as compared to nondiseased fish, supported the higher enzymes levels likely due to myopathy. The total inflammation scores were doubled as compared to the total necrosis scores in all HSMI fish which were considered as clinical sign of HSMI while necrosis being suggested as a secondary effect [13, 15]. The mean CK and LDH levels and total necrosis scores for chronic CMS fish were equal or lower to the nondiseased fish suggested no correlation to CMS fish and supported the hypothesis that increased enzymes levels identified in the HSMI fish were related to myopathy [30, 31]. The fish cages that experienced the CMS outbreak showed high inflammation scores as compared to other cages on the same farm that were not diagnosed with CMS, and both CMS and non-CMS fish had higher values of enzymes indicating that CK and LDH were not correlated to the chronic CMS fish histopathology scores.

The significantly positive correlations were identified with biochemical enzymes (CK and LDH) and histopathological changes in HSMI-affected Atlantic salmon [12, 24]. The histopathological scores for anatomically distinct areas of heart and skeletal muscle were correlated significantly with respective CK and LDH levels of the fish (P = 0.05). The serum enzymes (CK and LDH) correlations have been used previously to find significant relationships in great sturgeon (*Huso huso*) and rainbow trout (*Oncorhynchus mykiss*) [24, 37, 38]. The correlations between histopathology (inflammation and necrosis) and enzymes (CK and LDH) values were significant and positive, and suggested the HSMI disease

effects on blood biochemistry of Atlantic salmon and consistent with mammalian studies where blood biochemistry is changed in pathological conditions and used to predict the disease [29, 36]. The correlation of CK levels to HSMI histopathology appeared useful due to the release of CK after tissue injury and potential contribution in limited piscine blood biochemistry [28]. However, the CMS group showed non-significant correlations between histology and enzymes and suggested that blood biochemistry of Atlantic salmon might not be affected due to chronic CMS disease. Another reason for no correlation of chronic CMS fish might include the time of fish sampling which was conducted 6 months after acute phase of disease while biochemical enzymes (CK and LDH) release rapidly following tissue injury with peak levels for 10–12 days in human [28]. It was also supported by the fact that chronic CMS fish did not show signs (higher mortality levels) and severe histopathological lesions characteristic of acute phase of disease suggesting the late or chronic phase of disease. However, further studies are required to completely understand the CMS disease effects on the blood biochemistry. Histopathology is still a diagnostic method of choice in clinical case of HSMI even though the recent identification of a reovirus associated with the disease allows the possibility of identifying infected animals. However, asymptomatic Atlantic salmon have been identified with piscine reovirus and piscine myocarditis virus by RT-qPCR [17, 25]. However, it is a terminal procedure performed after the onset of disease and there has been as observed increase mortality level on farm, and mis-diagnosis may result with diseases such as PD and CMS presenting similar pathological changes to HSMI [12]. The blood biochemistry tests may prove useful and have been proved useful in the detection and diagnosis of metabolic disturbances in a number of diseases [39]. The use of serum enzymes to diagnose the cardiac diseases in humans is a common practice and a well-established method. The CK and LDH levels are affected in the cardiac diseases and serve as disease indicators in humans [26]. Previous attempts at measuring CK values were made in similar disease such as pancreas disease (PD) but were not correlated directly and extensively with histopathology [30, 31]. These studies have been shown the significantly increased CK levels and suggested the tissue damage as source of enzyme. HSMI fish exhibited higher mean histopathology scores and enzymes (CK and LDH) levels, and serum enzymes showed significant positive correlations to histopathology which supported the notion that CK and LDH levels were affected due to natural HSMI outbreak. The use of CK and LDH enzymes haematological levels for pathological changes at least for HSMI appeared promising and a potential contribution in the limited piscine blood biochemistry by identifying the enzyme ranges in the above-mentioned fish groups [22].

In conclusion, the present study measured the CK and LDH levels in diseased and nondiseased Atlantic salmon and correlated significantly to the histopathology of Atlantic salmon affected with natural HSMI outbreaks while being non-significant to chronic CMS. The significantly higher CK levels correlated positively and significantly to HSMI pathological changes, suggesting that the potential use of serum enzymes for screening HSMI is promising. The findings of the present study should be considered as a contribution to the more extensive research necessary to understand biological activities (enzymes) and the pathological changes of Atlantic salmon.

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Paper III

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Cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI)

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ABSTRACT

Heart and skeletal muscle inflammation (HSMI) is a disease of marine farmed Atlantic salmon where the pathological changes associated with the disease involve necrosis and an infiltration of inflammatory cells into different regions of the heart and skeletal muscle. The aim of this work was to characterize cardiac changes and inflammatory cell types associated with a clinical HSMI outbreak in Atlantic salmon using immunohistochemistry. Different immune cells and cardiac tissue responses associated with the disease were identified using different markers. The spectrum of inflammatory cells associated with the cardiac pathology consisted of mainly $CD3^+$ T lymphocytes, moderate numbers of macrophages and eosinophilic granulocytes. Proliferative cell nuclear antigen (PCNA) immuno–reaction identified significantly increased nuclear and cytoplasmic staining as well as identifying hypertrophic nuclei. Strong immunostaining was observed for major histocompatibility complex (MHC) class II in HSMI hearts. Although low in number, a few positive cells in diseased hearts were detected using the mature myeloid cell line granulocytes/monocytes antibody indicating more positive cells in diseased than non–diseased hearts. The recombinant tumor necrosis factor–α (TNFα) antibody identified stained macrophage–like cells and endothelial cells around lesions in addition to eosinophilic granular cells (EGCs). These findings suggested that the inflammatory response in diseased hearts comprised of mostly $CD3^+$ T lymphocytes and eosinophilic granular cells and hearts exhibited high cell turnover where DNA damage/repair might be the case (as identified by PCNA, caspase 3 and terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) reactivity).

1. Introduction

The occurrence of cardiac diseases in Norwegian Atlantic salmon (*Salmo salar*) aquaculture has increased in recent years as heart and skeletal muscle inflammation (HSMI) has become an increasingly important disease. Such disease outbreaks have increased from 54 (2004) to 162 cases reported in 2011 [1]. HSMI is a disease of marine farmed Atlantic salmon reported not only in Norway, but also in Scotland and more recently Chile. HSMI has a putative viral etiology and mainly affects heart and skeletal muscle. It is typically a disease of moderate mortality (~20%) but high morbidity (~100%) that typically affects fish 5–9 months after transfer to sea. Macroscopically pale hearts with loose texture, pericardial hemorrhages, ascities and pale/stained liver are observed without haematocrit changes. The cardiac and red skeletal muscles exhibit the most significant histopathological lesions [2]. Presently, HSMI can be diagnosed and differentiated from other similar cardiac diseases based upon histopathological changes and presents epi–, endo–, and myocarditis as well as a pronounced mononuclear cellular infiltration of both trabecular and compact layers of ventricular myocardium accompanied by myocytic necrosis, myositis and necrosis of red skeletal muscle [2–5]. HSMI appears to be transmissible in laboratory studies by injecting tissue homogenate from diseased to healthy fish [5]. Recently, a viral etiology was suggested where piscine reovirus was associated with HSMI, although Koch's postulates to date remains

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unfulfilled [6,7]. Lesions first appear and are more frequent in heart than skeletal muscle. Affected myocytes show signs of degeneration, loss of cardiomyocyte striation and eosinophilia, loss of skeletal muscle striation, vacuolation, centralized nuclei and karyorhexis. Inflammatory changes are more pronounced as compared to necrotic changes in heart and skeletal muscle [2,3,8].

The classification of pathological changes becomes more important in the absence of causative agent (s) particularly in case of HSMI where the aetiology needs to be confirmed. The MHC class II immunostaining has been performed with HSMI, although the nature of the inflammatory immune response has not been investigated or characterized [9]. Innate immunity is the first line of defense present in vertebrates against pathogens and mediates the response by activation of neutrophils, macrophages, mast cells, dendritic cells, and natural killer cells. These cells are capable of eradicating pathogens and transmit signals that amplify adaptive immune response in vertebrates [10]. Adaptive immunity is a crucial mechanism to protect the host from infections and represented mainly by helper T (Th) cells and cytotoxic T (Tc) lymphocytes defined by expression of CD4 and CD8 molecules respectively. These molecules serve as co–receptors and interacts specifically with either major histocompatibility class II or I to determine the discrete stage of T cell development within the thymus. Cytotoxic T lymphocytes bind to MHC class I that presents endogenous antigen via T cell receptor (TCR) and the CD8 co–receptor, and kills infected cells [11–13]. Presence of both cytotoxic and helper T cells in teleosts have been suggested by functional studies and supported by the expression of T cell receptors (TCR), CD8 and CD4 genes in teleost [14]. The TCR exhibits either α/β or γ/δ types in jawed fish non–covalently bound to CD3 molecules [14,15]. The CD3 molecules play an important role in signal transduction in TCR complex. Recently three subunits; CD3ζ, CD3γδ (forerunner of CD3γ and CD3δ in mammals) and CD3ε were cloned and sequenced in Atlantic salmon [16].

Cysteine–aspartic proteases (caspases) mediate immune responses (apoptosis, necrosis and inflammation) and are key component of apoptosis or programmed cell death. Caspases comprise of pro and catalytic domains and synthesized as inactive precursor molecule. Upon activation, the inactive pro–enzyme transfers into enzymatically–active heterotetrameric complex. At least 15 caspases have been identified in mammals while many important caspases have been identified in Atlantic salmon (reviewed in Ref. [17]). There are two main subcategories of caspases, effectors and initiators that mediate apoptosis by either extrinsic or intrinsic pathways. Caspase 3 belongs to the effectors group and can be initiated by both extrinsic and intrinsic pathways [17,18]. Terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) and caspase 3 immunostaining have been used as markers of irreversible apoptosis in chronic heart failure in humans [19]. Hypoxia inducible factor–1 (HIF1) is a heterodimer consisting of α–subunit (oxygen sensitive) and β–subunit (oxygen independent) and is one of the most important molecules involved in hypoxia, inflammation, apoptosis and influences adaptive immunity (reviewed in [10,20,21]. The oxidative stress is considered as one of major factors for causing damage to the myocardium and HIF is suggested as being essential for normal heart physiology and particularly plays important role in cardiac conditions such as ischemia and pressure overload [8,22]. Two TNFα genes of 246–amino acids were identified in Atlantic salmon [23] and being a part of innate immune response reacts to different forms of stress like infection, trauma, ischemia/reperfusion (I/R) [24]. A large amount of soluble TNFα is released by inflammatory stimuli (bacteria, virus, parasite and ischemia) from macrophages, lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue and may serve as biomarker for heart failure (reviewed in Ref. [24]). TNF can induce apoptosis or necrosis and receptors for TNF are present in almost all nucleated cells making the basis for a very complex cytokine network [23,25,26]. The proliferative cell nuclear antigen (PCNA) is a common marker for cellular proliferation and is widely accepted as a surrogate marker for DNA synthesis, marking PCNA cells in early G1 phase and S–phase of the cell cycle, DNA repair and apoptosis [27–30].

The aim of the present study was to characterize immunological responses and cardiac pathology associated with HSMI by immunohistochemistry [9]. Apoptosis and cell damage were studied using TUNEL and caspase 3 antibody, hypoxia transcription factor by HIF1α antibody, mitotic activity by PCNA antibody, proinflammatory cytokine TNFα by rTNFα antibody, granulocytes/monocytes by a marker expressed on mature myeloid cell lineage, T lymphocytes by CD3ε and CD8 antibodies, and antigen presenting cells by MHC class I and II staining.

2. Materials and methods

2.1. Antibodies

The antibodies used in this study included mab PCNA 10 identifying proliferative cell nuclear antigen (PCNA) (α–PCNA, Dako, Glostrup, Denmark) (1:150) [31], mammalian polyclonal caspase 3 (sc–7148, Santa Cruz Biotechnology, Santa Cruz, CA) (1:500), mammalian polyclonal hypoxia inducible factor (HIF1α) (sc–8711, Santa Cruz Biotechnology, Santa Cruz, CA) (1:200), Ø127 MHCII against salmon major histocompatibility complex (MHC) class II β chain (1:1000) [31,32], monoclonal rainbow trout granulocyte/monocytes antibody recognize mature myeloid cell lineage (1:2000), polyclonal anti–salmon CD3ε antibody (1:400) [33], polyclonal rabbit anti–trout TNFα antibody (1:500) [34], monoclonal antibodies Sasa CD8 alpha F1–29 and Sasa MHC I alpha F1–34 [35–37].

2.2. Atlantic salmon hearts

Atlantic salmon hearts were collected from 2 confirmed outbreaks of HSMI (The Norwegian Veterinary Institute, Norway) at early and peak mortality phases, and naive fish hearts were collected to serve as controls in this study. The control (non–diseased) fish were maintained from smolts for ~1 year at the Mørkvedbukta Research Station, University of Nordland, Bodø, Norway. The fish were kept in 2 m^3 tanks supplied with fresh ambient sea water (range 7–8 °C) and fed commercial fish pellets (Spirit, Skretting, Stavanger, Norway) 0.7% feed of their body weight three times weekly. All fish were sacrificed by a blow to head. The fish were examined by autopsy in addition to histological observation of the hearts and other vital organs (gills, liver, kidney, skeletal muscle and spleen) for any other abnormalities or signs of overt disease in the non–diseased fish. The diagnosis of the disease was made by histopathology of hearts and red skeletal muscle [5,9]. The number of heart sections range (3–10) for diseased group and (3–7) for non–diseased group were used for each antibody reaction to compare the results.

2.3. Immunohistochemistry on paraffin heart sections

Hearts were fixed in 10% neutral phosphate buffered formalin solution and processed by a standard paraffin wax protocol (dehydrated, embedded in paraffin and 3 μm thick sections). Immunostaining was performed by following the protocol described by Haugarvol et al. [31]. Briefly, 3 μm thick heart sections were cut and mounted on poly–L–lysine (Sigma–Aldrich, Norway) coated slides, dried at 50 °C for 30 min, deparaffinized in xylene

(three changes for 5 min each), rehydrated in an ethanol series (100%–80%, 10 min each) and finally rehydrated in distilled water (dH_2O). Antigen unmasking was undertaken by autoclaving the slides at 121 °C for 21 min in a glass box in 10 mM citrate buffer, pH 6.0 containing 0.1% Tween 20 (Sigma–Aldrich, Norway). Slides were kept at room temperature for 20 min and then washed twice with distilled water for 2 min each. All incubations were performed in a closed–lid humidity chamber. To inhibit endogenous peroxidase activity, the slides were incubated with 3% H_2O_2 (Sigma–Aldrich, Norway) in methanol for 10 min at room temperature. The sections were incubated in 5% bovine serum albumin (BSA) (Sigma–Aldrich, Norway) in phosphate buffered saline (PBS) (Sigma–Aldrich, Norway) for 1 h at room temperature. After removal of the BSA, slides were incubated with primary antibodies in 1.5% BSA in PBS overnight (~16 h) at 4 °C. Following morning, slides were washed three times with PBS with 0.1% Tween 20 (PBST) (Sigma–Aldrich, Norway), pH 7.4 for 5 min each. Slides were then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (sc–2020, donkey anti–goat or sc–3837, goat anti–rabbit, Santa Cruz biotechnology) in 1.5% BSA in PBS for 60 min at room temperature. Slides were washed three times with PBST for 5 min each. Slides were either incubated with 3, 3′–diaminobenzidine (DAB) or 3–amino–9–ethylcarbazole (AEC) (Sigma–Aldrich, Norway) for 5 min and then washed with distilled water (dH_2O) for 5 min. Slides were dipped in haematoxylin for 10 s for counterstaining and then passed through graded ethanol and xylene for 10 s each and mounted with poly–vinyl alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway). Negative controls were performed by replacing primary antibody with 1.5% BSA in PBS.

