Journal of Fish Diseases 2016, **39**, 41–54 doi:10.1111/jfd.12321

Coronary changes in the Atlantic salmon *Salmo salar* L: characterization and impact of dietary fatty acid compositions

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Abstract

Consumption of fatty acids from fishes is widely regarded as beneficial for preventing cardiovascular disorders. Nevertheless, salmonids themselves are victims of vascular diseases. As the pathogenesis and nature of these changes are elusive, they are here addressed using novel morphological and transcriptional approaches. Coronary arteries of wild Atlantic salmon *Salmo salar* L., ($n = 12$) were investigated using histological and immunohistochemical techniques, and RT-qPCR was employed to investigate expression of stretch-induced genes. In an experimental trial, fish were fed diets with different fatty acids composition, and histological features of the coronary arteries ($n = 36$) were investigated. In addition, the heart fatty acid profile ($n = 60$) was analysed. There were no differences in morphological or immunological features between wild fish and groups of experimental fish. Arteriosclerotic lesions consisted of smooth muscle cells in dissimilar differential stages embedded in considerable amounts of extracellular matrix in a similar fashion to what is seen in early stages of human atherosclerosis. No fat accumulations were observed, and very few inflammatory cells were present. In affected arteries, there was an induction of stretch-related genes, pointing to a stress-related response. We suggest that salmon may have a natural resistance to developing atherosclerosis, which corresponds well with their high investment in lipid metabolism.

Keywords: arteriosclerosis, early development, model organism, smooth muscle cells.

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Introduction

Salmonid fishes are widely regarded as beneficial supplements in human diet for the prevention of vascular diseases (Burr *et al.* 1989; GISSI-Prevenzione Investigators 1999; Gebauer *et al.* 2006). Nevertheless, salmonids themselves suffer from coronary vascular disorders consisting of neointimal proliferation of vascular smooth muscle cell (SMC) (Van Citters & Watson 1968; Moore, Mayr & Hougie 1976; Farrell *et al.* 1986; Kubasch & Rourke 1990; Saunders, Farrell & Knox 1992). Interestingly, the early changes in the vascular wall assumed to precede atherosclerosis in humans share similarities with the histological picture as seen in salmon arteriosclerosis (Robertson, Wexler & Miller 1961; House & Benditt 1981; Farrell 2002), suggesting a common starting point. Absence of fat deposits in salmonid coronary vessels is in contrast to the changes seen in advanced atherosclerosis in humans (Tabas, Williams & Borén 2007; Libby, Ridker & Hansson 2011), where these deposits have been suggested

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as the driving force for further pathological development. Studies of salmon coronary changes with their lack of fat accumulation could reveal important information for the early mechanisms of human coronary atherosclerosis.

Mammalian atherosclerosis including that of humans is characterized as a chronic inflammatory and immunological condition, where colocalization of macrophages and T lymphocytes is found to play a crucial part during advanced stages (Libby *et al.* 2011). T-cell involvement in salmon coronary disease has so far not been investigated due to lack of useful markers, but with such now at hand (Koppang *et al.* 2010), it is timely to address whether this characteristic also applies in salmonids. Another possible explanation for salmonid coronary disease is a mechanotransductional mechanism. The main salmonid coronary artery traverses the elastic and highly distensible bulbus arteriosus (Fig. 1a), resulting in cyclic tension of the vessel during increased workload (Saunders *et al.* 1992; Farrell 2002). In this study, we therefore wanted to investigate the occurrence of possible up-regulation of stretch-induced genes in the coronary lesions.

In addition to addressing immune cell involvement, we also aimed at characterizing other cell populations involved in coronary changes in the salmon. As previous investigations on such changes have not been supported by immunohistochemistry (IHC), we wanted to address the extent of endothelial and SMC involvement. In addition, an experimental study of different dietary oils representing different n-3/n-6 ratios was set up to compare the occurrence and the morphology of coronary changes between the dietary groups and wild salmon. This was made to assess the effect dietary oil manipulation, regarding the wild salmon group as a reference state.

Materials and methods

Wild fish

Animals and collection of tissue. Hearts from 12 wild, sexually mature Atlantic salmon, 3–14 kg,

