**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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## 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 CD3$\varepsilon^+$ 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 $\beta$ 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 $\beta$ chain in non–diseased hearts (Fig. 2H). The MHC class II $\beta$ 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 + $CoCl_2$ cell lysate) in the skeletal muscle by HIF1α antibody. (PC positive control, SM skeletal muscle, M PageRuler™ prestained protein ladder).

**Figure 1**

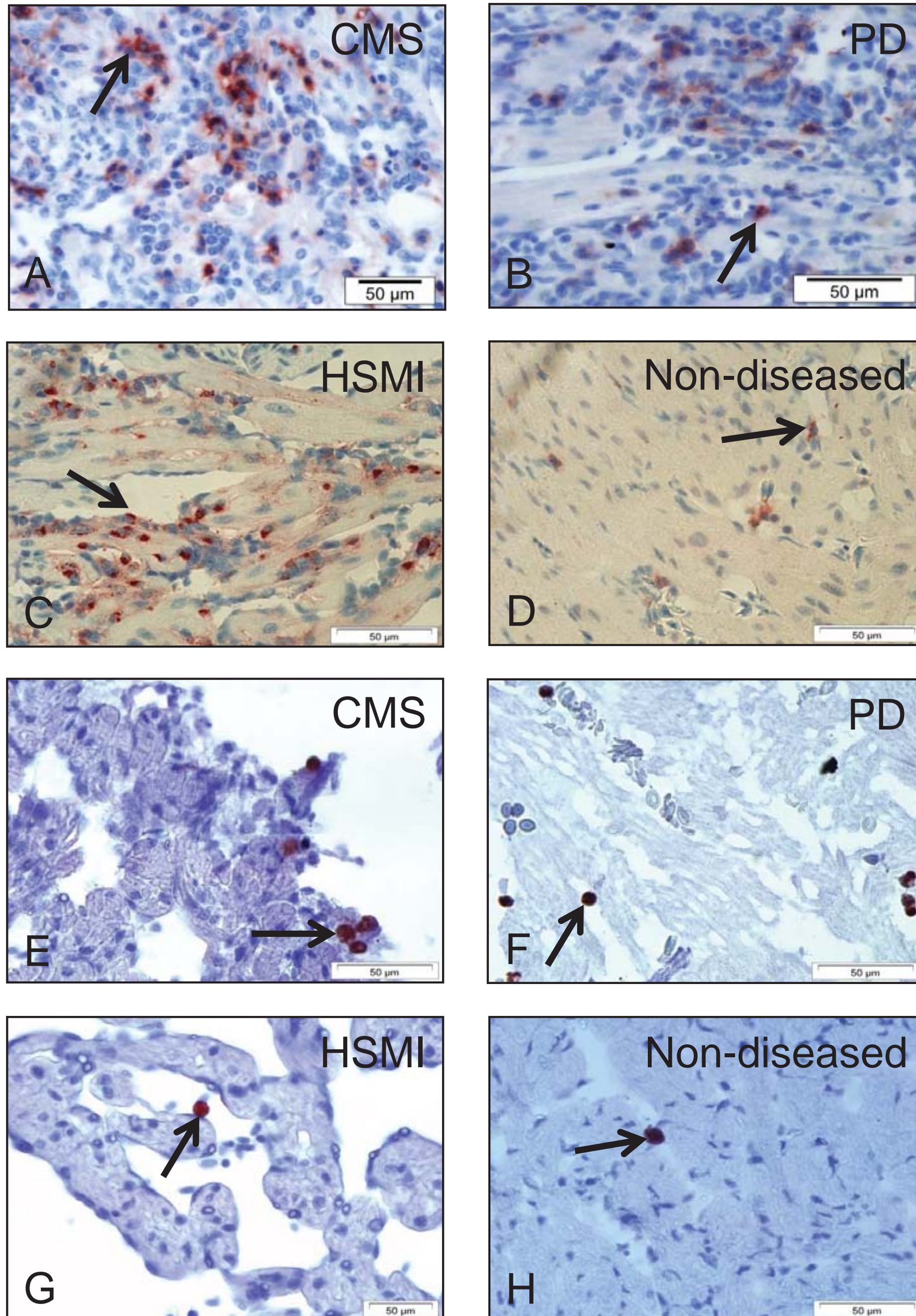


**Figure 2**

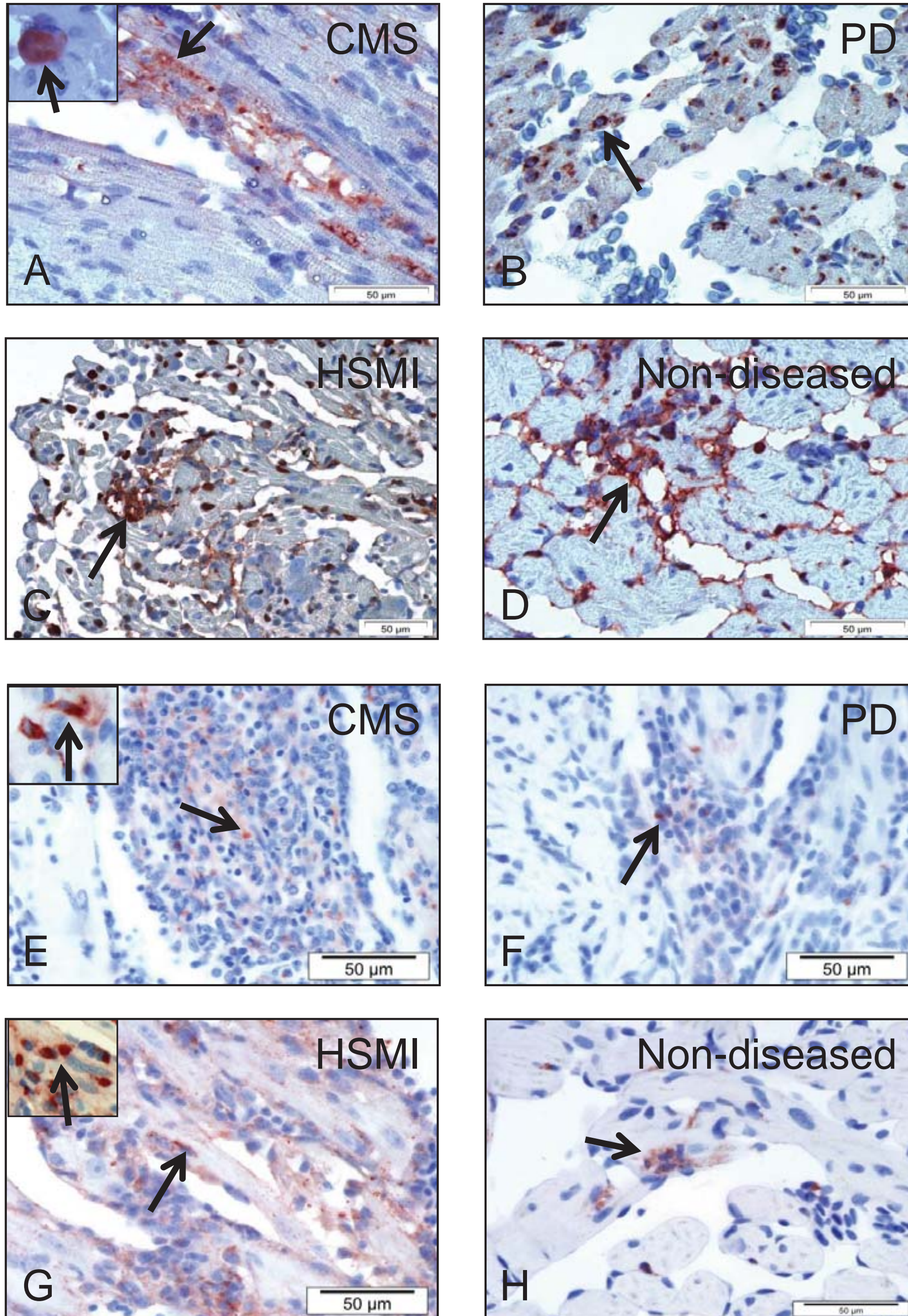


**Figure 3**

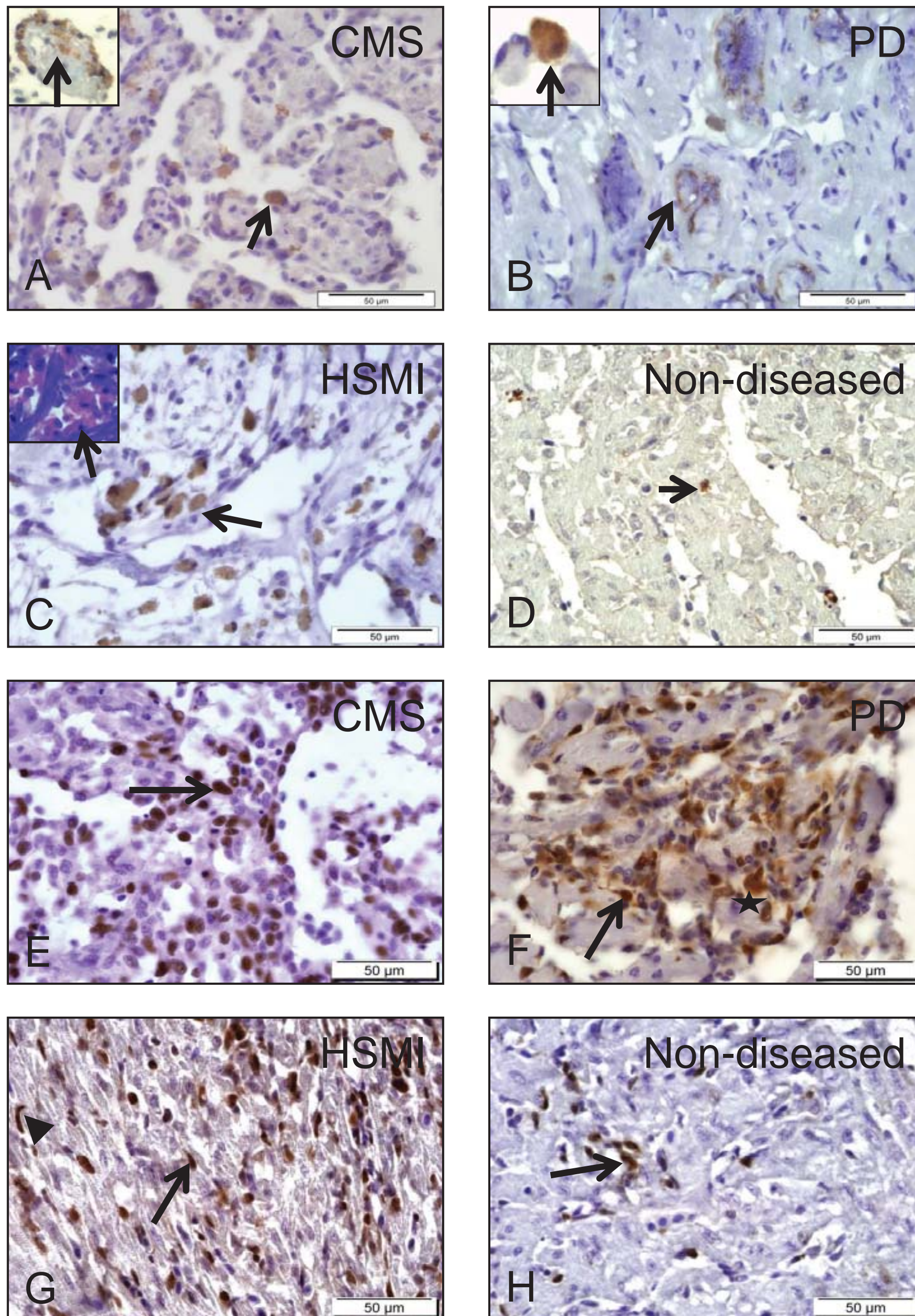
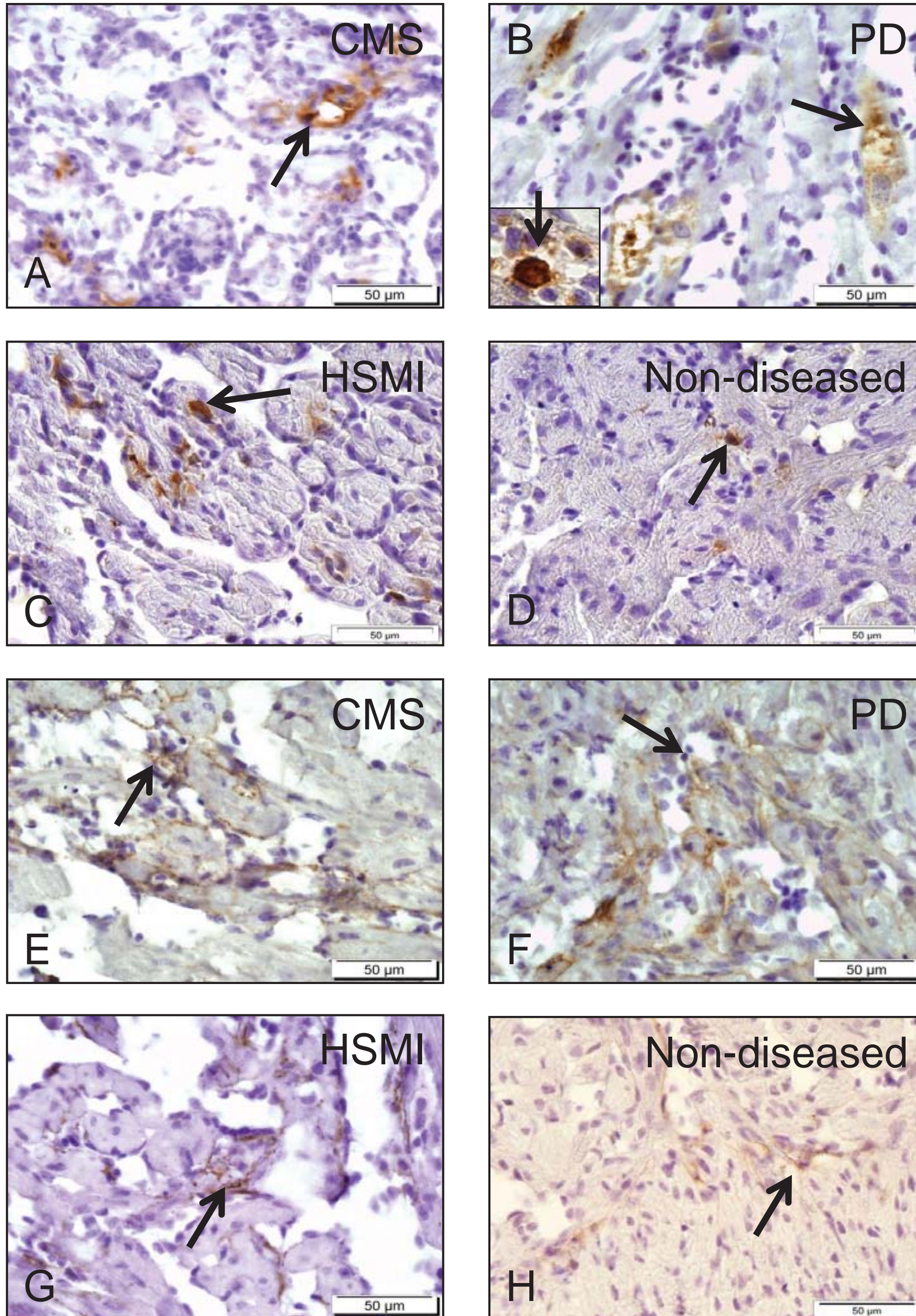