2.4. Immunohistochemistry using CD8 and MHC I mouse mabs

The protocol was followed according to the Tyramide Signal Amplification (TSA) Biotin system (PerkinElmer/NEN Life Science, Boston, MA, USA) for CD8 and MHC I antibodies. The above mentioned protocol in Section 2.3 was adopted until antigen retrieval step. Briefly slides were treated with 3% H_2O_2 (Sigma–Aldrich, Norway) in methanol for 10 min at room temperature followed by washed twice in PBS. Slides were blocked with TNB blocking buffer (0.1 M TRIS–HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent supplied in the TSA system) for 1 h followed by incubation of slides with primary antibodies in TNB buffer overnight (~16 h) at 4 °C. Slides were washed three times with PBS for 5 min each. To detect the primary antibodies, slides were incubated with HRP labeled anti–mouse secondary antibody (sc–3697, goat anti–mouse, Santa Cruz biotechnology) diluted in TNB blocking buffer for 1 h at room temperature. Three times PBS washing was performed as after primary antibody step. Slides were incubated with biotinyl tyramide amplification reagent followed by streptavidin–horseradish peroxidase (SA–HRP), both reagents were provided with the kit. Slides were visualized using 3–amino–9–ethyl carbazole (AEC; Sigma–Aldrich, Norway) as substrate. Slides were counter stained with haematoxylin for 10 s and mounted using ImmunoHistoMount (Sigma–Aldrich, Norway). Negative controls included the primary antibodies replaced with TNB blocking buffer and unamplified control (include all reagents except TSA reagents).

2.5. TUNEL staining

TUNEL was performed using 3 μm sections of heart tissues using an ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON® Int. Inc. USA). The sections were deparaffinized through a graded series of xylene and alcohol and then pretreated with freshly made IHC Select® Proteinase K (20 μg/ml) (Millipore) for 15 min at room temperature and washed twice in distilled water (dH_2O) for 2 min each. All incubations were performed in a closed–lid humidity chamber. The endogenous peroxidase was quenched by using 3% hydrogen peroxide (Sigma–Aldrich, Norway) in PBS for 5 min at room temperature. Slides were rinsed twice with dH_2O for 5 min each. The dH_2O was aspirated around the sections and immediately dipped in equilibration buffer at 75 μl 5 cm^{-2} for 10 s. The equilibration buffer was removed from slides and dipped in working strength TdT enzyme at 55 μl 5 cm^{-2} and incubated slides in a humidified at 37 °C for 1 h. The slides were dipped in working strength stop/wash buffer, agitated for 15 s and then incubated for 10 min at room temperature. The slides were washed with 3 changes of PBS for 1 min each and then dipped in anti–digoxigenin conjugate at 65 μl 5 cm^{-2} and incubated for 30 min at room temperature. Slides were washed with 4 changes of PBS for 5 min each. Sections were carefully blotted and dipped in peroxidase substrate at 75 μl 5 cm^{-2} and incubated for 3–6 min at room temperature. The sections were washed three times in dH_2O for 1 min each, then counter stained with 0.5% (W:V) methyl green (Sigma–Aldrich, Norway) for 10 min and washed in dH_2O. The slides were finally washed in 100% N–butanol (Sigma–Aldrich, Norway), dehydrated through graded alcohols and xylene for 2 min and mounted with poly–vinyl alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway). All reagents were provided with the kit or otherwise mentioned. Positive (provided with the kit) and negative control by replacing active TdT with equilibration buffer was performed. Slides were evaluated by light microscopy and apoptotic cells were identified by both positive staining and morphological signs of apoptosis.

2.6. Western blot

Western blot was performed to confirm the antibody specificity where non–salmonid homologous antibodies were used in immunohistochemistry against Atlantic salmon tissue. Approximately 30 mg of frozen tissue (kidney, spleen and skeletal muscle) was minced on ice and transferred to tubes containing RIPA buffer (150 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris–HCl at pH 8.0, 1% Triton X–100, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail (Complete ULTRA tablets, Mini *EASY*pack, Roche Diagnostics, GmbH, Germany) and sonicated until the tissue dissolved at 4 °C. The homogenate was centrifuged at 13,000 *g* for 30 min at 4 °C and the supernatant collected. The 50–100 μg tissue in each well and HeLa + CoCl2 cell lysate (sc–24679, Santa Cruz Biotechnology, Santa Cruz, CA) as positive control were separated by denaturing SDS–PAGE using 12% and 8% polyacrylamide gels for caspase 3 and HIF1α respectively by following the protocol described by Laemmli [38]. Samples were blotted on polyvinylidene fluoride (PVDF) membrane and incubated with caspase 3 (1:500) and HIF1α (1:100) antibodies and detected by enhanced chemiluminescence (Immobilon Western kit (Millipore)). The specificity of mab PCNA 10 identifying proliferative cell nuclear antigen (PCNA) (α–PCNA, Dako, Glostrup, Denmark) has already been demonstrated in Atlantic salmon [31].

2.7. Slide evaluation

A semi–quantitative scoring system was adopted for positive cell frequency that has been widely used [35,36,39] and graded as (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) where strong staining = severe diffuse staining, moderate staining = multifocal staining or many positive cells, weak staining = focal staining or few positive cells and no staining. The slides were evaluated by light microscopy using Olympus

microscope BX51 equipped with Cell^{B} software (Olympus Corporation, Tokyo, Japan). The scoring grades of diseased and non–diseased fish were compared by Mann–Whitney rank sum test using SigmaPlot (10.0™) and were considered statistically significant at P values ≤ 0.05.

3. Results

There were moderate numbers of CD3ε^{+} cells in the atria of diseased as compared to non–diseased hearts that showed low numbers of immunopositive cells (Fig. 1A and B). Diseased ventricles showed strong positive immunostaining as compared to non–diseased hearts that identified few CD3ε^{+} cells (Fig. 1C and D). The CD3ε immuno–reactivity was mostly identified in lymphocyte–like cells around areas with pathological changes and significantly different from non–diseased fish ($P = 0.002$). The non–significant low numbers of CD8^{+} cells were observed in both diseased and non–diseased atria (Fig. 1E and G) and ventricles (Fig. 1F and H), staining lymphocyte–like cells. A non–significant strong MHC class I^{+} immuno–reaction was observed in both

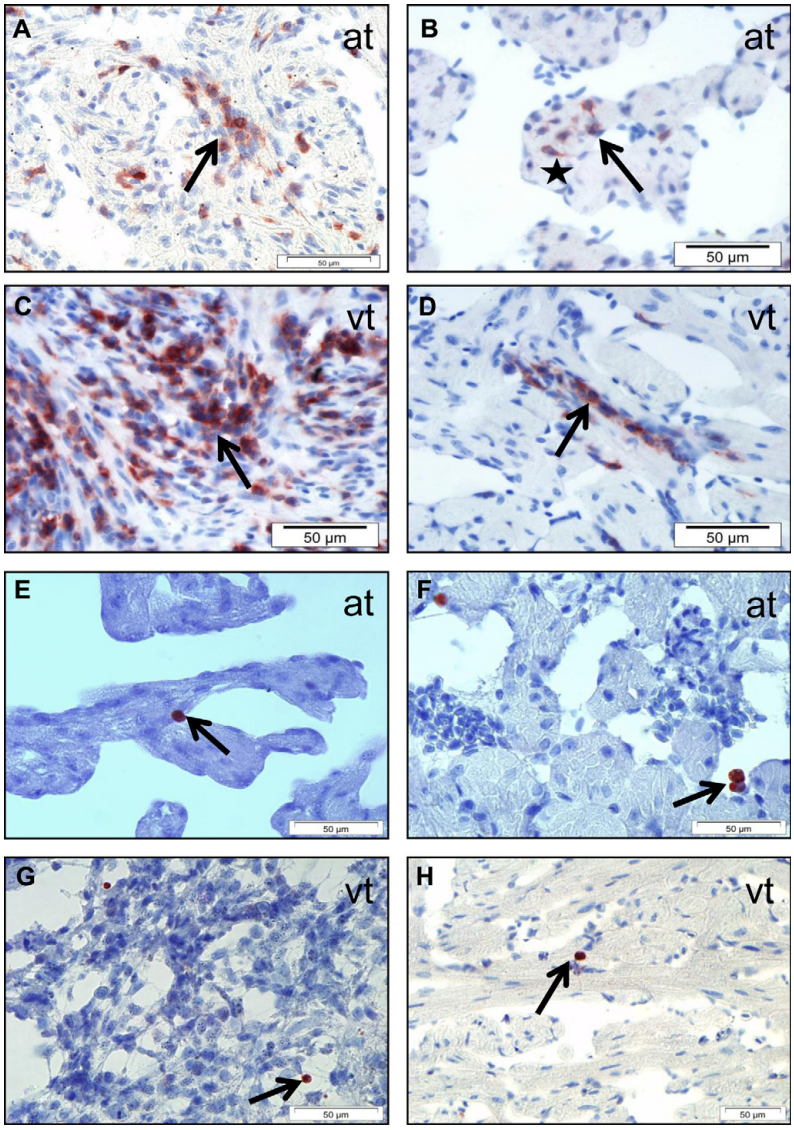

Fig. 1. Anti–CD3ε and CD8 immunostaining on diseased (A, C, E and G) and non–diseased (B, D, F and H) hearts of Atlantic salmon respectively. (A, C) Abundant CD3ε^{+} cells (arrow) in atrium (at) and ventricle (vt) of diseased salmon especially around inflamed areas. (B, C) Few CD3ε^{+} cells in atrium (at) and ventricle (vt) of non–diseased salmon. (B) Dendrite–like cells in the heart (star). (E, F, G and H) Few CD8^{+} lymphocytes–like cells in atrium (at) and ventricle (vt) of diseased and non–diseased Atlantic salmon.

diseased and non–diseased atria (Fig. 2A and B) and ventricles (Fig. 2C and D). MHC class I antibody identified myocardial and endothelial staining in diseased hearts as compared to non–diseased where endothelial staining was dominant. However, MHC class II β^{+} immunostaining was strongly positive in HSMI diseased hearts (Fig. 2E and G) and significantly different ($P \leq 0.001$) from low levels in non–diseased hearts (Fig. 2F and H). The immunopositive cells were found around focal affected areas in the endocardium and myocardium in addition to mononuclear lymphocyte–like cells (Fig. 2G and H). MHC class II β staining also identified dendrite–like cells in the diseased heart (Fig. 2E). The rTNFα^{+} cells showed moderate levels of immuno–reactivity in the diseased atria (Fig. 3A) and ventricles (Fig. 3C) which were significantly different ($P = 0.016$) from non–diseased fish where few positive cells were observed (Fig. 3B and D). The anti–rTNFα reaction identified macrophages (Fig. 3A, insert) and eosinophilic

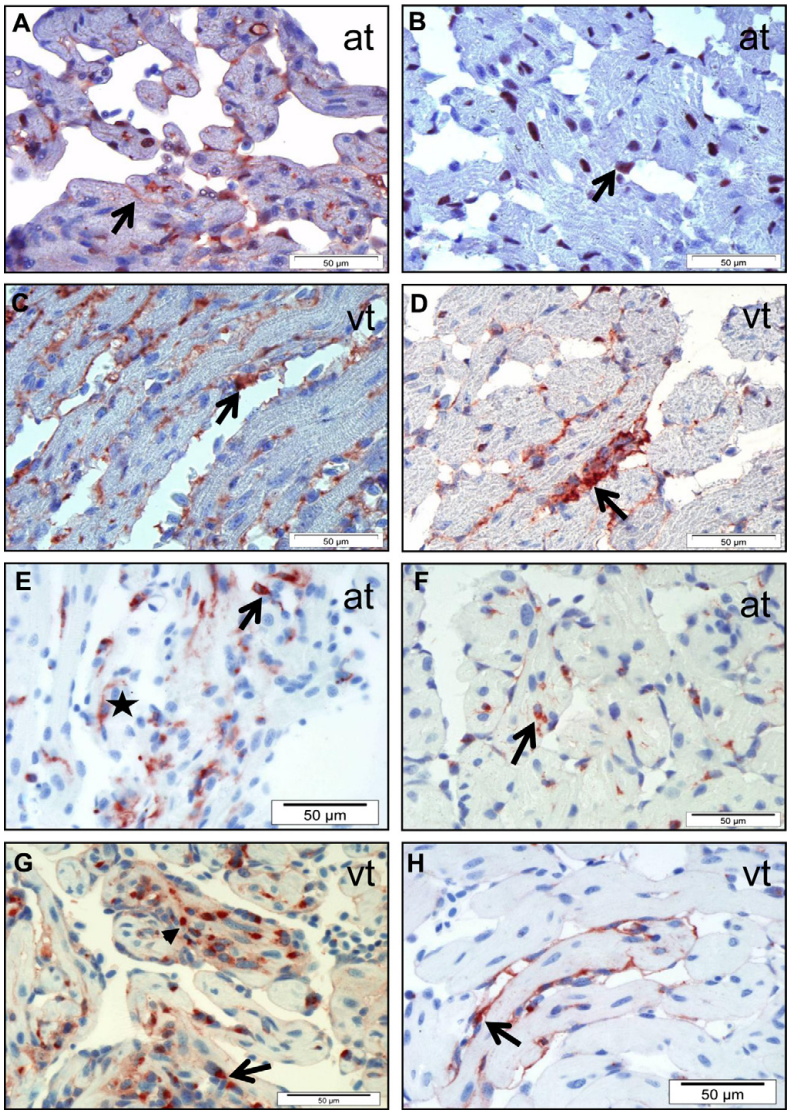

Fig. 2. MHC class I and II β chain immuno–reactivity of diseased (A, C, E and G) and non–diseased (B, D, F and H) hearts of Atlantic salmon respectively. (A, B, C and D) The strong levels of MHC class I immunostaining were observed (arrow) in atrium (at) and ventricle (vt) of diseased and non–diseased salmon. (E, G) MHC class II immuno–reaction showed moderate levels of staining (arrow) in atrium (at) while strong levels in the ventricle (vt) of diseased salmon especially around the inflamed areas with cytoplasmic staining of mononuclear lymphocytes–like cells (arrow head). Dendrite–like cells (star) identified in the heart. (F, H) Few MHC class II immuno–reactive cells were identified in atrium (at) and ventricle (vt) of non–diseased salmon.

