The effect of dietary n-3 polyunsaturated fatty acids on lipid composition and location of proteins in rat heart lipid rafts

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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in Biochemstry

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Abstract

Lipid rafts are microdomains in the cell membrane that have higher concentration of cholesterol and sphingolipids and are more tightly packed than the surrounding membrane. The location of proteins in lipid rafts in cardiomyocyte membranes is important for transmembrane signaling, as the rafts serve as a platform for signaling across membranes. Dietary n-3 long-chain polyunsaturated fatty acids (LC-PUFA) have been shown to lower the risk of cardiovascular diseases, potentially by affecting signal transduction across the cardiomyocyte membrane. Studies have shown that n-3 LC-PUFA alter the location of proteins in lipid rafts and their lipid composition in T-cells and other cell types. However, the effect of n-3 LC-PUFA on protein localization in lipid rafts in cardiomyocytes has not been investigated. The aim of this project was to study the effect of dietary n-3 LC-PUFA on the fatty acid (FA) composition and the location of raft markers and adrenergic receptors in lipid rafts in rat heart.

Lipid rafts were isolated on sucrose gradients from hearts of adult rats that had been fed a controlled diet enriched with fish oil (n-3 LC-PUFA) or safflower oil (n-6 PUFA; control group). Proteins and GM1 ganglioside were analyzed in 12 fractions of the sucrose gradient with western blot and dot blot technique, respectively. Cholesterol was measured with a spectrophotometric assay kit. Phospholipids were isolated from lipid rafts and their FA composition was analyzed with gas chromatography.

n-3 LC-PUFA levels were significantly higher in the lipid rafts from rats fed n-3 LC-PUFA than those fed n-6 PUFA. No significant difference was found in location of cholesterol and GM1 levels, or marker proteins, in lipid rafts between the n-3 and n-6 PUFA fed rats. Furthermore, there was no significant difference in quantity or localization of α_1- and β_1-adrenergic receptors in lipid rafts from the rat hearts between diet groups. However, a trend was observed towards a higher concentration of β_1-adrenergic receptors in lipid rafts from heart of rats fed n-3 LC-PUFA than those fed n-6 PUFA.

Ágrip

Himnuflekar eru örsvæði í frumuhimnu sem eru stífari og innihalda meira kólesteról og sphingólípíð en himnan umhverfis. Staðsetning próteina í himnuflekum í frumuhimnu hjartavöðvafruma er mikilvæg fyrir boðflutninga, en þættir ákveðinna boðflutningskerfa safnast saman á himnuflekasvæðum. Lækkuð dánartíðni vegna hjartasjúkdóma hefur verið tengd við neyslu n-3 langra fjölómettaðra fitusýra (L-FÓFS), en þær eru taldar vernda gegn hjartsláttartruflunum, hugsanlega með því að hafa áhrif á boðflutning í hjartavöðvafrumum. Rannsóknir hafa sýnt að n-3 L-FÓFS hafa áhrif á staðsetningu próteina í himnuflekum og á lípíðasamsetningu þeirra í T-frumum og fleiri frumugerðum. Áhrif n-3 L-FÓFS á staðsetningu próteina í himnuflekum í hjartavöðvafrumum hafa hins vegar ekki verið könnuð. Markmið þessa verkefnisins var að kanna áhrif n-3 L-FÓFS í fæði á fitusýrusamsetningu himnufleka og staðsetningu adrenergra viðtaka og einkennispróteina í himnuflekum í rottuhjörtum.

Himnuflekar voru einangraðir með spuna á sykurstyrkshalla úr hjarta fullorðinna rotta sem aldar voru á fóðri bættu með fiskolíu (n-3 L-FÓFS) eða körfublómaolíu (n-6 FÓFS; viðmiðunarhópur). Prótein voru greind í 12 hlutum af styrkhallanum með Western þerrun, gangliosíð GM1 með þerriblettun og kólesteról var mælt með ljósmælingu. Fosfólípíð voru einangruð úr himnuflekum og fitusýrusamsetning þeirra var greind með gasgreini.

n-3 L-FÓFS gildi voru marktækt hærri í himnuflekum rotta sem voru á n-3 FÓFS fóðri en hjá þeim rottum svo fengu n-6 FÓFS bætt fóður. Ekki var marktækur munur á kólesteról magni, GM1 magni né staðsetningu einkennispróteina himnufleka í himnuflekum á milli fóðurhópanna. Ekki var heldur marktækur munur á magni og staðsetningu α_1- og β_1-adrenegra viðtaka í himnuflekum í rottuhjarta á milli fóðurhópa. Hinsvegar, kom fram vísbending um það væri meira af β_1-adrenergrum viðtökum í himnu flekum úr hjörtum rotta sem fengu n-3 L-FÓFS sen þeirra sem fengu n-6 FÓFS.