**Figure 4**



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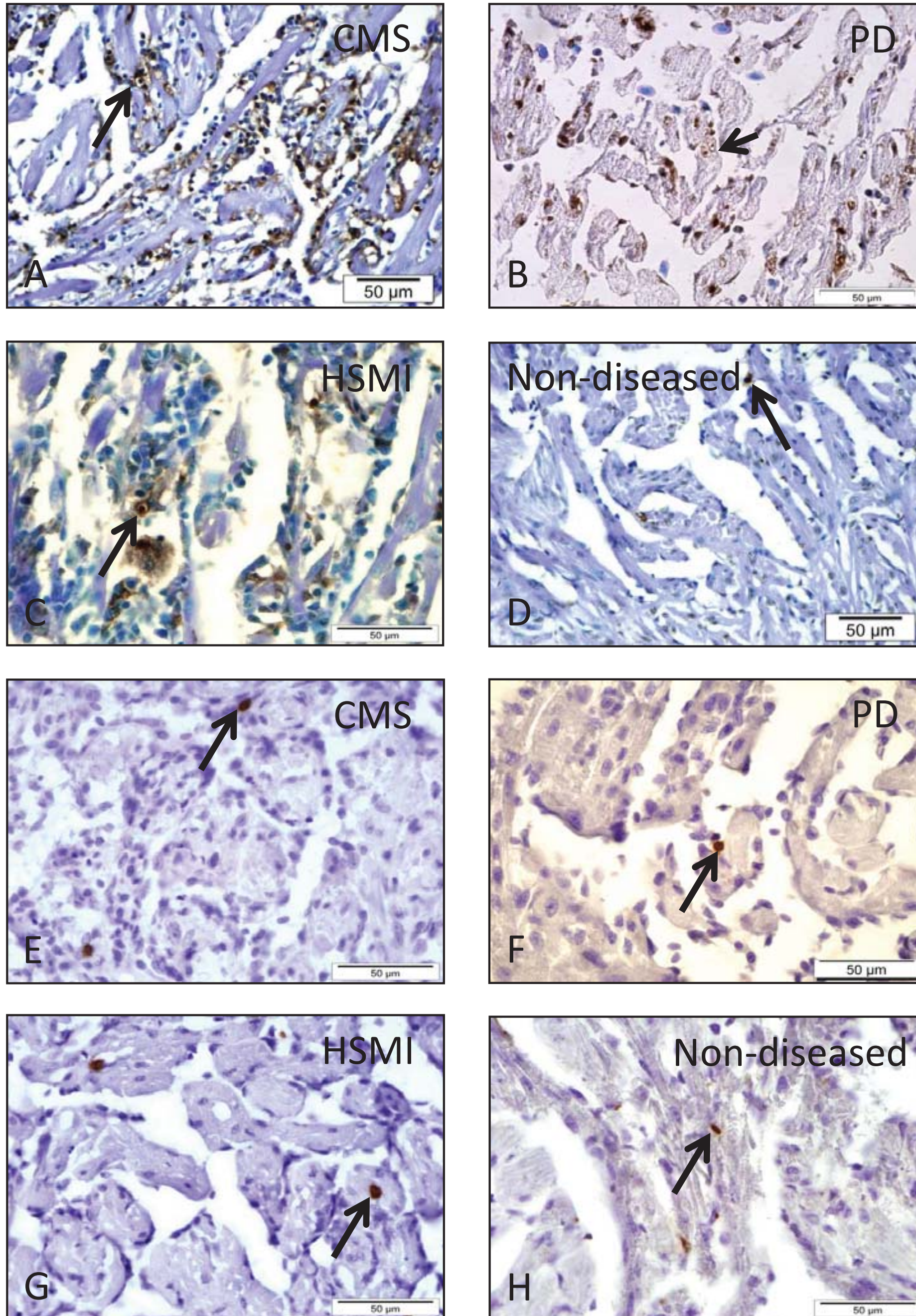