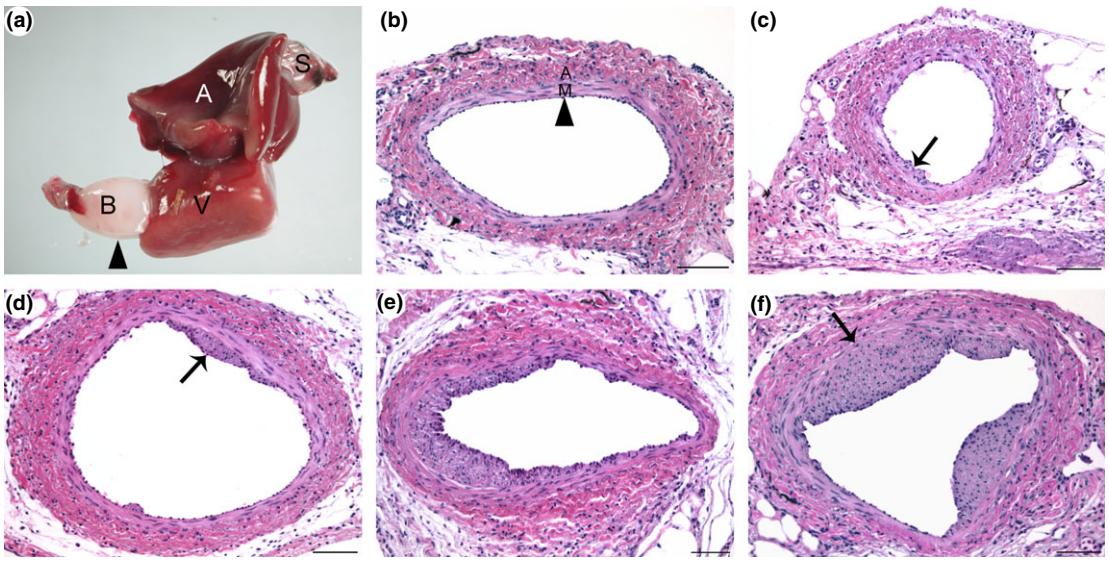

Figure 1 Classification scheme for grading of coronary lesions in Atlantic salmon for both wild and experimental fish. (a) Macroscopic overview of a heart from Atlantic salmon (S, sinus venosus; A, atrium; V, ventricle; B, bulbus arteriosus), with arrowhead indicating localization of the main coronary artery suspended to bulbus arteriosus. Haematoxylin and eosin-stained cross sections of the main coronary artery from experimental salmon, bars = 50 μm: (b) Lesion score 1; no proliferation of intima. A single layer of endothelium resting on the internal elastic lamina (arrowhead) (M, tunica media; A, tunica adventitia). (c) Lesion score 2; single cluster of intimal proliferation consisting of less than six cells (arrow). (d) Lesion score 3; intimal proliferation of more than six cells in a single cluster (arrow). Notice the loss of staining intensity of the ECM and the change of nuclei morphology of SMC in media beneath the intimal proliferation. (e) Lesion score 4; intimal proliferation affecting approximately 50% of the vessel wall with concomitant fragmentation of internal elastic lamina. (f) Lesion score 5; intimal proliferation occupying more than 50% of the vessel wall and near total depletion of media under lesions (arrow).

caught by dip net at Hellefossen in the river Drammenselva, Norway, autumn 2011, were included in the study. All animals were killed according to regulations for fish in aquaculture issued by the Norwegian Directory of Fisheries. Samples of bulbus arteriosus containing coronary artery were transferred to both 10% buffered formalin and 3% glutaraldehyde, snap-frozen in liquid nitrogen and stored at −70 °C until processing. Samples from ventricle and atrium were transferred to 10% buffered formalin.

Morphological investigations. Formalin-fixed tissue including the bulbus arteriosus, ventricle and atrium was processed for histological analysis using standard procedures and stained for haematoxylin and eosin, Martius scarlet blue, van Gieson's, Alcian blue and Periodic acid schiff (Bancroft & Gamble 2008). Cryosections (10 μm) were stained with Oil red O for fat. Transversally dissected samples of bulbus arteriosus with associated coronary vessels were fixed in 3% glutaraldehyde in 0.1 m cacodylate buffer and processed for transmission electron microscopy (TEM) or scanning electron microscopy (SEM) as described previously (Koppang *et al.* 2005).

For IHC, formalin-fixed, paraffin-embedded tissues including bulbus arteriosus, ventricle and atrium were processed for IHC investigations as described previously (Koppang *et al.* 2003) with some modifications. Heat-induced epitope retrieval was performed at 121 °C for 10 min. Labelled polymer-HRP anti-mouse (Dako EnVison+ System-HRP; Dako) was used for monoclonal antibodies, and labelled polymer-HRP anti-rabbit (Dako EnVison+ System-HRP; Dako) was used for polyclonal antibodies. Substrates used are listed in Table 1, and Mayer's haematoxylin was used for counterstaining. Primary antibodies were chosen to determine whether the neointimal cells resembled SMC and further to distinguish between different phenotypes of SMC. Also, the integrity of the endothelium and the presence of inflammatory cells were investigated. Antibodies used are listed in Table 1. Myocardium from the same individuals on identical sections was used as an internal positive control. The same protocols, but without primary antibody, were used as negative controls.

Gene expression analysis. To obtain detectable amounts of RNA from graded lesions, 4-μm cryosections of frozen coronary vessels were cut and transferred to microscope slides for grading, while the subsequent 32 μm of the coronary vessel specimen was collected in a tube and stored at −20 °C until RNA extraction. This procedure was repeated until sufficient amount of tissue for

Table 1 List of primary antibodies used for immunohistochemical analysis

Antibody	Reactivity	Manufacturer/ reference
Actin, muscle-specific Ab-6, clone MSA06/HUC-1 (mouse monoclonal, IgG1/kappa). DAB	Ab-6 reacts with α-smooth muscle as well as α-skeletal and α-cardiac (sarcomeric) isoform of actin, thus being a pan-muscle actin marker	Thermo Fisher Scientific (Cat.#: MS-1296-P)
Antismoothelin, clone R4A/MAB3242 (mouse monoclonal, IgG1). DAB	MAB3242 reacts with smoothelin, a cytoskeletal protein exclusively found in smooth muscle cells (SMC) in a differentiated state, and it is useful to discriminate fully differentiated, contractile SMCs from proliferative SMCs	Chemicon (Cat.#: MAB3242)
Anti-PCNA, clone PC10 (mouse monoclonal, IgG2a/kappa). AEC	PC10 reacts with cells in proliferative phase in late G1 or early S phase, where PCNA constitute an essential factor during DNA replication, recombination and repair	Dako (Cat.#:M0876)
Anti-Atlantic salmon endothelium-specific monoclonal antibody, (clone 10E4, IgM). AEC	MAb 10E4 binds on the surface of all types of Atlantic salmon endothelials, but also to red blood cells	Aamelfot *et al.* (2013)
MHC class II β (rabbit polyclonal). AEC	Reactive against the β-chain of the MHC class II complex in Atlantic salmon	Koppang *et al.* (2003)
Anti-CD3ε (rabbit polyclonal). AEC	Reactive against the CD3ε-chain of the CD3 complex, being highly specific maker during all developmental stages of T cells in Atlantic salmon	Koppang *et al.* (2010)

Antibody (Ab), immunoglobuline G (IgG), 3,3′-diaminobenzidine (DAB), proliferating cell nuclear antigen (PCNA), 3-amino-9-ethyl carbazol (AEC), major histocompatibility complex (MHC).