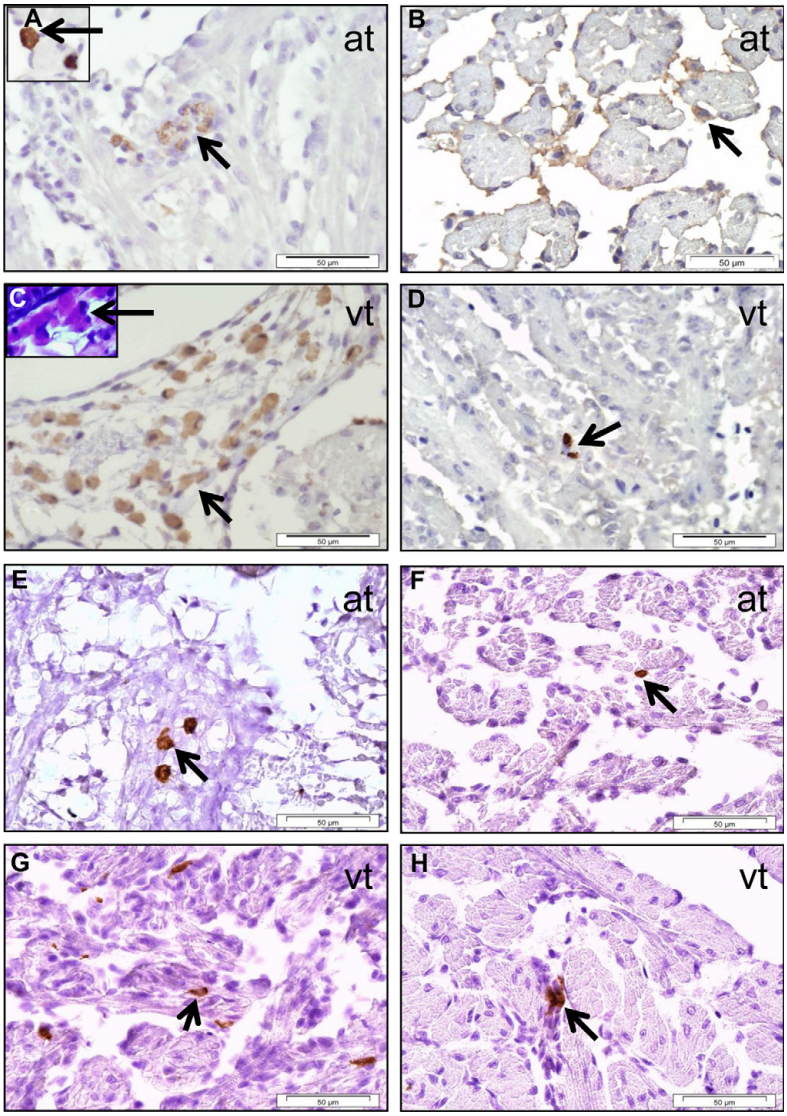

Fig. 3. Anti rTNFα and mature myeloid cell line granulocytes/monocytes immunostaining on diseased (A, C, E and G) and non–diseased (B, D, F and H) hearts of Atlantic salmon respectively. (A, C) Moderate levels of rTNFα^{+} cells were identified in atrium (at) and ventricle (vt) of diseased salmon (B, D) while few positive cells observed in non–diseased hearts. (A and C)The insets showed macrophage–like and eosinophilic granulocytes (H&E) respectively. (E, F, G and H) Few granulocyte^{+} cells were present in diseased and non–diseased hearts.

granular cells in the diseased hearts where the scattered granules were easily seen (Fig. 3A, C and C, insert). Overall, mature myeloid like cell line antibody against granulocytes/monocytes immunostaining was non–significant and low in numbers (Fig. 3E and G) but still could be differentiated with more positive cells present in diseased as compared to non–diseased hearts (Fig.3F and H). TUNEL staining showed moderate levels of staining for diseased hearts (Fig. 4A and C) as compared to low levels of positive cells identified in non–diseased hearts (Fig. 4B and D). Similarly there was moderate levels of PCNA immuno–reaction in HSMI diseased hearts (atrium and ventricle) (Fig. 4E and G) and significantly different ($P = 0.012$) from a low number of positive cells in non–diseased hearts (Fig. 4F and H). PCNA showed distinctive nuclear and cytoplasmic staining (Fig. 4E and G) as well as identified hypertrophic nuclei (Fig. 4E, insert). There were non–significant low levels of HIF1α^{+} cells in both diseased (Fig. 5A and C) and non–diseased hearts (Fig. 5B and D). The non–significant low levels of caspase 3 immunopositive cells were

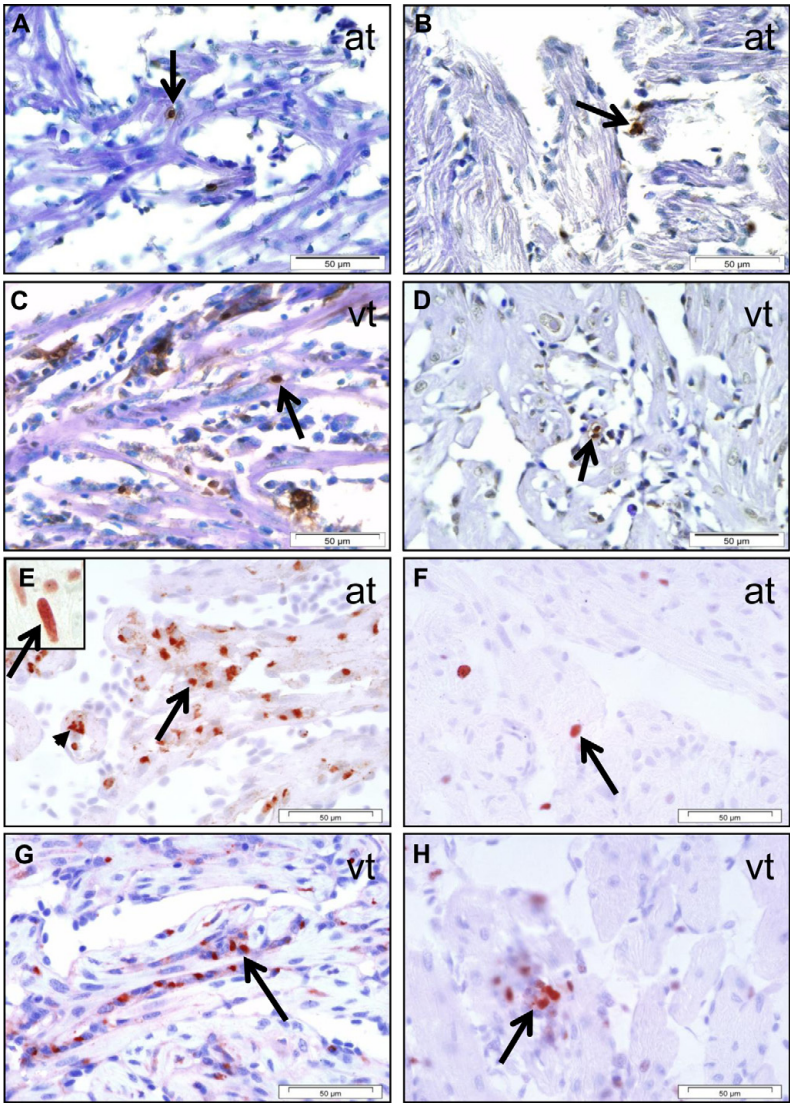

Fig. 4. TUNEL and PCNA immunostaining for detection of apoptosis and cell proliferation on diseased (A, C, E and G) and non–diseased (B, D, F and H) hearts of Atlantic salmon respectively. (A, C) Low and moderate levels of staining were identified in atrium (at) and ventricle (vt) for TUNEL staining in HSMI hearts respectively (B, D) while low numbers of apoptotic cells were identified in non–diseased hearts. (E, G) Strong $PCNA^{+}$ immuno–reactive cells in diseased atrium (at) and ventricle (vt), and (F, H) low levels of $PCNA^{+}$ cells were identified in the atrium (at) and ventricle (vt) of non–diseased salmon hearts. The hearts presented with granular nuclear (arrow) and cytoplasmic (arrow head) staining. (E) The inset showed hypertrophic nucleus identified by PCNA antibody.

observed in diseased hearts (Fig. 5E and G) while few positive cells were seen in non–diseased hearts (Fig. 5F and H). The caspase 3 staining was mostly endothelial presumably in areas of necrosis (Fig. 5E, F and G).

As shown in Fig. 6, Western blot analysis of caspase 3 antibody identified procaspase 3 (~40 kDa) and caspase 3 (~12 kDa) bands while the middle band may correspond to p20 (~21 kDa) which was evident in the kidney. The HIF1α antibody identified a band of ~70 KDa in the skeletal muscle (Fig. 6).

4. Discussion

Heart and skeletal muscle inflammation (HSMI) is considered as a disease with more inflammatory changes than necrotic changes [2,3] and this study was aimed to identify and characterize the different cell types that infiltrated the infected fish hearts, the majority of which appear mononuclear and distinctively lymphocyte–like by H&E staining. There were very few $CD8^{+}$ cells in the diseased or non–diseased hearts, staining lymphocyte–like

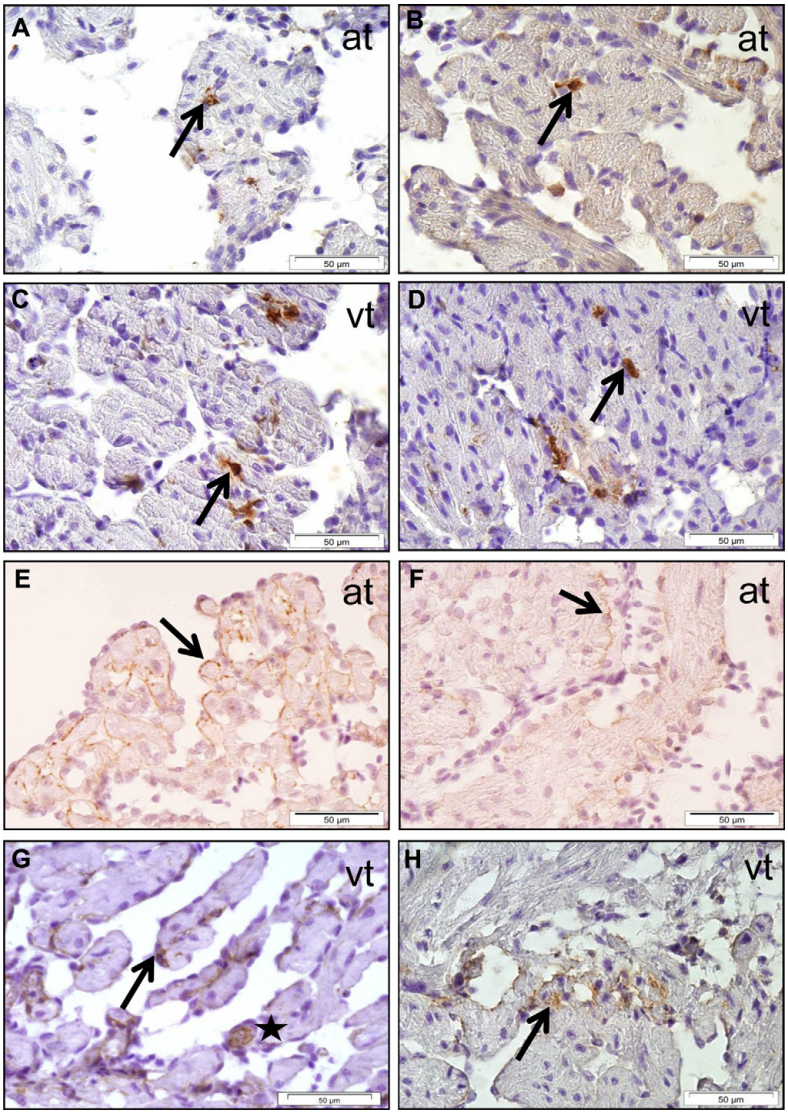

Fig. 5. Anti–HIF1α and caspase 3 immunostaining on diseased (A, C, E and G) and non–diseased (B, D, F and H) hearts of Atlantic salmon respectively. (A, B, C and D) The low numbers of HIF1α^{+} cells were identified in diseased and non–diseased atria (at) and ventricles (vt). (E, F, G and H) The low levels of endocardial immunostaining were observed for caspase 3 in diseased and control hearts. (G) The caspase 3 immunostaining appeared predominantly in affected areas (star).

cells and in line with Hetland et al. [36]. The low numbers of CD8^{+} cells have been suggested to have protective roles in the immune defense of Atlantic salmon against infectious salmon anaemia virus (ISAV) [36]. There were, however, strong levels of staining with the anti–CD3ε antisera identified in the diseased hearts (HSMI) and specifically in affected areas with inflammatory foci and degenerative myocytes. The staining was significantly different from non–diseased hearts where only a few CD3ε^{+} cells could be observed. There were more CD3ε^{+} cells present in ventricles as compared to atria in diseased hearts. The low number of CD8^{+} cells and higher number of CD3ε^{+} cells suggested these inflammatory lymphocyte–like cells were predominantly CD3ε^{+} T cells in HSMI disease similar to those identified in gill associated lymphoid tissue of salmon [33,7]. Anti–MHC class I staining showed a strong positive staining reaction in mononuclear cells, myocardium and endothelium in diseased hearts as compared to non–diseased hearts where endothelial staining was dominant, consistent with the findings from other viral diseases of Atlantic salmon [35,36,40]. MHC class I molecules are abundantly expressed in nearly all known cell types [40]. Besides the presentation of antigenic

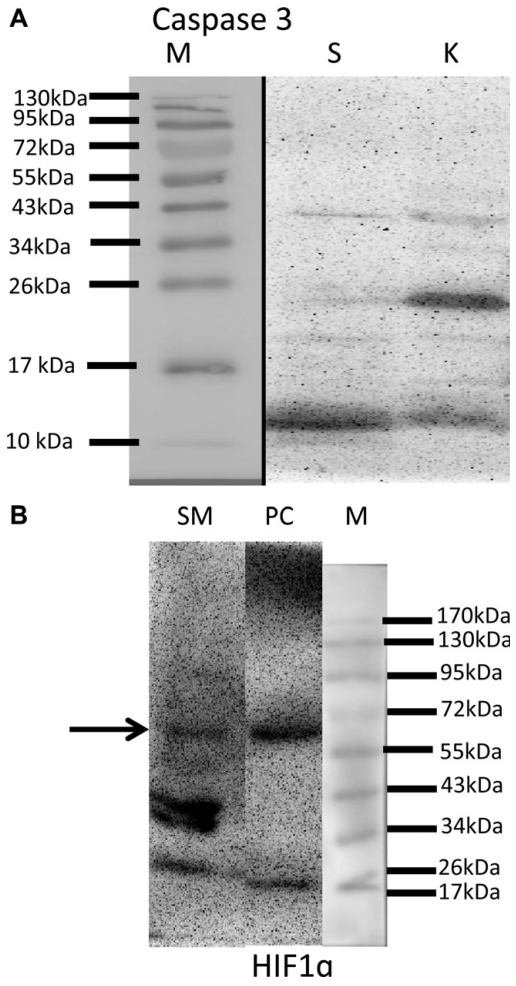

Fig. 6. Western blot analysis of different tissues of Atlantic salmon by using mammalian polyclonal antibodies (Caspase 3 and HIF1α). (A) The procaspase−3 (~40 kDa), caspase 3 (~12 kDa) and p20 (~21 kDa) bands were identified in the kidney while first two were visible in spleen. (K Kidney, S spleen, M PageRuler™ prestained protein ladder (Fermentas GmbH, Norway). (B) A band of same size (~70 kDa) was identified in skeletal muscle and positive control (HeLa + CoCl2 cell lysate) by using HIF1α antibody. (PC positive control, SM skeletal muscle and M PageRuler™ prestained protein ladder).