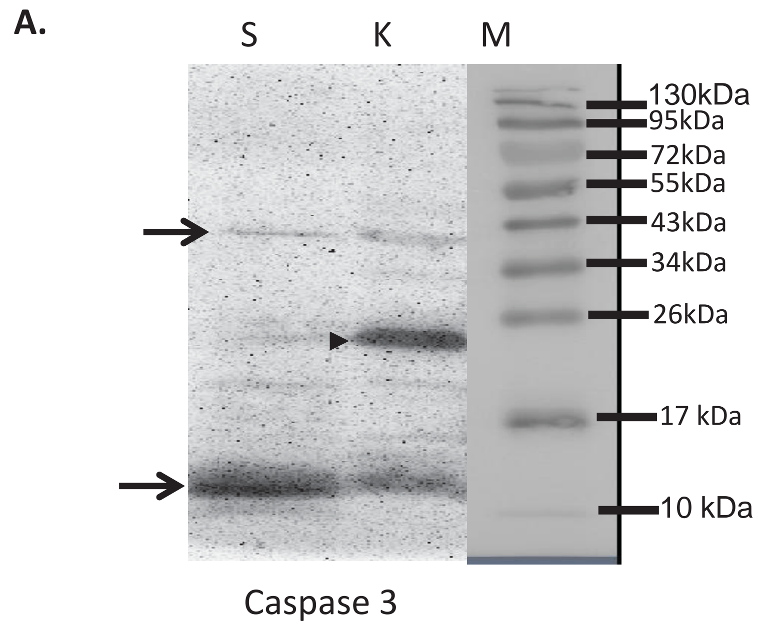


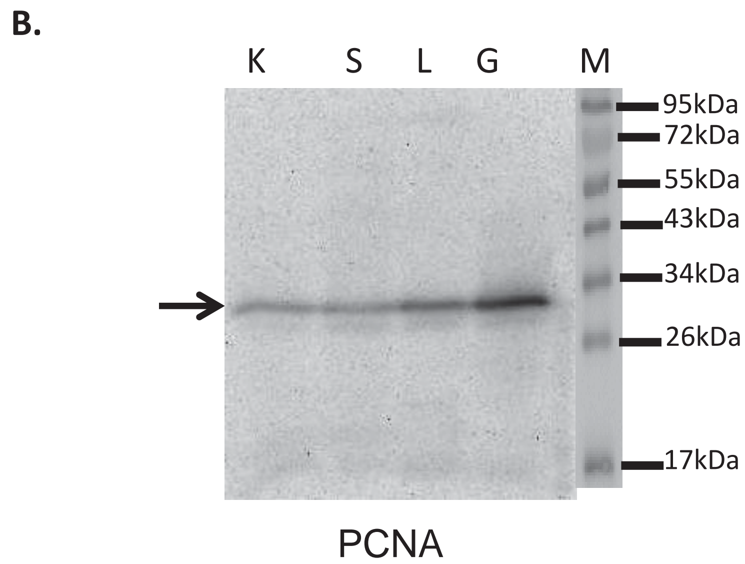
**Figure 6**

**A.**




Caspase 3

**B.**




PCNA

**C.**