RNA extraction was collected. For practical reasons, the material was divided into three groups, namely negative, moderate and pronounced, corresponding to grade 1 ($n = 3$), grade 2–3 ($n = 3$) and grade 4–5 ($n = 2$) after classification scheme described previously (Seierstad, Poppe & Larsen 2005a) (Fig. 1), respectively. Materials from each of the three groups were pooled, and RNA isolation was performed using QIAzol® Lysis Reagent (QIAGEN) followed by RNA clean-up on NucleoSpin® RNA II (Macherey & Nagel) and measurement of final RNA concentration using BioSpec-nano Spectrophotometer (Shimadzu). The extracted RNA was used directly for cDNA synthesis with M-MLV Reverse transcriptase (Promega). Quantitative real-time PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) and carried out in an ABI 9700 PCR machine (Applied Biosystems) according to the producer's instructions. The following PCR profile was used: (i) denaturation at 95 °C for 10 min, (ii) 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 60 °C for 1 min. All samples not reaching threshold value after 40 cycles were regarded as negative. The level of expression was normalized using EF1a as the reference gene (Olsvik *et al.* 2005). Primers and hydrolysis probes used in the TaqMan assay are given in Table 2.

Experimental fish

Dietary trial and collection of tissue. An experimental dietary trial including four groups was conducted to assess the impact of different dietary oils with respect to coronary lesions. A detailed description of this trial is given elsewhere (Liland *et al.* 2012). Lasting for 28 weeks, a control group was fed a diet with 100% fish oil (FO), and in the three experimental groups, 80% of the FO was replaced either with olive oil (OO) or rapeseed oil (RO)(representing intermediate n-3/n-6 ratios), or soya oil (SO) (low in n-3/n-6 ratio) (Table 3). Mean weight at the start of the dietary trial was 815 ± 28 g. The mean final weight of all the dietary groups (3399 ± 76 g) did not differ significantly. At the end of the trial, animals were killed according to regulations as stated before, and samples were collected for morphological investigations ($n = 36$) and fatty acid analysis ($n = 60$).

Histology. Morphological investigations were conducted as described for the wild fish. Grading was performed blinded, meaning that the operator did not know what group each individual belonged to during grading. The severity of the changes was scored from 1 to 5 (Fig. 1) (Seierstad *et al.* 2005a). Differences in lesion score between the different dietary groups were evaluated using chi-square test implemented in Prism® (version 6.02; GraphPad Software) at a 95% confidence level.

Analysis of fatty acid composition of hearts. Hearts ($n = 15$) were randomly sampled from each of the four diet groups, and lipids were extracted

Table 2 Primers and probes used for RT-qPCR assay

Gene	Source/accession number	Sequence (5′–3′)
EF1a	NM001141909.1	F – TGCCCCTCCAGGATGTCTAC R – CACGGCCCACAGGTACTG P – FAM-AAATCGGCGGTATTGG-MGB
CD3 zeta	NM_001123646.1	F – AACAGGGATCCAGAGAGTGCTG R – AAGGGACGTGTAAGTGTCGTCA P – FAM-ACGGCACGCGATAATCGCAGGA-BHQ1
MHC II	X70167.1	F – CCACCTGGAGTACACACCCAG R – TTCCTCTCAGCCTCAGGCAG P – FAM-TCCTGCATGGTGGAGCACATCAGC-BHQ1
CTGF	NP001133471.1	F – TCTCACTCTCTCCTACCTGGCTG R – CTCACACCAGGGGTACACTG P – FAM-AGGAGTGCAGTGGGCAGTGTAGTTG-BHQ1
Cyr61	NP001133449.1	F – GTGGAATCTGCCGTGCTAAG R – CTTGCAGTTAGGCTGGAAGC P – FAM-TCCGTTCTGGTAAATCCTGCTGTTG-BHQ1

Primers (400 nM each) and hydrolysis probes (100 nM) used for RT-qPCR assay on coronary vessels of Atlantic salmon, investigating expression of inflammatory mediated genes and stretch-induced genes. Elongation factor 1 alpha (EF1a), black hole quencher (BHQ), minor groove binding (MGB), cluster of differentiation (CD), major histocompatibility complex (MHC), connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (Cyr61).

Table 3 Dietary proximate composition (g 100 g^{-1}) and fatty acid composition (area %) of the four experimental diets

Gene	FO	OO	RO	SO
Fat	34.1	33.6	34.5	33.2
Protein	44.1	40.8	41.3	40.6
Dry matter	93.0	94.0	93.0	93.0
Ash	5.3	5.2	5.2	5.2
14:00	6.8	1.5	2.0	1.7
16:00	15.9	12.4	7.8	14.8
18:00	2.9	3.3	2.5	3.8
Total SFAs	26.8	18.0	13.2	21.2
16:1n-7	5.9	1.9	1.8	1.5
18:1n-7	2.0	2.2	2.7	1.6
18:1n-9	11.9	50.3	42.7	21.5
20:1n-9	5.6	1.4	2.4	1.4
22:1n-1	10.2	2.0	2.8	2.2
Total MUFAs	38.5	58.4	53.5	28.6
18:2n-6	2.7	12.1	15.8	37.5
20:3n-6	0.9	1.0	1.1	1.3
20:4n-6	0.5	0.1	0.2	0.1
Total n-6	4.7	13.2	17.2	39.0
18:3n-3	1.4	4.1	7.8	4.4
18:4n-3	2.6	0.5	0.7	0.6
20:4n-3	0.7	0.2	0.2	0.2
20:5n-3	8.1	1.9	2.5	2.0
22:6n-3	10.0	2.3	3.0	2.4
Total n-3	24.9	9.5	14.8	10.1
n-3/n-6	5.3	0.7	0.9	0.3
LA/LNA	1.9	2.9	2.0	8.5

FO, fish oil; OO, olive oil; RO, rapeseed oil; SO, soya bean oil. Saturated fatty acid (SFA); monounsaturated fatty acid (MUFA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); polyunsaturated fatty acid (PUFA); linoleic acid (LA) and α-linolenic acid (LNA).

using chloroform/methanol (2:1, v/v), filtered, saponified and methylated using 12% BF_3 in methanol. Fatty acid composition of total lipids was analysed using methods described by Lie & Lambertsen (1991) and Torstensen *et al.* (2004). All samples were integrated using the software Chromeleon® version 6.8 (Thermo Scientific) connected to the gas–liquid chromatography. Amount of fatty acid per gram sample was calculated using 19:0 methyl-ester as the internal standard. The fatty acid composition data were evaluated in STATISTICA® (version 8.0; Statsoft) using one-way ANOVA followed by a *post hoc* Tukey test with a level of $P < 0.05$ considered to be significant.