peptides to cytotoxic T lymphocytes, MHC class I molecules are also responsible for other biological functions including cell−to−cell communication and receptor−mediated trans−membrane signal transduction (reviewed in Ref. [41]. The strongly positive staining observed using MHC class II β antisera was mostly confined to regions of tissue damage and mononuclear cell infiltrations. Being antigen presenting molecules, MHC class II has been shown to be expressed on the leucocyte lineage (dendritic cells, macrophages, B cells and T cells) and epithelial cells [31,42]. Previously, MHC class II^+ cells have been suggested as T lymphocytes with HSMI [9]. The present study concurs with this interpretation and MHC class II cytoplasmic staining was confined to the myocardium, endocardium and mononuclear inflammatory cells. The MHC class II β chain antisera has been used for morphological studies before and identified specific staining for epithelial cells, multinucleated giant cells (MGC), macrophages and dendrite−like cells [43–45]. The MHC class II has been suggested to be involved in immune cell trafficking and antigen presentation in Atlantic salmon affected with amoebic gill disease [45]. The common regulatory mechanisms have been suggested for MHC class I and II expression in Atlantic salmon [46]. These findings may support the present study where strong levels of staining for both MHC class I and II with HSMI were observed. Moreover, the significantly increased expression of MHC class II^+ cells have been reported in Atlantic salmon affected with granulomatous uveitis and amoebic gill disease [43,45].

There were significant moderate levels of $rTNF\alpha^+$ cells in the diseased hearts while few cells were identified in the non−diseased hearts. The staining was mostly confined to macrophages and eosinophilic granule cells (EGCs) as well as in endocardium. EGCs were easily identified with their scattered granular appearance and were also $rTNF\alpha^+$ in the *bulbus arteriosus*. TNFα enhances EGCs recruitment in conjunction with LPS [47] but as an analogue of mast cells [48], are likely to be involved in enhancing T cell activation and release of TNFα similar to that seen in mammalian mast cell [49,50]. TNFα is known to be produced by macrophages in rainbow trout and mammalian eosinophilic granulocytes that were also suggested as antigen presenting cells [51–53]. Few functional studies have been conducted in teleosts and one of the *in vitro* study in rainbow trout showed that recombinant TNFα (rTNFα) enhanced the leucocyte migration and phagocytic activity. This study used the same recombinant rainbow trout TNFα antibody which has been shown specific by Western blot [34]. Another *in vivo* study in gilthead seabream showed rapid recruitment of phagocytic granulocytes to the injection site and the induction of granulopoiesis in the head kidney by rTNFα [54]. These studies may explain the possible reasons of increased number of macrophages/granulocytes due to increased expression of rTNFα that might attract macrophages/EGCs to the site of action in HSMI hearts than non−diseased hearts. The use of monoclonal rainbow trout granulocytes/monocytes antibody recognized mature myeloid like cells in the hearts. There was overall low signal strength in immuno−reactive cells and in addition, only few positive cells were identified in the diseased hearts. However, they could be differentiated from non−diseased hearts where very few cells were stained. This staining was also supported by rTNFα antibody which also stained moderate numbers of granular cells including EGCs in HSMI affected as compared to non−diseased hearts. It reflected that the inflammatory response was not primarily comprised of granulocytes in HSMI affected hearts. TUNEL identified non−significant moderate levels of staining for apoptotic cells in diseased hearts while caspase 3 presented with low levels of staining in the diseased hearts due to the fact that HSMI is a disease of more inflammation than necrosis/apoptosis [2,3,8] and in line with Grammes et al. [55]. Western blot analysis confirmed the specificity of caspase 3 antibody and identified procaspase 3 (~40 kDa) and caspase 3 (~12 kDa) bands and in line with Chiou et al. [56] while the middle bands (~21 kDa) may correspond to the p20 that is formed by the cleavage of procaspase 3 to caspase 3 in mammals [57]. The p20 band was not strong in immunoblot analysis of spleen tissue and might be explained by the rapid clearing of cells containing activated caspase 3 and thus contributed little to total proteins extracted from the tissue [58]. Over expression of caspase 3 cDNA into fish cell cultured cells and zebrafish resulted in extensive apoptosis and ceramide generation [59]. It might suggest the direct role of caspase 3 in inducing apoptosis. Environmental stresses such as UV light, heat shock and γ−irradiation triggered apoptosis and caspase 3 activity in

zebrafish embryo [60]. The presence of apoptotic cardiomyocytes in diseased and non–diseased hearts has been shown in many mammalian studies but higher in diseased groups than normal (reviewed in Ref. [61]). Necrosis being rapid in nature while energy demanding apoptosis may co–determine the final degree of lethal myocardial injury after ischemia and reperfusion in mammals (reviewed in Ref. [62]). The mammalian reovirus has also been shown to induce apoptosis in heart and central nervous system (reviewed in Ref. [63]) while recently the chum salmon reovirus has been shown to induce apoptosis and macrophage modulation in salmonid cell culture [64]. The caspase 3 antibody identified lower number of positive cells than TUNEL staining in the hearts affected with chronic heart failure [19]. Although not pathognomic for reovirus infections, the cellular responses seen with HSMI, the putative agent of which is the piscine reovirus (PRV) are associated with that seen with other reovirus–caused pathological changes (mammals and chum salmon) [63,64].

Being a highly conserved eukaryotic protein, PCNA showed significant strong granular staining suggesting the S–phase of the cell cycle while the significant increased cell proliferation has been described in many fish species in normal and diseased conditions [30,65–70]. The significant increase in $PCNA^+$ cells suggested an increased cell turnover and recruitment with amoebic gill disease in Atlantic salmon [45,71]. The pronounced and extensive staining of cardiomyocytes in HSMI affected hearts suggested active myocardial proliferation. A few $PCNA^+$ cells were also identified in the non–diseased hearts. This is to be expected since the heart is a dynamic organ capable of remodeling [8]. We suggest that the HSMI hearts were more active with increased cell division in the disease condition. PCNA staining also demonstrated the presence of hypertrophic nuclei in HSMI affected hearts supporting cardiac hypertrophy. PCNA has been suggested as marker of cell proliferation in human myocardial hypertrophy, myocarditis, valvular heart disease, ischemic heart disease and cardiomyopathy [72–74]. The low levels of $HIF1\alpha^+$ cardiomyocytes were identified in diseased and non–diseased hearts. Reports suggested that HIF1α expression may lead to cardiac degeneration, dysfunction and leading to heart failure in humans [75,76]. The presence of HIF1α staining in normal organs has been proposed their physiological roles in tissue homeostasis which may explain the low levels of HIF1α staining in normal hearts in this study. Recently, hypoxia has been quantified in Atlantic salmon and suggested its possible involvement in vertebral column deformity (VCD) [77]. Moreover, TNFα which is rapidly released after infection, has been suggested to activate HIF1 in mammals and may explain the expression of HIF1α in HSMI hearts [20].

There were significantly higher numbers of positive cells identified with antibodies such as CD3ε, MHC class II, rTNFα and PCNA in HSMI hearts. In addition, strong levels of CD3ε and MHC class II staining suggested the mononuclear lymphocyte–like cells as activated T helper cells but require further studies for confirmation. This study characterized the involvement of CD3ε lymphocytes, macrophages and granulocytes (identified by rTNFα and anti–granulocytes antibodies), and antigen presenting molecules (identified by MHC class I and II staining) in the hearts of Atlantic salmon affected with HSMI. The lymphocytic responses dominated the inflammation over granulocytic infiltrates. The increased number of $PCNA^+$ cells with increased expression of caspase 3 and TUNEL suggested an induction of cell and tissue repair occurring and apoptosis in the HSMI hearts.

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Paper IV

Comparative cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI), cardio-myopathy syndrome (CMS) and pancreas disease (PD)

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Key words: Atlantic salmon, CMS, HSMI, immunohistochemistry, PD.

Abbreviations

AEC: 3–amino–9–ethyl carbazole, BSA: bovine serum albumin, Caspase: cysteine–dependent aspartate protease, CHF: chronic heart failure, CMS: cardiomyopathy syndrome, DAB: 3, 3'–diaminobenzidine, EGC: eosinophilic granular cells, dH_2O: distilled water, H&E: haematoxylin and eosin, HIF: hypoxia inducible factor, HRP: horseradish peroxidase, HSMI: Heart and skeletal muscle inflammation, MHC: major histocompatibility complex, PBS: phosphate buffered saline, PBST: PBS with 0.1% Tween 20, PCNA: proliferative cell nuclear antigen, PD: pancreas disease, PVA: polyvinyl alcohol, PVDF: polyvinylidene fluoride, rt–TNF: recombinant rainbow trout tumor necrosis factor, SDS: sodium dodecyl sulfate, SDS–PAGE: sodium dodecyl sulfate– polyacrylamide gel electrophoresis, TCR: T cell receptors, TUNEL: terminal deoxynucleotidyl transferase nick–end labeling

Abstract

Heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD) are diseases of marine farmed Atlantic salmon (*Salmo salar*) which commonly affect the heart in addition to the skeletal muscle, liver and pancreas. The main findings of these diseases are necrosis and inflammatory cells infiltrates affecting different regions of the heart. In order to better characterize the cardiac pathology, study of the inflammatory cell characteristics and cell cycle protein expression was undertaken by immunohistochemistry. Immunohistochemistry was performed on paraffin embedded hearts from confirmed diseased cases applying specific antibodies. The inflammatory cells were predominantly $CD3^+$ T lymphocytes while few eosinophilic granulocytes were identified. The PD diseased hearts exhibited moderate hypoxia inducible factor–1α (HIF1α) immuno–reaction that suggested tissue hypoxia while recombinant tumor necrosis factor–α (rTNFα) antibody identified putative macrophages and eosinophilic granular cells (EGCs) in addition to endocardial cells around lesions. There were strong to low levels of major histocompatibility complex (MHC) class II immunostaining in the diseased hearts associated with macrophage–like and lymphocyte–like cells. The diseased hearts expressed strong to low levels of apoptotic cells identified by caspase 3 and terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) staining. The strong signals for proliferative cell nuclear antigen (PCNA) and TUNEL, and moderate levels of caspase 3 immuno–reactivity suggested a high cell turnover where DNA damage/repair might be occurring in the diseased hearts.

1. Introduction

The marine farmed Atlantic salmon (*Salmo salar*) are prone to numerous cardiac disorders including aplasia or hypoplasia of the septum transversum, abnormal location and shape of heart (Poppe and Seierstad, 2003), arteriosclerosis (Poppe et al., 2007; Farrel, 2002), ventricular hypoplasia (Poppe and Taksdal, 2000) and specific viral diseases such as cardiomyopathy syndrome (CMS) (Poppe and Ferguson, 2006; Poppe and Seierstad, 2003; Ferguson et al., 1990; Brun et al., 2003; Grotmol et al., 1997), heart and skeletal muscle inflammation (HSMI) (Kongtorp et al., 2004, 2006) and pancreas disease (PD) (Christie et al., 1998; Taksdal et al., 2007; Nelson et al., 1995).

HSMI is a disease of marine farmed Atlantic salmon that mainly affects heart and red skeletal muscle. It is characterized as disease of low mortality (20%) but with high morbidity (100%) that affects fish mostly after 5 to 9 months transfer to sea and has been reported from Norway, Scotland and Chile (Kongtorp et al., 2004a; Ferguson et al., 2005). HSMI is diagnosed by histopathological investigation with changes including epicarditis, mononuclear cell infiltration in both spongy and compact layers of ventricle and necrotic myocytes. Affected myocytes show signs of degeneration, loss of striation and eosinophilia, vacuolation, central nuclei localisation and karyorhexis (Poppe and Ferguson, 2006; Kongtorp et al., 2004, 2006). Recently, a viral etiology was suggested where piscine reovirus was associated with HSMI, nevertheless, Koch's postulates remained unfulfilled (Wiik–Nielsen et al., 2012; Palacios et al., 2010).

Pancreas disease (PD) was first described in Atlantic salmon in Norway in 1989 (Poppe et al., 1989) although an associated alpha virus was first isolated from diseased

Atlantic salmon from the west coast of Norway in 1998 (Christie et al., 1998). During last decade, PD has emerged to become a major economical and animal welfare problem for farmed Atlantic salmon in Europe. It has been reported from different regions of Europe such as Ireland, Scotland, UK, Spain, Italy and Norway (Ferguson et al., 1990; Christie et al., 1998; Taksdal et al., 2007; Poppe et al., 1989; McVicar, 1987; Rowley et al., 1998; Graham et al., 2003, 2007; Crockford et al., 1999). The mortality ranges from 1 to 42 % in natural outbreaks and outbreak lasts for 3–4 months (McLoughlin et al., 2002; Christie et al., 2007). The fish show inappetence, lethargy, yellow faecal casts and increased mortality. The acute phase of PD at 2–14°C lasts up until 10 days with inflammatory lesions in pancreas and heart as dominating features. This is followed by a sub–acute phase 10–21 days after onset of clinical signs with lesions in pancreas, heart and muscles, and a chronic phase after 21–42 days with lesions in muscles as dominating feature, and then subsequently a recovery phase (McLoughlin et al., 2002, Taksdal et al., 2007). The pathological changes involve severe losses of exocrine pancreas, cardiac and skeletal myopathies, epicarditis, focal gliosis of brain stem, white skeletal muscle degeneration and functionally unknown cells in kidney with cytoplasmic eosinophilic granules (Taksdal et al., 2007; Christie et al., 2007).