Results

Morphological investigations

On a morphological basis, it was not possible to distinguish between coronary vessels from wild and experimental fish; thus, they are described together. Further, the different dietary groups showed no differences in lesion scores (Fig. 2).

There were changes in the coronary arteries in all 12 wild salmon individuals and in nearly all individuals in the experimental groups. The lesions ranged from a single cell layer of myointimal proliferation (Fig. 1c) to proliferation that was several cell layers thick and occupied over half of the arterial lumen (Fig. 1f). The media lying immediately abluminal to the normal intima were characterized by a dense extracellular matrix (ECM) that embedded fusiform elongated SMC nuclei with a circular orientation to the long axis of the vessel. In the media subjacent to small- and medium-sized neointimal lesions, SMC nuclei adopted a more rounded and less dense morphology (Fig. 1e,f). The appearance of rounded nuclei may be a result of a change in cell orientation from a circular to a longitudinal orientation to the long axis of the vessel. Within the intima, cell nuclei appeared rounded and dense. Thus, nuclei in the media beneath lesions resembled a transition stage compared with those seen in unaffected media and neointimal proliferations.

In HE-stained sections, ECM of normal media displayed a more eosinophilic staining compared with ECM deposited in the neointima. Also, the connective tissue of the media underneath intimal lesions displayed a markedly looser organization than in normal intima, resembling a loosely woven fibrillar meshwork (Fig. 1d–f). Following increased size of neointimal proliferations, the thickness of the media was decreased. Ultimately, in large lesions, fragmented lamina elastica was in direct contact with the adventitia (Fig. 1f). On several occasions, longitudinally orientated SMCs crossed the fragmented lamina elastic interna from the media into the intima, as also observed in TEM preparations (Fig. 3a).

Special staining revealed trabecular connective tissue orientated from the media towards the neointima. The total content of ECM gradually decreased towards the luminal surface. Van Gieson's staining revealed a trabecular pattern of collagen radiating through neointima tapering off towards the lumen (Fig. 4a). Martius scarlet blue staining confirmed this pattern (Fig. 4b). In contrast, staining for acidophilic proteoglycans gave a more uniform pattern throughout lesions as seen with Alcian blue (Fig. 4c) and periodic acid schiff (Fig. 4d). In all these investigations, there was a tendency for stronger staining of the neointimal

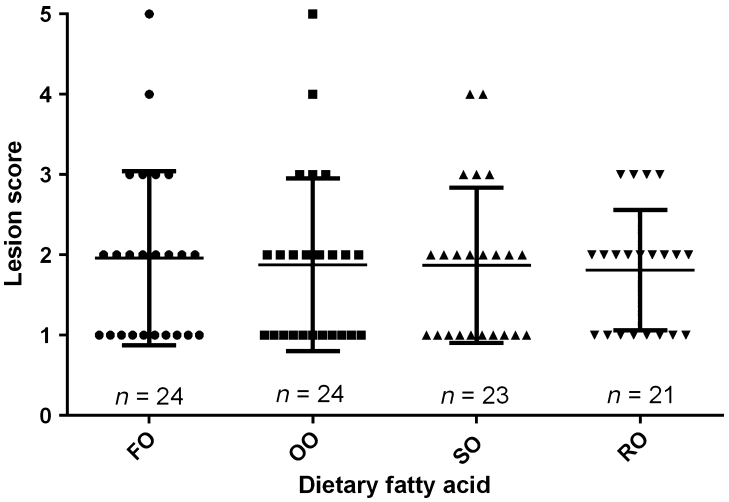

Figure 2 Coronary lesion scores for the different dietary groups. Mean and standard deviation indicated by bars. No significant differences were found between the different dietary groups when evaluated by chi-square test at a 95% confidence level. Fish oil (FO), olive oil (OO), rapeseed oil (RO), soya bean oil (SO).

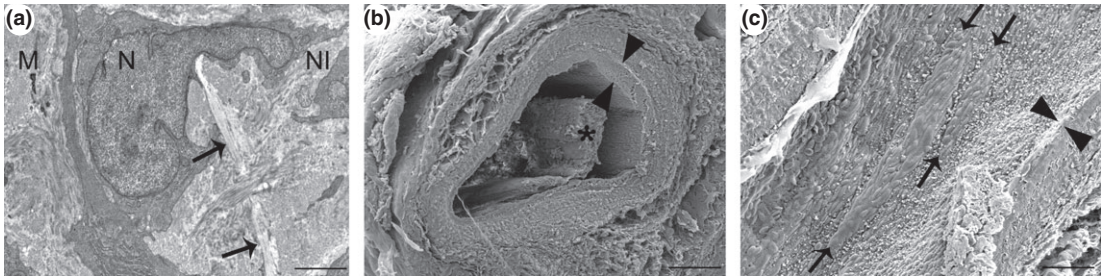

Figure 3 Ultrastructural characterization of neointimal coronary proliferations in wild Atlantic salmon. (a) TEM of a smooth muscle cell crossing the internal elastic lamina (arrows) (M, media; N, nucleus; NI, neointima, bar = 2 μm). (b) SEM of a transversal section of a coronary vessel with lesion score 4. Arrowheads delineate the neointima (asterisk; blood clot in the vessel lumen, bar = 100 μm). (c) SEM of a longitudinal section of a coronary vessel with estimated lesions of score 1–2 (delineated by arrows), demonstrating the profound change in endothelial cell morphology yet forming a continuous layer (arrowhead; unaffected intima, bar = 100 μm).

ECM for acidophilic composition and weaker staining for collagen compared with media ECM. Importantly, Oil Red O staining of cryosections showed no evidence of fat deposits in any of the investigated groups.

In IHC, neointimal cells reacted avidly for actin and smoothelin, with a pattern revealing differences in the orientation of SMCs between medial and neointima (Fig. 5a,b). Media and neointima labelled homogenously against actin (Fig. 5a). However, smoothelin labelled the luminal parts of neointimal proliferations more avidly than deeper parts towards the media, revealing a staining gradient throughout the lesion (Fig. 5b). Invariably, SMC-positive labelling was delineated from the lumen by a single layer of continuous endothelium (Fig. 5d).

A proliferative cell nuclear antigen (PCNA) antibody was used to investigate for cell proliferation in the coronary lesions. In all neointimal lesions, a layer of immunoreactive cells was observed close to the vascular lumen (Fig. 5c) including the endothelium. Although present in scarce amounts, proliferating cells in media were invariably accompanied by rearrangement of architecture, and these cells were situated close to the elastic lamina. No proliferative nuclei were seen in normal-appearing media. Strongest labelling of SMC was seen in neointima close to media, while PCNA-labelled cells towards the lumen resembled endothelial cells. Labelling with the Atlantic salmon endothelium-specific antibody confirmed the continuity of a single layer of endothelium covering the lesions (Fig. 5d).

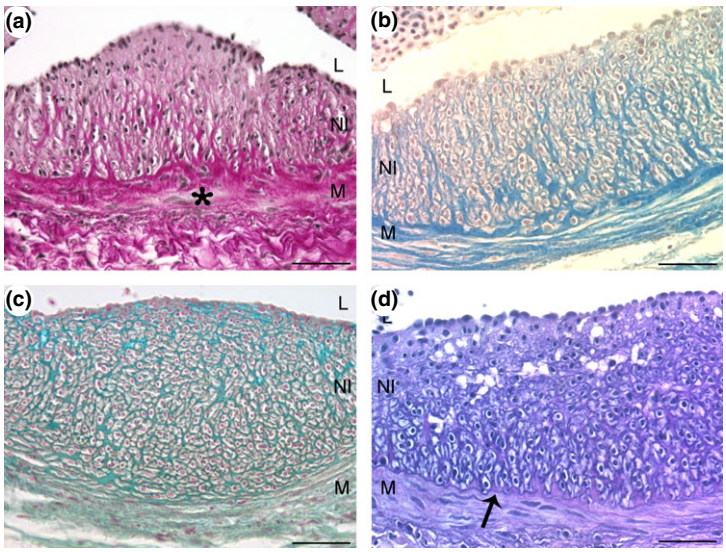

Figure 4 Special staining of coronary lesion extracellular matrix in wild Atlantic salmon (M, media; NI, neointima; L, vascular lumen, bars = 5 μm). (a) Trabecular pattern of collagen (red) traversing from media to basal part of neointima, tapering off towards the lumen. Note, the change in orientation of SMC underneath lesions (asterisk) (van Gieson's staining). (b) Same pattern as in (a), with collagen staining blue (nucleated erythrocytes in the left corner, Martius scarlet blue staining). (c) Uniform staining for acid mucins and proteoglycans (blue) throughout the neointimal proliferation, with stronger staining intensity than in the media. Note, the nearly depleted media layer and absence of internal elastic lamina (Alcian blue staining). (d) Same as in (c), with acid mucins staining magenta and glycoprotein, proteoglycans and neutral mucins staining blue. Here, the media and internal elastic lamina (arrow) are partly intact (periodic acid schiff staining).

Only a few $CD3^+$ cells (T cells) were detected (Fig. 5e). The results of MHC class II immunolabelling reflected these results as only a few labelled cells were occasionally identified (Fig. 5f).

Gene expression analysis

In agreement with IHC, expression levels of CD3 and MHC class II did not increase with increasing lesion score. However, there was a trend with increasing level of stretch-induced genes with the increasing lesion score (Fig. 6). The expression level of cysteine-rich angiogenic inducer 61 (Cyr61) increased over threefold from negative to pronounced lesions, while the concomitant expression level of connective tissue growth factor (CTGF) increased over sevenfold.

Heart fatty acid composition

Heart fatty acid composition (Table 4) generally reflected the dietary fatty acid composition (Table 3). However, 22:6n-3 levels were conserved in hearts showing no statistical differences between FO-fed fish and RO- and OO-fed fish. SO-fed fish, however, contained significantly less DHA compared with the other three groups. The n-3/n-6 ratio varied considerably in the hearts ranging from 5.5 in hearts from FO-fed fish to 0.9 in hearts from SO-fed fish. RO- and OO-fed salmon had both intermediate n-3/n-6 ratios, thus reflecting the dietary fatty acid composition.

Discussion

The scientific community has from the 1930s been intrigued by vascular changes in salmonids, not the least because consumption of such fish is regarded to be beneficial for the prevention of coronary disease in humans. However, salmonids themselves show onset of coronary changes at an early age, and in sexually mature fish, the occlusion of the vessel lumen may be close to total (Farrell 2002). Normally, foam cells do not appear during salmonid coronary changes, though occasionally, they may be observed (Koppang *et al.* 2007). Due to lack of immunohistochemical studies, the nature of these changes has remained elusive.