Cardiomyopathy syndrome (CMS) is a cardiac disease with a suggested totiviral etiology of Atlantic salmon that mainly affects atrium and trabecular ventricle without involvement of skeletal muscle. It shares similar features with HSMI where both diseases cause myocarditis (Poppe and Ferguson, 2006; Palacios et al., 2010; Ferguson et al., 1986; Løvoll et al., 2010). It was first reported in late–1980s in the cultured Atlantic salmon in Norway (Amin and Poppe, 1989; Ferguson et al., 1990) and also subsequently reported in the wild salmon (Poppe and Seierstad, 2003). Recently a piscine myocarditis virus (PMCV) belonging to family totiviridae was proposed as causative agent for cardiomyopathy (Wiik–

Nielsen et al., 2012; Løvoll et al., 2010; Haugland et al., 2011) and viral etiology was also supported by two separate challenge trials (Bruno and Noguera, 2009; Fristvold et al., 2009). Histopathological findings include necrosis and inflammation of trabecular myocardium of the ventricle and atrium, epicarditis and a cellular infiltrate includes mono–nuclear leucocytes. The rupture of the atrium or sinus venosus was also reported at terminal stages of CMS (Ferguson et al., 1990; Poppe and Ferguson, 2006). CMS may occur in adult salmon after 12–18 months of sea transfer and causes economic losses up to € 8.8 million annually in Norway (Ferguson et al., 1990; Brun et al., 2003). The number of HSMI outbreaks has been increased three times from 2004 (54 outbreaks) to 2010 (162 outbreaks) while PD has re–emerged from the beginning of the year 2000 (11 outbreaks) to 2011 (89 outbreaks) and CMS remained at uniform pattern with 53 outbreaks in 2010 in Norway (Bornø et al., 2011; Marta et al., 2012).

The heart is the common organ involved in all three of these apparently similar cardiac diseases and gives us an opportunity to characterize putative cardiac immunological differences using cell and cell cycle protein markers applying immunohistochemistry. Atlantic salmon as with other teleosts exhibits both innate and adaptive immune responses (Koppang et al., 2007; Nam et al., 2003; Liu et al., 2008; Moore et al., 2005). Initially, the innate immune system responds to pathogens include the actions of neutrophils, macrophages, mast cells, dendritic cells, and natural killer cells. These cells participate in the eradication of the pathogens and transmit signals that amplify adaptive immune response (Eltzschig and Carmeliet, 2011). Adaptive or cellular immunity comprises of the recognition of cell surface MHC–peptide complex by T lymphocytes. This system helps to protect the host from infections and represented mainly by helper T (Th) and cytotoxic T (Tc) lymphocytes defined by the expression of specific markers CD4 and CD8 respectively. MHC molecules interact with either CD4/TCR/CD3 or CD8/TCR/CD3 complex on antigen presenting cells where

CD3 serves as important trigger of T cell activation (Wang et al., 2009; Sun et al., 1995; Salter et al., 1989). TNFα as part of innate immune response reacts rapidly to different forms of stimuli such as bacteria, virus, parasitic infections, trauma, and ischemia/reperfusion (I/R) (reviewed in (Kleinbongard et al., 2011)). The cytokines and specially TNFα are capable of activating HIF1 that has been suggested to be involved in inflammation, apoptosis and influences adaptive immunity (reviewed in (Eltzschig and Carmeliet, 2011; Gale and Maxwell, 2010; Dehne and Brune, 2009)). Oxidative stress is one of the major factors causing damages to myocardium, and HIF1α is suggested to be important in physiological and pathological conditions (Poppe and Ferguson, 2006; Hopfl et al., 2004; Huang et al., 2004). Apoptosis or programmed cell death is an important process to remove damaged or unnecessary cells ensuring normal development of multicellular animals. Caspases (cysteine–dependent aspartate protease) are capable of mediating immune responses (apoptosis, necrosis and inflammation) and are key players in apoptosis (Takle and Andersen, 2007). Terminal deoxynucleotidyl transferase nick–end labeling (TUNEL) is used for *in situ* detection of cleaved DNA. Taken together, TUNEL and active caspase 3 immunostaining have been suggested as better approaches for detection of apoptosis in chronic heart failure (CHF) in humans (De–Boer et al., 2000). Proliferative cell nuclear antigen (PCNA) is suggested to be most commonly used marker for cellular proliferation and is widely accepted as a surrogate marker for DNA synthesis. PCNA is a marker for cells in different phases of the cell cycle, DNA repair and apoptosis (Soonpaa and Field, 1998; Eldridge and Goldsworthy, 1996; Chapman and Wolgemuth, 1994; Panday and Wang, 1995).

The present study was aimed to characterize and differentiate the immunological responses associated with apparently similar diseases (CMS, PD and HSMI) by immunohistochemistry. The markers for apoptosis and cell damage were studied using

TUNEL and polyclonal antibodies against caspase3, hypoxia transcription factor by HIF1α, mitotic activity by PCNA, pro–inflammatory cytokine TNFα by anti–recombinant trout TNFα, as well as monoclonal antibodies against salmonid granulocytes/monocytes by a marker expressed on mature myeloid cell lineage, T lymphocytes by CD3ε and CD8, and antigen presenting cells by MHC class I and II staining.

2. Materials and Methods

2.1. Hearts

Atlantic salmon hearts were collected from confirmed outbreaks of HSMI (n = 3–10), CMS (n = 3–7) and PD (n = 3–7). All of the HSMI, CMS and PD outbreaks were diagnosed and confirmed by National Veterinary Institute, Oslo, Norway (NVI) using PCR (for pancreas disease virus) and histopathology. Hearts from naive fish were collected as controls (n = 3–7). The control (non–diseased) fish were maintained from smolts for ~1 year at the Mørkvedbukta research station, University of Nordland, Bodø, Norway in 2 m^3 tanks supplied with fresh ambient sea water (range 7 – 8°C) and fed with commercial fish pellets (Spirit, Skretting, Stavanger, Norway) 0.7% of their body weight three times weekly. Fish were regularly monitored for mortalities and aberrant behavior. All fish were sacrificed by a blow to head in accordance with national regulations for research animals. The fish were examined by autopsy in addition to histological observation of the hearts and other vital organs including such as gills, liver, kidney and spleen for other abnormalities or signs of overt disease (Table 1). The diagnosis of the diseases was confirmed during the study by histopathology, a method of choice for the above mentioned diseases (Kongtorp, 2008; Poppe and Ferguson, 2006). Diseased and non–diseased hearts were fixed in 10% neutral phosphate

buffered formalin solution and processed by a standard paraffin wax protocol (dehydrated, embedded in paraffin and 3 µm thick sections).

2.2. Antibodies

The antibodies used in this study included polyclonal rabbit proliferative cell nuclear antigen (PCNA) (sc–7907, Santa Cruz Biotechnology, Santa Cruz, CA) 1:150, polyclonal rabbit caspase 3 (sc–7148, Santa Cruz Biotechnology, Santa Cruz, CA) 1:500, polyclonal goat hypoxia inducible factor (HIF1α) (sc–8711, Santa Cruz Biotechnology, Santa Cruz, CA) 1:200, polyclonal rabbit anti–salmon major histocompatibility complex (MHC) class II β chain 1:1000 (Koppang et al., 2003), mab 21 anti–rainbow trout granulocytes/monocytes antibody recognizes a marker expressed on mature myeloid cell lineage 1:2000, polyclonal rabbit anti–salmon CD3ε antibody 1:400 (Koppang et al., 2010) and polyclonal rabbit anti–trout tumor necrosis factor–α (TNFα) antibody 1:500 (Zou et al., 2003). Anti–salmon mouse monoclonal antibodies included Sasa CD8 alpha F1–29 and Sasa MHC I alpha F1–34 (Hetland et al., 2010, 2011; Olsen et al., 2011).

2.3. Immunohistochemistry on Atlantic salmon hearts

Immunohistochemistry was performed on the heart sections by following the protocol described by Haugarvoll et al. (2008). The heart sections were mounted on poly–L–lysine (Sigma–Aldrich, Norway) coated slides, dried at 50º C for 30 min, deparaffinized in xylene using three changes for 5 min each, followed by graded ethanol baths from 80% to absolute alcohol for 10 min each and rehydrated in distilled water (dH_2O). Antigen retrieval was performed by autoclaving the slides at 121ºC for 20 min in a box in 10 mM citrate buffer, pH

6.0 containing 0.1% Tween 20 (Sigma–Aldrich, Norway). Slides were kept at room temperature for 20 min and then washed twice with distilled water for 2 min each. All incubations were performed in a closed–lid humidity chamber. To inhibit endogenous peroxidase activity, the slides were incubated with 3% H_2O_2 (Sigma–Aldrich, Norway) in methanol for 10 min at room temperature. The sections were incubated in 5% bovine serum albumin (BSA) (Sigma–Aldrich, Norway) in phosphate buffer saline (PBS) (Sigma–Aldrich, Norway) for 1 h at room temperature. After removal of the BSA, slides were incubated with primary antibodies in 1.5% BSA in PBS overnight (~16 hours) at 4 ºC. Following day, slides were washed three times with PBS with 0.1% Tween 20 (PBST) (Sigma–Aldrich, Norway), pH 7.4 for 5 min each. After washing, slides were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (sc–2020, donkey anti–goat or sc–3837, goat anti–rabbit, Santa Cruz biotechnology) in 1.5% BSA in PBS for 60 min at room temperature. Slides were washed three times with PBST at room temperature for 5 min each. Slides were incubated with 3, 3'–diaminobenzidine (DAB; Sigma–Aldrich, Norway) or 3–Amino–9–ethylcarbazole (AEC; Sigma–Aldrich, Norway) for 5 min and then washed with distilled water for 5 min. Slides were dipped in haematoxylin for 10 sec for counterstaining and then passed through graded ethanol and xylene for 10 sec each and mounted with poly–vinyl alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway). Negative controls included primary antibody replaced with 1.5% BSA in PBS.

2.4. Immunohistochemistry using anti–salmon CD8 and MHC class I mouse mabs

The immunohistochemistry was performed by following Tyramide Signal Amplification (TSA) Biotin system (PerkinElmer/ NEN Life Science, Boston, MA, USA) for CD8 and MHC class I antibodies. The protocol was performed as described in section 2.3 until antigen retrieval. The slides were treated with 3% H_2O_2 (Sigma–Aldrich, Norway) in

ethanol for 10 min at room temperature followed by washed twice in PBS to avoid endogenous peroxidase. The sections were blocked with TNB blocking buffer (0.1 M TRIS–HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent supplied in the TSA system) for 1 hour at room temperature followed by incubation of slides with primary antibodies in TNB buffer overnight at 4 ºC. Following morning slides were washed three times with PBS for 5 min each. Slides were incubated with HRP labeled anti–mouse secondary antibody (sc–3697, goat anti–mouse, Santa Cruz biotechnology) diluted in TNB blocking buffer for 1 hour at room temperature to detect the primary antibodies. Three times PBS washing was performed as mentioned above after secondary antibody step. Slides were incubated with biotinyl tyramide amplification reagent followed by streptavidin–horseradish peroxidase (SA–HRP) (provided with the kit). Slides were incubated for 5 min with 3–amino–9–ethyl carbazole (AEC; Sigma–Aldrich, Norway) substrate. Slides were counterstained with haematoxylin for 10 sec and mounted using ImmunoHistoMount (Sigma–Aldrich, Norway). The negative controls were performed by replacing primary antibodies with TNB blocking buffer and unamplified controls that included all reagents except TSA reagents.

2.5. Western blot

The frozen tissues such as kidney, liver, gills, skeletal muscle and spleen (approx. 30 mg) were minced on ice and transferred to tubes containing RIPA buffer (150 mmol l^{-1} Nacl, 50 mmol l^{-1} Tris–Hcl at pH 8.0, 1% Triton X–100, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail (cOmplete ULTRA tablets, Mini *EASY*pack, Roche Diagnostics, GmbH, Germany) and homogenized by ultrasonic device at 4°C. Proteins samples (50–100 µg) and HeLa + CoCl2 cell lysate (sc–24679, Santa Cruz Biotechnology) as positive control were separated by denaturing SDS–PAGE using 12% polyacrylamide gels for

PCNA and caspase 3 antibodies and 8% polyacrylamide gel for HIF1α antibody by following the protocol described by Laemmli (1970). Samples were blotted on polyvinylidene fluoride (PVDF) membrane by following Koppang et al. (2003) and tested for mammalian polyclonal PCNA (1:1000), caspase 3 (1:500) and HIF1α (1:100) antibodies. The secondary antibodies (1:5000) conjugated to horseradish peroxidase (HRP) (sc–2020, donkey anti–goat or sc–3837, goat anti–rabbit, Santa Cruz biotechnology) were used and detected by enhanced chemiluminescence (Immobilon Western kit (Millipore)).

2.6. TUNEL staining

TUNEL staining was performed by following ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON® Int. Inc. USA). Briefly, the hearts were deparafinised through graded series of xylene and alcohol and then pretreated with freshly made IHC Select® Proteinase K (20 µg ml^{-1}) (Millipore) for 15 min at room temperature and washed twice in distilled water (dH_2O) for 2 min each. The endogenous peroxidase was quenched with 3% hydrogen peroxide (Sigma–Aldrich, Norway) in PBS for 5 min at room temperature. Slides were rinsed twice with dH_2O for 5 min each. Slides were carefully aspirated and immediately dipped in equilibration buffer at 75 µl 5 $cm^{-2}$for 10 s. The working strength TdT enzyme was used as 55 µl 5 cm^{-2} and incubated in a humidified at 37°C for 1 hour. Slides were dipped in working strength stop/wash buffer, agitated for 15 s and then incubated for 10 min at room temperature. The slides were washed with 3 changes of PBS for 1 min each and then dipped in anti–digoxigenin conjugate at 65 µl 5 cm^{-2} and incubated for 30 min at room temperature in a humidified chamber. Slides were washed with 4 changes of PBS for 5 min each. Sections were carefully blotted and dipped in peroxidase substrate at 75 µl 5 cm^{-2} and incubated for 3–6 min at room temperature. Sections were washed thrice in

dH_2O for 1 min each. Slides were counter stained with 0.5% (W:V) methyl green (Sigma–Aldrich, Norway) for 10 min and washed three times in dH_2O. Finally, the slides were washed in 100% N–butanol (Sigma–Aldrich, Norway). Positive control (provided with the kit) and negative control by replacing TdT with equilibration buffer was performed. Slides were dehydrated in a graded series of alcohols, cleared in xylene for 2 min and mounted with poly–vinyl alcohol (PVA) mounting media, pH 8.2 (Histolab, Oslo, Norway). All reagents were either supplied with the kit or mentioned. The apoptotic cells were identified by both positive staining and morphological signs of apoptosis.

2.7. Slides evaluation

The slides were evaluated by light microscopy using Olympus microscope BX51 equipped with Cell^B software (Olympus Corporation, Tokyo, Japan). A semi–quantitative scoring system for positive cell frequency was adopted that has been widely used (Hetland et al., 2010, 2011; Koo et al., 2009) and graded as (no staining = –, weak staining = +, moderate staining = ++, strong staining = +++) where strong = severe, diffuse staining; moderate = multifocal staining or many positive single cells; low = focal staining or few positive cells; and no staining. A variation in staining intensity and frequency was present between different antibodies.

3. Results

Strong levels of CD3ε^+ immunoreactivity were found in HSMI–diseased hearts (Fig. 1C) as compared to moderate levels in CMS– and PD–affected hearts, and low levels

identified in non–diseased hearts (Fig. 1A, B and D). Immunoreactivity was mostly identified around areas with pathological changes. There were low levels of cytotoxic T (CD8) positive cells in both diseased and non–diseased hearts (Fig. 1E, F, G and H). The CD8 immunostaining appeared cytoplasmic in the mono–nuclear cells (lymphocytes–like cells). Strong levels of MHC class I immunoreactivity was identified in both diseased and non–diseased hearts (Fig. 2A, B, C and D) where immunostaining was myocardial as well as endocardial in diseased hearts (Fig. 2A, B and C) as compared to predominantly endocardial localization in non–diseased hearts (Fig. 2D). The MHC class I immuno–reaction was also identified in macrophage–like cells in diseased heart (Fig. 2A, insert). However, anti–MHC class II β chain immunostaining showed moderate reaction in CMS–affected hearts (Fig. 2E), low levels to no staining in PD–affected hearts (Fig. 2F) while strong levels observed in HSMI–affected hearts (Fig. 2G). There was low reactivity of MHC class II β chain in non–diseased hearts (Fig. 2H). The MHC class II β chain antibody identified dendrite–like cells (Fig. 2E, insert) and lymphocyte–like cells (Fig. 2G, insert), and staining pattern was myocardial (Fig. 2E and F) as well as endocardial (Fig. 2G) which was obvious especially around lesioned areas. Moderate levels of immunoreactivity were found using rTNFα antibody in CMS–, PD– and HSMI–affected hearts (Fig. 3A, B and C) while low levels identified in non–diseased hearts (Fig. 3D). Immuno–reaction was predominantly confined to eosinophilic granular cells (Fig. 3C, insert), macrophage–like cells (Fig. 3B, insert) and in areas surrounding lesions (Fig. 3A, insert, B and C). Strong to moderate immuno–reactivity resulting from PCNA positive cells were observed in all three investigated heart diseases (Fig. 3E, F and G) while focal low levels of immuno–reactivity were identified in non–diseased hearts (Fig. 3H). PCNA immunostaining was cytoplasmic as well as nuclear where hypertrophic nuclei were also identified in diseased hearts. The HIF1α immuno–reaction identified moderate levels of positive cells, especially in damaged cardiomyocytes, in PD–

affected hearts (Fig. 4B) and low levels to no staining in CMS–, HSMI–affected and non–diseased hearts (Fig. 4 A, C and D). Moderate levels of caspase 3 immunoreactivity were identified in CMS– and PD–affected hearts (Fig. 4E and F) while low levels to no immunostaining were found in HSMI–affected and non–diseased hearts respectively (Fig. 4G and H). TUNEL staining identified strong levels of apoptotic cells in CMS–affected hearts (Fig. 5 A), moderate levels of positive cells in PD– and HSMI–affected hearts (Fig. 5 B and C) and low to no staining in non–diseased hearts (Fig. 5D). The mab 21 for granulocytes/monocytes staining identified low levels of immuno–reaction in diseased (Fig. 5E, F and G) and non–diseased hearts (Fig. 5H).

Western blot analysis was performed to show the specificity of mammalian polyclonal antibodies (PCNA, caspase 3 and HIF1α antibodies). Three subunits were identified with caspase 3 antibody. Procaspase 3 (~40 KDa) and caspase 3 (~12 KDa) bands were identified in both tissues while middle band of ~21 KDa that may correspond to P20 subunit was visible in kidney (Fig. 6A). PCNA antibody showed single band (~30 KDa) in all tissue samples (Fig. 6B) while HIF1α antibody gave a band at ~70KDa (Fig. 6C). The immunohistochemistry findings of three diseased (HSMI, CMS and PD) and non–diseased hearts were summarized (Table 2).

4. Discussion

In this study, we have addressed immunopathological features of heart manifestations in CMS–, PD–, and HSMI–affecting Atlantic salmon. So far, only very limited information on the pathological responses of these apparently similar cardiac diseases have been available, and this study might be a useful addition in order to provide differential diagnostic criteria.

Disease outbreaks involving cardiac manifestations have increased dramatically in farmed Atlantic salmon during last decade (Bornø et al., 2011). This study was aimed to identify, characterize and differentiate the inflammatory cell populations, responses and cell cycle proteins associated with cardiac pathological changes in the above mentioned diseases. The inflammatory cells infiltrating different cardiac compartments appeared to be mono–nuclear and lymphocyte–like as identified with H&E staining. The cytotoxic T cells (CD8) staining (Hetland et al., 2010, 2011; Olsen et al., 2011) identified few lymphocyte–like cells in diseased and non–diseased hearts. However, CMS–affected hearts identified with more $CD8^+$ cells as compared to other diseased and non–diseased hearts. The low levels of $CD8^+$ cells in diseased hearts have been suggested to have protective roles in Atlantic salmon (Hetland et al., 2011). Anti–CD3ε antibody identified large numbers of T cells in the diseased hearts with the majority of these cells being localized in areas of inflammatory foci, while few $CD3\varepsilon^+$ cells were present in non–diseased hearts. The inflammatory cell population comprised of predominantly $CD3^+$ T lymphocytes in the investigated diseased hearts.

There were strong levels of MHC class I staining in diseased and non–diseased hearts and in line with previous findings that MHC class I are abundantly expressed in nearly all known cell types (Dijkstra et al., 2003). The MHC class I staining identified macrophage–like cells in addition to myocardial and endocardial staining where later was dominant in non–diseased hearts (Dijkstra et al., 2003; Hetland et al., 2010). However, the MHC class II staining identified strong levels of staining in HSMI– and moderate levels of staining in CMS–affected diseased hearts. The MHC class II staining was mostly confined to inflammatory foci and identified lymphocyte–like and macrophage–like cells. In addition, myocardial and endocardial staining was evident in the hearts by MHC class II antibody. As an antigen presenting molecule, MHC class II has been shown to be expressed on cells of the

leucocyte lineage (dendritic cells, macrophages, B and T cells) and epithelial cells (Haugarvoll et al., 2008; Glimcher and Kara, 1992). Previously, cells stained with MHC class II have been suggested as T lymphocytes, epithelial cells, multinucleated giant cells (MGC), macrophages and dendrite–like cells (Koppang et al., 2003a, 2004). The MHC class II cells has been indicative of immune cell trafficking and suggested to be involved in antigen presentation in Atlantic salmon affected with amoebic gill disease (Morrison et al., 2006). The common regulatory mechanisms have been suggested for the expression of MHC class I and II in Atlantic salmon (Koppang et al., 1999). Recently, a CMS transcriptomic study identified the up–regulation of T–lymphocytes (CD3 and CD8), MHC and apoptotic genes, and also correlated with histopathological changes (Timmerhaus et al., 2011). The increased presence of MHC class II^+ cells have been described in granulomatous uveitis and amoebic gill disease of Atlantic salmon (Koppang et al., 2004; Morrison et al., 2006). These studies were in line with the strong to moderate levels of MHC class II staining observed in diseased hearts in the present study. The strong to moderate levels of $CD3^+$ and MHC class II^+ cells in diseased hearts suggested the cardiac inflammatory cells as activated T helper cells; however this assumption requires further assessment for confirmation.

The rTNFα antibody identified eosinophilic granulocytes (EGCs), macrophage–like cells and endocardial staining where EGCs were easily identified with their scattered granular appearance. Eosinophilic granulocytes were also identified in bulbus arteriosus of diseased and non–diseased hearts. Moderate levels of rTNFα staining were observed in all investigated diseased hearts. TNFα is a proinflammatory cytokine that is one of the very first responses to pathological insult and is produced by activated macrophages and T cells at site of infection/inflammation (see reviews (Van Snick, 1990; Moller and Villiger, 2006; Brouckaert et al., 1993)). TNFα enhances EGCs recruitment in conjunction with LPS (Olszewski et al.,

2007) but as an analogue of mast cells (Reite and Evensen, 2006; Qin et al., 2001), are likely to be involved in enhancing T cell activation and release of TNFα similar to that seen in mammalian mast cell (Hogan et al., 2008; Rothenberg and Hogan, 2006). Recently Wee et al. (2011) have shown that TNFα regulates the lymphocyte trafficking in sheep. TNFα has been produced by macrophages in rainbow trout and mammalian eosinophilic granulocytes which were capable of antigen presentation (Qin et al., 2001, Hogan et al., 2008; Rothenberg and Hogan, 2006). There is a general lack of functional studies in teleosts, but one of the *in vitro* study suggested that the recombinant TNFα (rTNFα) enhanced the leucocyte migration and phagocytic activity in rainbow trout (Zou et al., 2003). The current study identified macrophage–like cells and eosinophilic granulocytes by using the same rTNFα antibody (Zou et al., 2003). In addition, García–Castillo et al. (2004) have demonstrated the rapid recruitment of phagocytic granulocytes to the injection site and the induction of granulopoiesis in the head kidney by rTNFα. The presence of macrophages/eosinophilic granulocytes in the current study was suggested to be activated/migrated in the hearts due to above mentioned diseases (CMS, PD and HSMI) of Atlantic salmon but further studies are required to confirm this assumption. The mature myeloid cell lineage granulocytes/monocytes staining were low in all diseased and non–diseased hearts supporting the lymphocytic ($CD3^+$ T cells) nature of infiltrating cells in diseased hearts. The mab 21 was produced to recognize a marker on granulocytes/monocyte cells, but not against B–cells, T–cells, thrombocytes or erythrocytes progenitors. The lymphocytic response dominated the inflammation over granulocytic infiltrates.

Western blotting confirmed the cross reactivity of mammalian polyclonal antibodies to Atlantic salmon tissue. Western blot analysis of caspase–3 antibody identified procaspase–3 (~40 kDa) and caspase 3 (~12 kDa) bands and in line with Chiou et al. (2009) while the

middle bands (~21 kDa) may correspond to the p20 that is formed by the cleavage of procaspase–3 to caspase–3 in mammals (Chiou et al. 2009; Fernandes–Alnemri et al., 1994). The p20 form was not detected by immunoblot analysis of spleen tissue and might be explained by the rapid clearing of cells containing activated caspase 3 and thus contributed little to total proteins extracted from the tissue (Krajewska at al., 1997). The polyclonal PCNA antibody identified a single band of ~30 kDa. PCNA is a highly conserved eukaryotic protein suggesting the cell division. This study identified strong to moderate levels of PCNA staining in diseased hearts and in agreement with previous studies (Zenker et al., 1987; Ortego et al., 1995). In addition, significant increase in the number of $PCNA^{+}$ cells has been suggested as high cell turn over and recruitment in Atlantic salmon affected with amoebic gill disease (Morrison et al., 2006; Adams and Nowak, 2003). The active myocardial hyperplasia was indicated in diseased hearts due to pronounced and extensive immunostaining of cardiomyocytes. The low levels of PCNA staining in non–diseased hearts may be explained by the dynamic nature of hearts capable of remodeling (Becker et al., 2011; Poss, 2007; Sun et al., 2009; Vornanen et al., 2002; Poppe and Ferguson, 2006). PCNA staining suggested high cell division activity in diseased hearts and also identified hypertrophic nuclei. In humans, PCNA has been suggested as a marker of cell proliferation in myocardial hypertrophy, myocarditis, valvular heart disease, ischemic heart disease and cardiomyopathy (Matturi et al., 1997, 2002; Arbustini et al., 1993).

The specificity of polyclonal HIF1α antibody was demonstrated. HIF1α antibody identified a band of ~70 kDa that was in line with rainbow trout HIF1α (Soitamo et al. 2001). The HIF1α showed moderate levels of staining in PD–infected hearts, while low levels were identified in CMS– and HSMI–infected hearts. HIF1α is a transcriptional factor that is upregualted under hypoxia, and moderate levels of HIF1α staining in PD–infected hearts

suggested possible hypoxic hearts (Hopfl et al., 2004; Gale and Maxwell., 2010; Dehne and Brune, 2009). HIF1α staining in diseased hearts was also supported by the fact that HIF1α is involved in inflammation, apoptosis and can influence adaptive immune response. However, induced hypoxia has not shown any effect on the severity of PD in Atlantic salmon that was suggested due to either low levels of hypoxia or fish acclimatized to the hypoxic conditions (Anderson et al., 2010). The caspase 3 and TUNEL staining identified strong to moderate levels of apoptotic cells in diseased hearts. The caspase 3 identified cytoplasmic staining in cardiomyocytes and in line with De–Boer et al. (2000) and Krajewska at al. (1997). The CMS and PD are the cardiac diseases identified with more degenerative changes as compared to HSMI where inflammatory changes dominated in the heart (Kongtorp et al., 2004, 2004a; Grammes et al. 2012). These findings were pointing the high cell turn over in the hearts where DNA damage/repair might be the case (as identified by PCNA, caspase 3 and TUNEL reactivity) (Table 2) (Ferguson et al., 1986; Poppe and Ferguson, 2006; Kongtorp et al., 2004, 2006). In addition, the transcriptomic profile of CMS–affected fish has shown the upregulation of apoptosis genes, supporting the strong to moderate levels of apoptosis staining in diseased hearts (Timmerhaus et al., 2011).

Comparatively, the current investigation identified the strong levels of CD3 and MHC class II immunostaining in HSMI–affected hearts as compared to moderate to low levels in CMS– and PD–affected heart. The moderate levels of HIF1α immuno–reactivity identified in PD–affected hearts as compared to low levels of staining in CMS– and HSMI–affected hearts. The strong levels of PCNA immuno–reaction identified in CMS–affected hearts as compared to moderate levels in HSMI– and PD–affected hearts. The moderate levels of caspase 3 immunostaining identified in CMS– and PD–affected hearts as compared to low levels of staining in HSMI–affected hearts. TUNEL staining identified strong levels in CMS–affected

hearts as compared to moderate levels in PD– and HSMI–affected hearts. However, there were no differences for CD8, MHC class I, TNFα and granulocytes/monocytes staining between the three investigated diseases. The CD3, MHC class II, PCNA, TNFα, caspase 3, HIF1α and TUNEL staining were confined to the lesioned areas in the diseased hearts, pointing to the pathological changes and appeared promising in the identification of lesioned areas in the investigated diseased hearts. The strong levels of TUNEL– and $PCNA^{+}$ cells with moderate levels of caspase 3 and HIF1α staining suggested an induction of cell and tissue damage/repair occurring in the diseased hearts. In conclusion, the immunohistochemical approach appeared promising to identify and differentiate the cardiac immunological responses at least for the given investigated diseases (HSMI, CMS and PD).

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Table 1. Main tissues affected and histopathological lesions appeared in the investigated diseases (Cardiomyopathy syndrome, CMS, Heart somatic muscle inflammation HSMI and pancreas disease PD. (compiled from Kongtorp et al. (2004)).

Tissue	**Lesions description**	**CMS**	**HSMI**	**PD**
Heart	Epicarditis	+	+	+
	Compact–myocarditis and degeneration	–	+	+
	Spongy–myocarditis and degeneration	+	+	+
Skeletal muscle	Inflammation and degeneration	–	+	+
Liver	Necrosis of hepatocytes	–	–	+
Pancreas	Necrosis of exocrine tissue.	–	–	+

Table 2. Comparative scoring of different immunohistochemical markers in the hearts of Atlantic salmon (*Salmo salar* L.). HSMI: heart and skeletal muscle inflammation, CMS: cardiomyopathy syndrome, PD: pancreas disease and control: non–diseased fish.