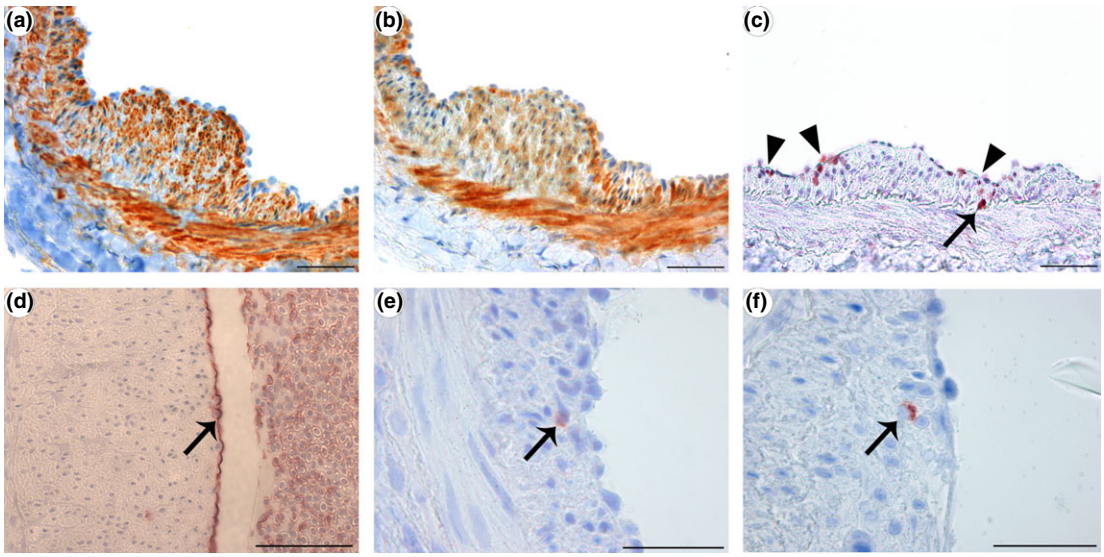

Figure 5 Immunohistochemistry of neointimal proliferations in wild Atlantic salmon. (a) Labelling against actin appears homogenous throughout the media and neointima (bar = 10 μm) (b) While being homogenous throughout the media, labelling against smoothelin reveals a gradient throughout the neointimal proliferations with stronger labelling towards the vascular lumen (bar = 10 μm). (c) Most smooth muscle cells labelling for PCNA are primarily found in the neointima, with the strongest labelling nuclei found towards media (arrow). Considerable amounts of endothelial cells were also labelled (arrowhead) (bar = 10 μm). (d) Labelling for endothelial cells confirms the continuity of a single layer of endothelium covering the proliferation (arrow, nucleated erythrocytes are seen in the vascular lumen, bar = 10 μm (e) A single CD3-positive cell in neointimal proliferation (arrow, bar = 5 μm). (f) A single MHC class II positive cell in a neointimal proliferation (arrow, bar = 5 μm).

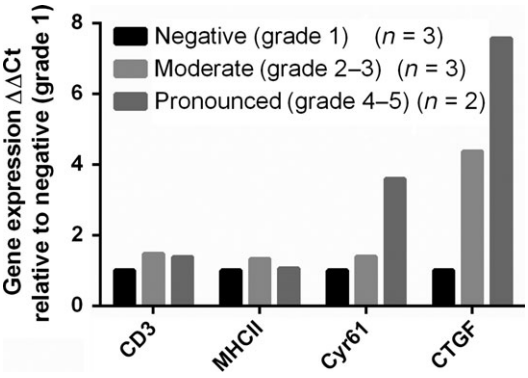

Figure 6 Gene expression assay of neointimal proliferations in wild Atlantic salmon. Graphically presentation of the RT-qPCR study of segmentally dissected coronary vessels. There was no response in selected immune-related genes, but a tendency of up-regulation of stretch-induced genes. Cluster of differentiation (CD), major histocompatibility complex (MHC), cysteine-rich angiogenic inducer 61 (Cyr61), connective tissue growth factor (CTGF).

Here, we have applied a number of methods and markers for the characterization of coronary changes in both wild and experimental farmed Atlantic salmon that have not previously been used. In the experimental salmon, we further investigated the impact of diets containing different fatty acids with respect to heart fatty acid profile and lesion occurrence. Our results show that the coronary lesions as found in wild and experimental salmon cannot be distinguished on a morphological basis, and further, that morphological changes in experimental salmon fed oils from different lipid sources cannot be distinguished from one another with the methods applied. In mammals, there has been an increasing focus on the possible influence of gut microbial conversion of different food stuffs to atherogenic components (Wang *et al.* 2011; Koeth *et al.* 2013), and also, bacteraemia is a well-known contributor to cardiovascular diseases (Kiechl *et al.* 2001; Lehr *et al.* 2001). The same might be the case with marine bacteria in Atlantic salmon even though no differences were seen between the different feed recipes in this study. The lack of dietary oil impact on the occurrence, nature and severity of coronary lesions is in accordance with other experiments investigating effects of cholesterol (Farrell *et al.* 1986) and vegetable oils on coronary health (Seierstad *et al.* 2005b, 2008). While the significantly

Table 4 Relative fatty acid composition (area%) of Atlantic salmon hearts fed either FO-, RO-, OO- or SO-based diets for 28 weeks. Data are presented as mean ± SD of the three replicate analysis (with 5 fish hearts pooled prior to fatty acid analyses in each replicate) in each of the four diet groups

	FO	RO	OO	SO
Sum saturated FA	23.8 ± 0.3[a*]	18.7 ± 0.9[c]	20.3 ± 0.4[b]	21.9 ± 1.0[b]
Sum monoene FA	25.0 ± 2.4[b]	31.1 ± 2.9[a]	35.5 ± 3.2[a]	22.0 ± 3.1[b]
18:2n-6	3.8 ± 0.3[c]	9.4 ± 0.8[b]	8.5 ± 0.1[b]	22.2 ± 3.9[a]
Sum omega-6	7.3 ± 0.6[c]	13.9 ± 0.6[b]	13.1 ± 0.3[b]	29.3 ± 3.3[a]
20:5n-3	8.5 ± 0.4[a]	5.8 ± 0.7[b]	4.5 ± 0.2[c]	3.9 ± 0.8[c]
22:6n-3	23.9 ± 3.0[a]	21.4 ± 2.2[a]	20.0 ± 2.5[a]	16.5 ± 4.9[b]
Sum omega-3	39.9 ± 2.4[a]	34.6 ± 2.7[a]	29.6 ± 2.6[b]	25.3 ± 5.5[b]
n-3/n-6	5.5 ± 0.2[a]	2.5 ± 0.3[b]	2.3 ± 0.2[b]	0.9 ± 0.3[c]

[*]Different letters indicate statistical differences between dietary groups, tested by one-way ANOVA. FO, Fish oil; OO, olive oil; RO, rapeseed oil; SO, soya bean oil.

different dietary fatty acid profiles were reflected in the heart fatty acid profiles, lesion score did not differ among the different experimental groups. This study showed that neither n-3/n-6 ratio nor high levels of omega-6 fatty acids increased the risk for developing coronary lesions in Atlantic salmon when total dietary EPA + DHA was 1.4%. We have demonstrated that coronary changes mainly consist of SMCs in an undifferentiated proliferative state, embedded in ECM. Importantly, neither inflammatory cells nor endothelial cells seem to be involved in lesion development. Also, the contributory role of arterial fat accumulation seems to be absent. This finding might explain the apparent lack of immunological reaction in the changes. Further, we demonstrated up-regulation of stretch-induced genes in dissected vessels, supporting a mechanistic-induced pathogenesis as previously proposed (Farrell 2002).