Antibodies	**Hearts**			
	HSMI	**CMS**	**PD**	**Control**
CD3ε	+++	++	++	+
CD8	+	+	+	+
MHCI	+++	+++	+++	+++
MHCII	+++	++	+/–	+
mab21	+	+	+	+
rTNFα	++	++	++	+
PCNA	++	+++	++	+
HIF1α	+	+	++	+/–
Caspase 3	+	++	++	+/–
TUNEL	++	+++	++	+

Strong staining = +++, moderate staining = ++, focal staining = +, no staining = –.

Figure legends

Figure 1. Anti–CD3ε and CD8 immunostaining in CMS–, PD–, HSMI–affected and non–diseased hearts of Atlantic salmon. (A and B) Moderate and (C) strong levels of $CD3\varepsilon^+$ cells (arrow) were identified in the diseased hearts especially around inflamed areas. (D) Low levels of $CD3\varepsilon^+$ cells were also present in non–diseased heart. (E, F, G and H) Few $CD8^+$ cells were identified in diseased and non–diseased Atlantic salmon hearts.

Figure 2. MHC class I and II β chain immunoreactivity in diseased (CMS, PD and HSMI) and non–diseased hearts of Atlantic salmon. (A, B, C and D) The strong levels of MHC class I staining was observed (arrow) in diseased and non–diseased salmon hearts. (A and B) The diseased hearts exhibited both myocardial and endocardial staining while (D) later was dominant in non–diseased hearts. The inset shows (A) MHC class I^+ macrophage–like cell. (E) MHC class II staining showed moderate levels of staining (arrow) in CMS–affected hearts while (G) strong levels of staining was identified in HSMI–affected hearts especially around inflamed areas. (F and H) There was low to no staining in PD–affected and non–diseased hearts. The insets show (E) MHC class II^+ dendrite–like cells and (G) lymphocyte–like cells.

Figure 3. Anti–rTNFα and PCNA immunostaining in diseased (CMS, PD and HSMI) and non–diseased hearts of Atlantic salmon. (A, B and C) Moderate levels of staining was observed for $rTNF\alpha^+$ cells in CMS–, PD – and HSMI–affected hearts (D) while low levels of staining observed in non–diseased hearts. The insets show (A) myocardial staining, (B) macrophage–like cells and (C) eosinophilic granulocytes (H&E). (E, F and G) Strong to moderate levels of PCNA positivity was observed in CMS–, HSMI– and PD–affected hearts (H) while low levels of $PCNA^+$ cells were identified in non–diseased hearts. The hearts

presented granular nuclear (arrow) and cytoplasmic (star) staining. Hypertrophic nuclei observed in diseased heart (arrow head).

Figure 4. Anti–HIF1α and caspase 3 immunostaining in diseased and non–diseased hearts of Atlantic salmon. (B) The moderate level of HIF1α^{+} cells were identified in PD–affected hearts while (A, C and D) low levels to no staining was identified in CMS–, HSMI–affected and non–diseased hearts. The inset shows (B) mono–nuclear cell. (E and F) The moderate levels of staining observed for caspase 3 in CMS– and PD–affected hearts while (G and H) low levels to no staining were identified in HSMI–affected and non–diseased hearts.

Figure 5. TUNEL and mature myeloid cell lineage granulocytes/monocytes immunostaining for detection of apoptosis and granulocytes/monocytes in diseased (CMS, PD and HSMI) and non–diseased hearts of Atlantic salmon. (A) The strong levels of staining were identified for TUNEL in CMS–affected hearts (B and C) while moderate levels of staining were identified in PD– and HSMI– affected hearts. (D) Low levels of apoptotic cells were identified in non–diseased hearts. (E, F, G and H) Low levels of immuno–reaction were identified for granulocytes/monocytes in diseased and non–diseased salmon hearts.

Figure 6. Western blot analysis of polyclonal antibodies such as (a) caspase 3, (b) PCNA and (3) HIF1α. Atlantic salmon tissues were separated by denaturing SDS–PAGE electrophoresis in 12% (caspase 3 and PCNA) and 8% polyacrylamide gels. Bound antigens were detected using enhanced chemiluminescence. (A) The caspase 3 antibody identified procaspase–3 (~40 kDa), caspase–3 (~12 kDa) and p20 (~21 kDa) bands in the kidney while first two were visible in spleen. (K Kidney, S spleen, M PageRuler™ prestained protein ladder (Fermentas GmbH, Norway) (B) PCNA antibody identified single band (~30 kDa) in all four tissues. (G

gills, K Kidney, L liver, S spleen, M PageRuler™ prestained protein ladder). (C) The HIF1α antibody identified a band of same size (~70 kDa) to the positive control (HeLa + CoCl2 cell lysate) in the skeletal muscle by HIF1α antibody. (PC positive control, SM skeletal muscle, M PageRuler™ prestained protein ladder).

Figure 1

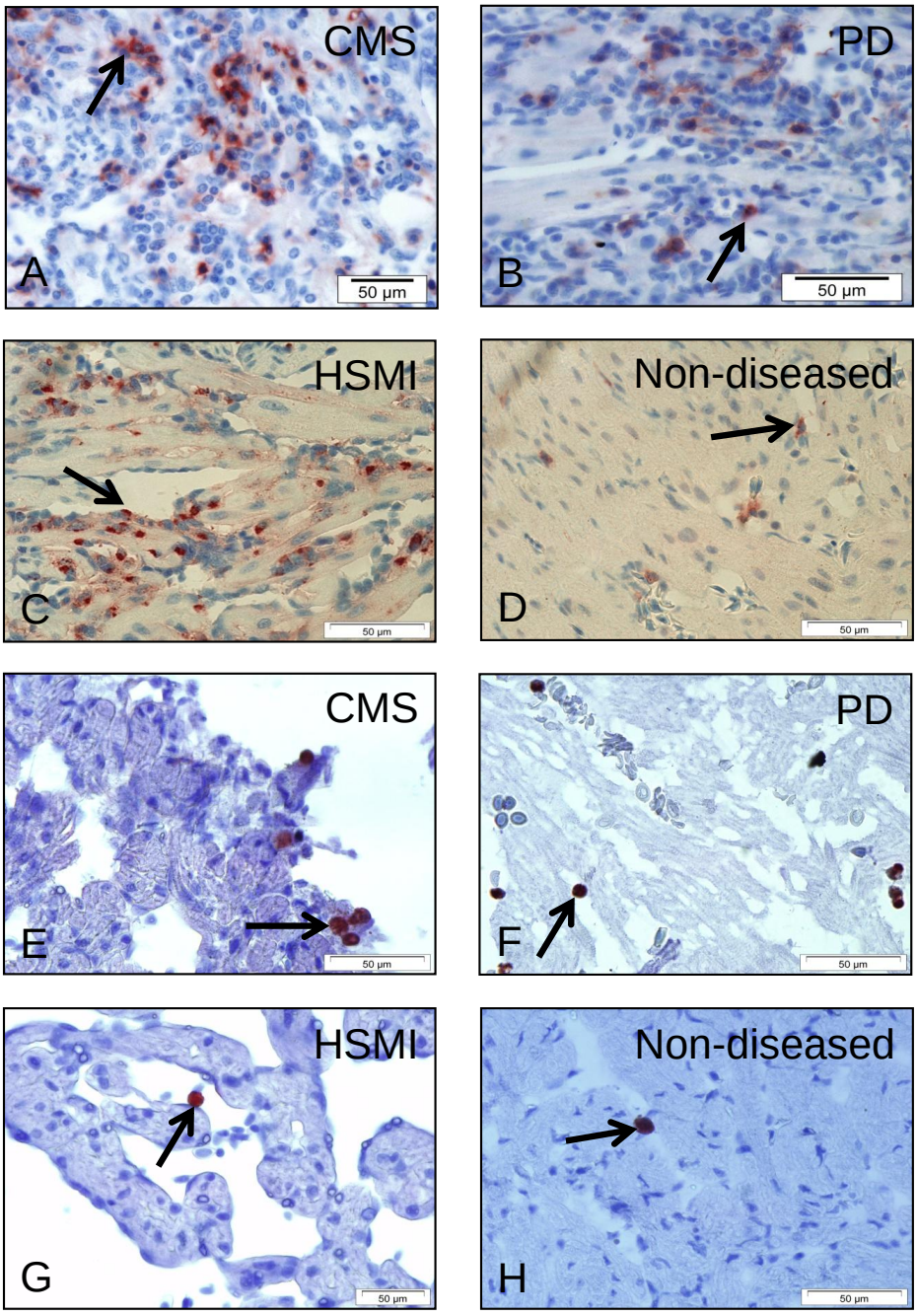
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Figure 2

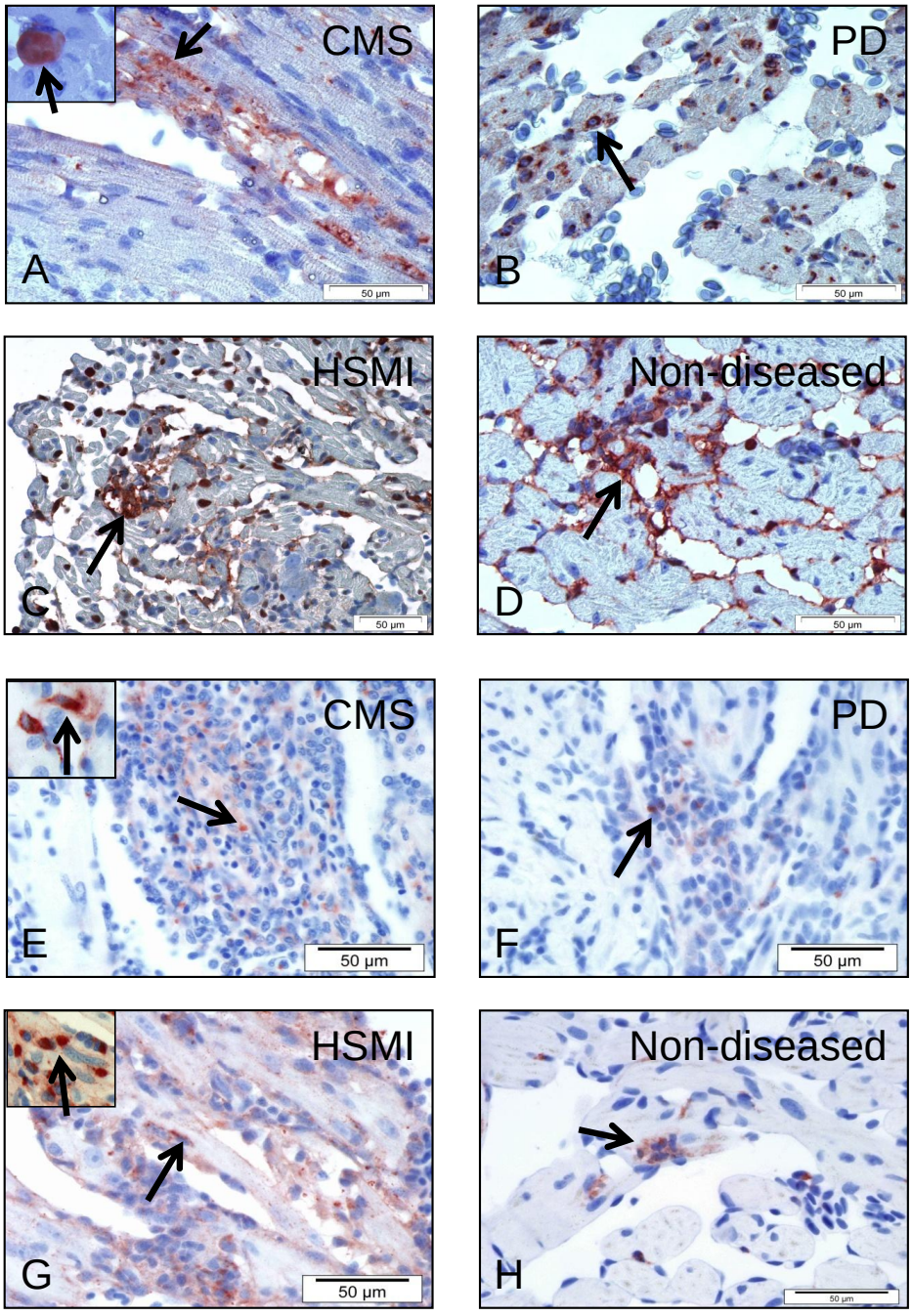
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Figure 3

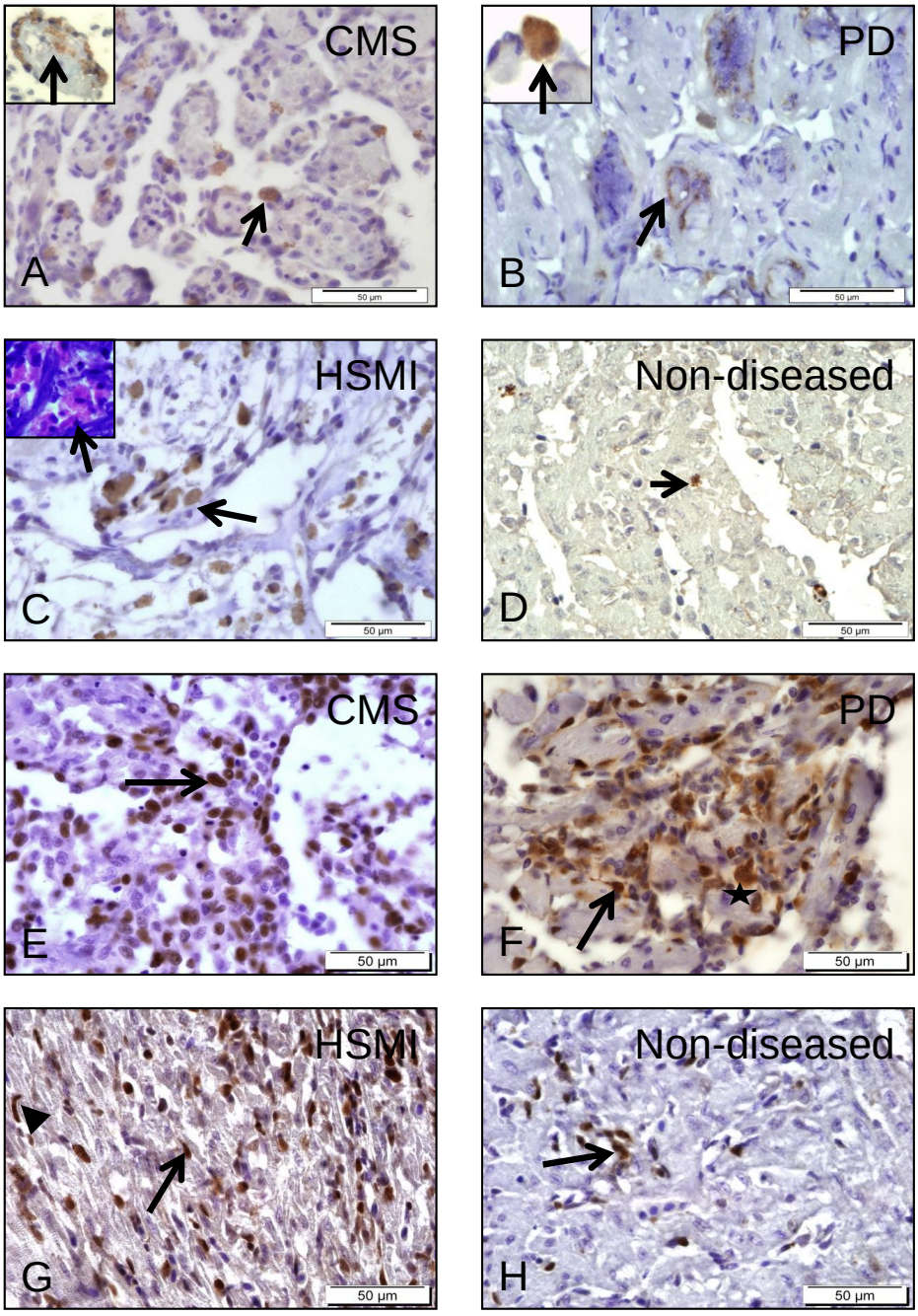
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Figure 4

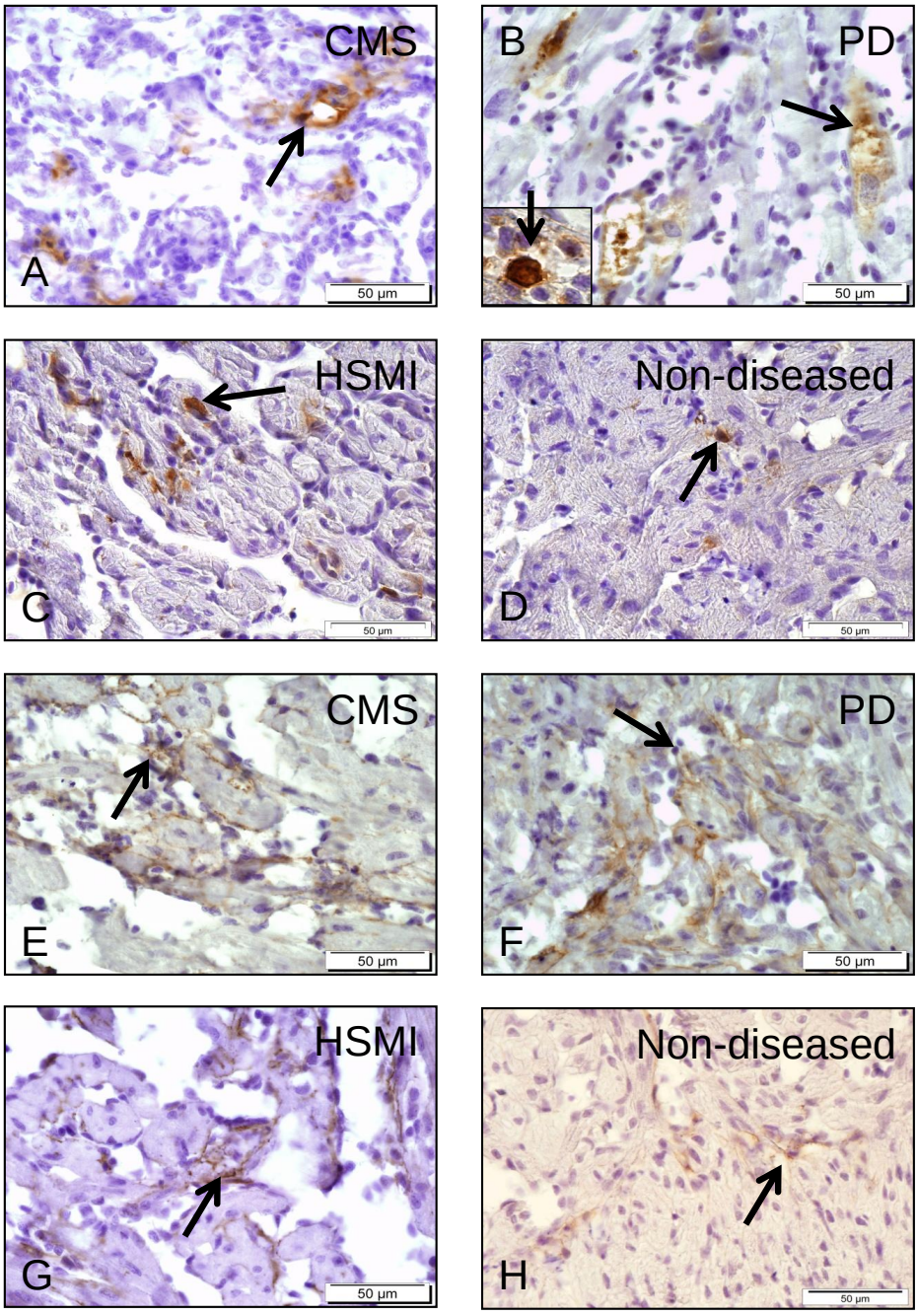
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Figure 5

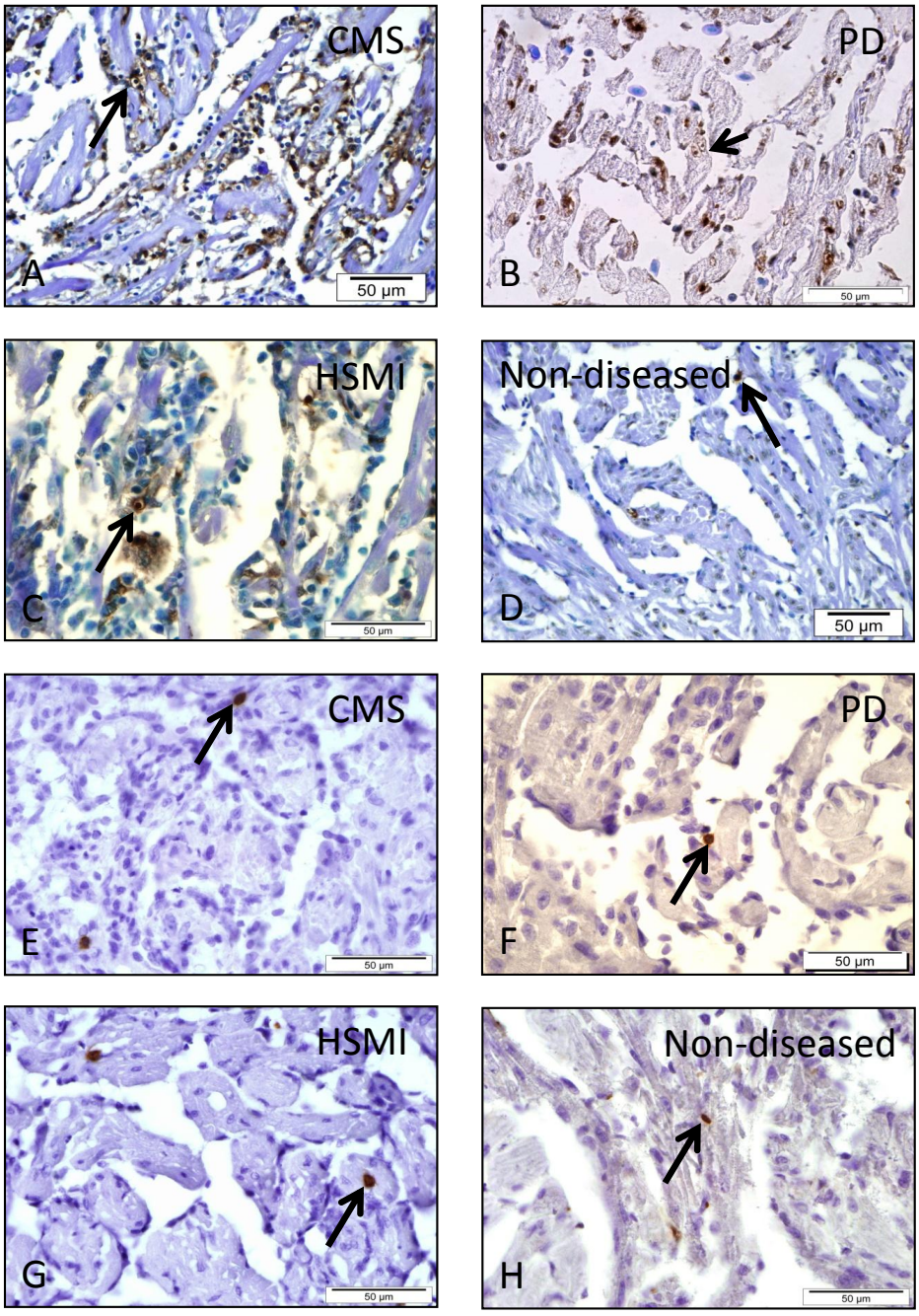
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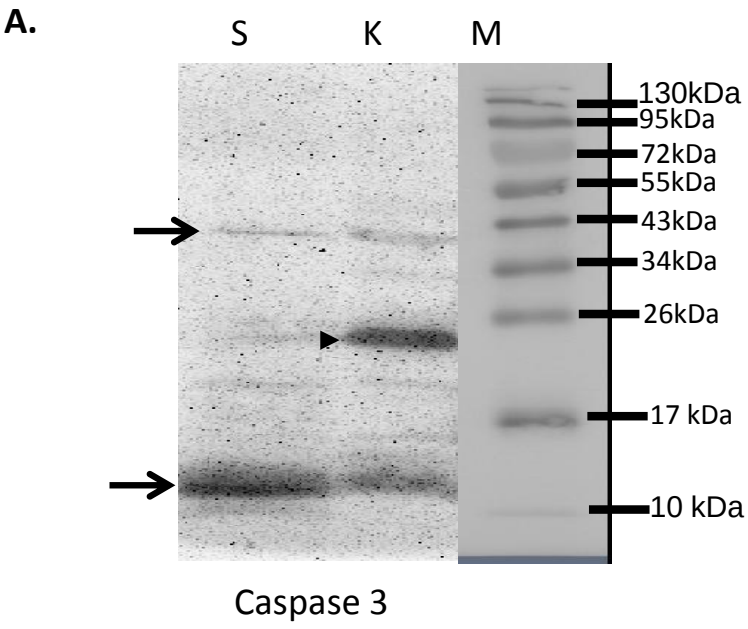
Figure 6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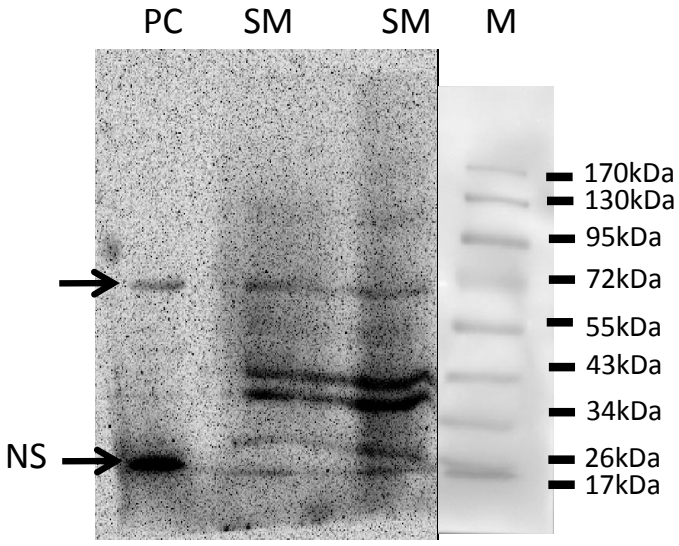
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List of previously published PhD dissertations for PhD in Aquaculture,

University of Nordland

No. 1 (2011)

Chris Andre´ Johnsen

Flesh quality and growth of farmed Atlantic salmon (*Salmo salar* L.) in relation to feed, feeding, smolt type and season

ISBN: 978–82–93165–00–2

No. 2 (2012)

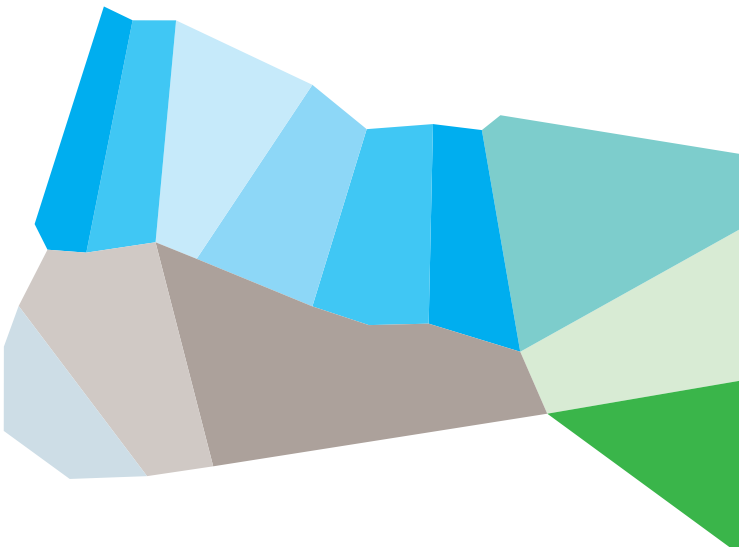
Jareeporn Ruangsri

Characterization of antimicrobial peptide in Atlantic cod

ISBN: 978–82–93165–01–9

UNIVERSITY OF
NORDLAND

Heart is the powerhouse of circulatory system. The present thesis focuses on the Atlantic salmon heart and related diseases. Viral fish diseases are a serious challenge to Atlantic salmon aquaculture in Norway and increasing numbers of heart malformations have been reported in Atlantic salmon. The investigated cardiac diseases included cardiomyopathy syndrome (CMS), pancreas disease (PD) and recently identified heart and skeletal muscle inflammation (HSMI). In spite of this importance, little was known about the Atlantic salmon heart anatomy (pacemaker cells), biomarkers and heart responses to these diseases. The heart beat initiates in autonomous pacemaker cells and propagate impulses to other parts of the heart. This thesis identified and characterized the pacemaker tissue located at the junction of sinus venosus and atrium in Atlantic salmon. Except PD, the other two diseases are diagnosed with increased mortality levels at farms and cause huge economical losses to farmers. There was a need for better tools to diagnose sick fish before sudden death. Present work identified the potential biomarkers to predict these cardiac diseases by blood tests non-lethally (without killing) as compared to conventional fish slaughter method (histopathology). Additionally, immunopathological responses were identified and provided a supplementary tool to predict these above mentioned cardiac diseases. It is noteworthy these apparently similar cardiac diseases exhibit differences in the immunopathological responses in the Atlantic salmon heart.



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