We have demonstrated and confirmed that vascular SMCs play a prominent role in the pathogenesis of arteriosclerosis in salmonids as they do in atherosclerosis in mammals. Unlike many other cells including heart and skeletal muscle cells, SMCs are not terminally differentiated even in adult organisms and may exhibit a striking degree of plasticity in response to local environmental cues (Owens, Kumar & Wamhoff 2004). A distinction between contractile and synthetic SMCs has been introduced (Chamley-Campbell, Campbell & Ross 1979; Shanahan & Weissberg 1998). Contractile SMCs represent a non-migratory subset, characterized by low rates of proliferation and turnover. In contrast, synthetic SMCs display a high propensity towards migration and ECM production, playing a critical role in repair in the vascular system (Owens *et al.* 2004). Even though this plasticity is important in reparative processes, the continual influence of different abnormal environmental signals on the vascular wall can lead to prolonged phenotypic switching and acquisition of characteristics that can contribute to development of vascular lesions. There is evidence that SMC can under such conditions take on a proinflammatory phenotype, both expressing cell adhesion molecules recruiting different leucocytes and secreting proinflammatory cytokines (Nilsson 1993; Orr *et al.* 2010). Vascular SMCs express multiple markers indicative of their relative state of differentiation, but no single marker may conclusively identify them to the exclusion of other cells (Owens *et al.* 2004). Most, if not all, SMC markers are expressed, at least transiently, in other cell types during development, tissue repair or different states of disease. With this background, we tested several SMC markers in our material as such investigations have previously not been addressed. We here report that actin and smoothelin are excellent markers in this regard. While actin is a pan-muscle filament, smoothelin is regarded as highly specific to SMCs. In addition to being a highly selective linage marker for SMCs, smoothelin has also been proposed as a differentiation marker, being specific for highly differentiated, contractile SMCs (van der Loop *et al.* 1997; Krämer *et al.* 1999; van Eys, Niessen & Rensen 2007). We report a marked immunolabelling gradient against smoothelin throughout intima, with stronger labelling adjacent to the lumen and a gradual decrease towards the media. Overall, SMC in the media displayed stronger labelling than neointimal SMCs. This difference could be due to the different orientation of contractile fibres. Likewise, difference of staining intensity within the neointimal lesions could in part be caused by a dilution effect of ECM juxtamedialy displacing the

contractile apparatus. Even so, there was weaker labelling also in close proximity to the cell nuclei deep in the neointima, giving a strong indication of a different phenotype of SMCs in this region. This is in accordance with synthetic phenotype of vascular SMCs inhabiting the deeper part of the lesions close to the media, while contractile phenotypes are reforming in the outermost part. This organization would be in agreement with the neointimal SMCs being recruited from the media. Also, strongest PCNA labelling of SMC was seen in the neointima close to the media, indicating cell replication in this region.

In humans, development of coronary intimal thickening, referred to as diffuse intimal thickening, is proposed as the initial step in atherosclerosis formation (Nakashima *et al.* 2002). Such changes are initially characterized by neointimal proliferation of SMCs without concomitant fat deposits and inflammatory cell recruitment and occur at an early age (Doran, Meller & McNamara 2008) in regions known to be prone to later development of atherosclerosis (Nakashima *et al.* 2002). Similarly, coronary lesions have been demonstrated in juvenile salmonids (McKenzie *et al.* 1978; Kubasch & Rourke 1990; Seierstad *et al.* 2008). An important anatomical difference between human and salmon arteries is that while human intima harbours some SMCs (Nakashima, Wight & Sueishi 2008; Libby *et al.* 2011), normal salmon intima does not (Robertson *et al.* 1961; Farrell 2002). Interestingly in our material, salmon arteriosclerotic neointimal SMCs adopted the same orientation as seen in humans, with a longitudinal orientation in the neointima compared with a circumferential orientation in the media. The origin of neointimal SMCs is somewhat controversial, and different sources have been discussed (Owens *et al.* 2004). Our results indicate that in salmon, SMCs in the media serve as an important recruiting reservoir. This was suggested by the appearance of SMC-marker positive cells budding through the internal elastic lamina as seen in TEM and IHC, the fragmentation of the internal elastic lamina and, finally, the depletion of medial SMCs beneath large neointimal lesions. The latter observation could also result from mechanical compression exerted by the overlaying lesion as noted previously (Seierstad *et al.* 2005b), or because of nutritional depletion resulting from increased diffusion length.

Smooth muscle cell contributes to several mechanisms during human atherosclerosis formation both in early and in late stages of lesion development. One key point in this regard is the production of ECM (Doran *et al.* 2008). Further, modified ECM produced by synthetic SMCs has been proposed as a pivotal requirement for the initiation and development of atherosclerosis (Fogelstrand & Borèn 2012), with retention and modification of low-density lipoprotein (LDL) (Williams & Tabas 1995; Tabas *et al.* 2007). Charge and spatial characteristics of ECM are of major importance for retention of lipids within the vessel wall (Doran *et al.* 2008), and it can thus serve as a depot for proatherogenic molecules (Rekhter 1999). In the present study, we saw a gradient throughout the neointima with collagen localized to the basal layers, while acidic proteoglycans was found uniformly throughout lesions. Even though further identification of ECM was not undertaken in this study, we suggest that comparison of charge properties of salmon versus human ECM could offer a novel insight into the contribution of ECM in the development of atherosclerosis. Accumulation of LDL in the vessel wall seems to be a critical step in the development of the inflammatory response leading to atherosclerosis in mammals (Andersson, Libby & Hansson 2010; Libby *et al.* 2011; Fogelstrand & Borèn 2012). Indeed, the presence of T lymphocyte clones specifically recognizing oxidized LDL in atherosclerotic lesions has been confirmed (Semme *et al.* 1995). Most of these belong to the Th1 subset of CD4-positive T lymphocytes (Huber *et al.* 2001; Profumo *et al.* 2012), which through an interferon (INF)-γ associated pathway promotes atherosclerotic lesion formation. Interesting, this effect is counteracted by the Th2 subset of CD4-positive T lymphocytes (Huber *et al.* 2001; Engelbertsen *et al.* 2013). In the present study, both early and advanced coronary lesions of Atlantic salmon contained very few inflammatory cells. Based on the lack of intimal fat accumulation as shown in this and other studies (McKenzie *et al.* 1978; House & Benditt 1981; Seierstad *et al.* 2005b), there must be a different pathogenesis giving rise to the severe changes observed. Overall, teleost fishes are hyperlipidemic and hypercholesterolaemic as compared with mammals [42], with total plasma cholesterol levels up to five times higher than normal mammalian levels [5]. So why is there no fat accumulation in salmon

coronary lesions? One fundamental difference to mammals is blood pressure, with teleost fish having typically one-fourth of that normally seen in mammals (Babin & Vernier 1989). Indeed, increased blood pressure is recognized as an important risk factor for coronary health disease in humans (MacMahon *et al.* 1990). Another striking feature is the species difference in serum lipoproteins. High-density lipoprotein (HDL) is the predominant lipoprotein in the blood of teleost fish, with most plasma cholesterol transported by HDL as a result of this abundance (Babin & Vernier 1989). Also, fish LDL contains more triacylglycerols and less cholesterol esters than human LDL. In contrast to humans where the majority of plasma cholesterol is found in the LDL fraction, most of the circulation cholesterol is in the HDL fraction in fish (Farrell *et al.* 1986) including Atlantic salmon (Jordal, Lie & Torstensen 2007). Interestingly, it has been demonstrated that cholesterol-enriched LDL has increased affinity for arterial wall proteoglycans (Flood *et al.* 2004). High levels of HDL compared with LDL are known to be of benefit, with HDL exerting an athero-protective effect (Tall 2008). Thus, salmon species may be naturally resistant against vascular fat accumulation.

The mechanism responsible for salmonid coronary lesion formation has so far remained unknown even though there is a growing consensus regarding a mechanical aetiology owing to the great expandability of bulbus arteriosus from which the main coronary artery is suspended (Farrell 2002). In this study, we investigated whether increase in lesion size was accompanied with increased expression of the stretch-inducible genes Cyr61 and CTGF. These dynamically expressed matricellular proteins are involved in production and modification of the ECM (Bornstein & Sage 2002; Leask & Abraham 2006). Both Cyr61 and CTGF are structurally related but functionally distinct multimodular proteins that are expressed in different organs and tissues only during specific developmental or pathological events, and they are known to be highly expressed in several mechanical stress-related pathological changes (Chaqour & Goppelt-Struebe 2006). In humans, both Cyr61 and CTGF have been shown to promote neointimal hyperplasia after vascular injury (Jun & Lau 2011). We found a trend towards a several fold increase in these stretch-induced genes with increasing lesion size in the wild-caught fish, which is in agreement with what would be expected with a mechanotransductional mechanism. These observations are the first experimental evidence supporting the stress-response theory for lesion development in fish.

In most fish species other than salmonids, coronary vascular changes seem to be absent (Farrell 2002). Thus, salmonids are interesting model organisms for future research, both for studies addressing stress-induced initiation of coronary disease, but also as a knock-out model for addressing mechanisms related to fat retention and inflammation. As the salmon full-sequenced genome will be available soon, experimental possibilities may be vast. From the fish farming industry's perspective, our results are also interesting, as they show that substitution of marine with different vegetable oil and protein sources in commercial feasible recipes has no apparent effect on coronary health, despite significant differences in heart n-3/n-6 ratios in the different dietary groups. Given the growing shortage on marine feed sources forcing the commercial fish feed industry to rely on vegetable fat and protein sources to an increasing extent (Tacon & Metian 2008), this is an important finding from an animal welfare perspective.

This work shows that early manifestations in salmon coronary vascular disease are similar to that of humans with respect to cellular composition and morphological appearance. However, while such changes continue with fat accumulations and inflammatory cell infiltration in humans, this is not the case in salmonid fish. Here, the main cellular component consists of proliferating SMC that produce an ECM. This process is not influenced by dietary fatty oils but seems to be initiated by vascular stress similar to the initiation of human coronary lesions finally resulting in atherosclerosis. This study underscores the importance of vascular stress in the onset of coronary disorders. The characterization of the coronary changes in salmonid species may assist in identifying factors leading to inflammation and fat accumulations in humans.

Sources of funding

The study was funded by the Research Council of Norway (RCN) ('SAFE FEED, SAFE AND HEALTHY SEAFOOD Contaminants, nutrients and health challenges in the novel production of

farmed Atlantic salmon', project number 199626) and Skretting ARC.

Acknowledgements

A great thank to the Sport Fishermen's Club at Hellefossen, river Drammenselva, Norway, for providing wild salmon, and to Mr. Steinar Stølen, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Norway, for excellent technical support on scanning electron microscopy. The authors are indebted to Dr. Lars Austbø for his contribution to the RT-qPCR assay and Associated Professor Dr. David Griffiths for his contribution to Fig. 1a, both at the Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences, Norway.

Publication History

Received: 22 August 2014
Revision received: 9 November 2014
Accepted: 15 September 2014

This paper was edited and accepted under the Editorship of Professor Ron Roberts.

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