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Abbreviations

AA	Arachidonic acid
AC	Adenylyl cyclase
ALA	α-linolenic acid
AT_1-R	Angiotensin II type 1 receptor
B-ARK1	β–adrenergic receptor kinase
BHT	Butylated hydroxytoluene
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophospate
CVD	Cardiovascular diseases
DHA	Docohexaenoic acid
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FO	Fish oil
GC	Gas chromatography
GM1	Ganglioside monosialotetrahexosyl
GPCR	G-protein-coupled receptor
HRP	Horseradish peroxidase
IL-2R	Interleukin 2 receptor
LA	Linoleic acid
LC	Long-chain
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MUFA	Monounsaturated fatty acid
N_2	Liquid nitrogen
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI3K	Phosphatidylinositol-3-kinase
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinyldifluoride
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
TLC	Thin layer chromatography
VLDL	Very low-density lipoprotein
β-blockers	Beta adrenergic blocking agents

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1. Introduction

From the settlement Icelanders have been using fish oil for many purposes. Fish oil, mainly cod liver oil (*is. lýsi*), was one of the main export products from Iceland and it is known that around the year 1100, Icelanders used fish oil as a currency to pay the tithe. It was mostly used as an illuminant, but fishermen applied fish oil to their clothes to make them waterproof and consumed it to keep them warm out at sea in cold weather. Fish oil was also used for storage of fish food. Later it was discovered that fish oil could have a positive effect on health. Around 1900 Icelandic school children were given fish oil at school as it was known to be rich in vitamins. In the 1960s it was discovered that fish oil probably had greater health benefits than just as a vitamin supplier (*4*).

Today it is common knowledge that consumption of fish oils is beneficial, not only because of its A and D vitamin content, but also because they are a rich source of n-3 long-chain polyunsaturated fatty acids (PUFA). n-3 LC-PUFA were named the essential FAs when it was discovered that they were essential for normal growth in young children and animals (*5*). They are essential for growth and function of nervous tissue. Recent studies suggest that n-3 LC-PUFA has a role in neurogenesis, neurotransmission, and protection against oxidative stress (*6*), and infants that were given fish oil supplement were found to show improvement in immune function maturation (*7*).

Extensive studies have shown that n-3 LC-PUFA are essential for humans and can have multiple beneficial effects, for example by lowering heart rate and improving vascular function (*8, 9*). It has also been shown that n-3 LC-PUFA consumption lowers plasma triglycerides in blood and decrease inflammation (*10, 11*). Increased consumption of n-3 LC-PUFA reduces hepatic very low-density lipoprotein (VLDL) synthesis which is thought to reduce subsequently FA availabilty for triglyceride synthesis (*12*). n-3 LC-PUFA has been demonstrated to have a protective effect against cardiovascular diseases (CVD), including coronary artery disease (CAD), myocardial infarction (MI), and stroke. These cardioprotective benefits have been observed with daily consumption of as little as

25 g of fatty fish which is high in n-3 LC-PUFA. This amount is equivalent to ≥1 fish meal weekly or even monthly (*13*).

It has been shown that dietary n-3 LC-PUFA target the lipid membrane and in particular the signaling proteins in the lipid membrane that contribute to inflammation signaling (*14, 15*). n-3 LC-PUFA change the FA composition in the lipid membrane and it has been shown that the lipid raft microdomains, are generally a target for those alterations (*16-19*).

1.1 Biomembranes

Biomembranes are separating membranes that work as selective barriers around cell or cell compartments, and are essential for all eukaryotic cells. They consist of a lipid bilayer with embedded proteins, which can constitute up to 50 % of the membrane content. The cell membrane maintains the crucial difference in between the cytosol and the extracellular environment and defines the boundaries of the cell. The ionic difference across the biomembranes makes it possible for the cell to *e.g.* synthesize the energy-transferring compound adenosine triphosphate (ATP). In cell membranes of muscle and nerve cells the ionic difference over biomembranes is used to produce and transmit electrical signals.

The lipids in biomembranes consist of glycerophospholipids, sphingolipids and sterols. Phospholipids have a hydrophobic (non-polar) end and a hydrophilic (polar) end (Figure 1.B). The hydrophobic ends cluster together while the hydrophilic parts stick out forming a bilayer (Figure 1.A). Proteins that are embedded in the lipid membrane have a hydrophobic part that enables them to move within the membrane.

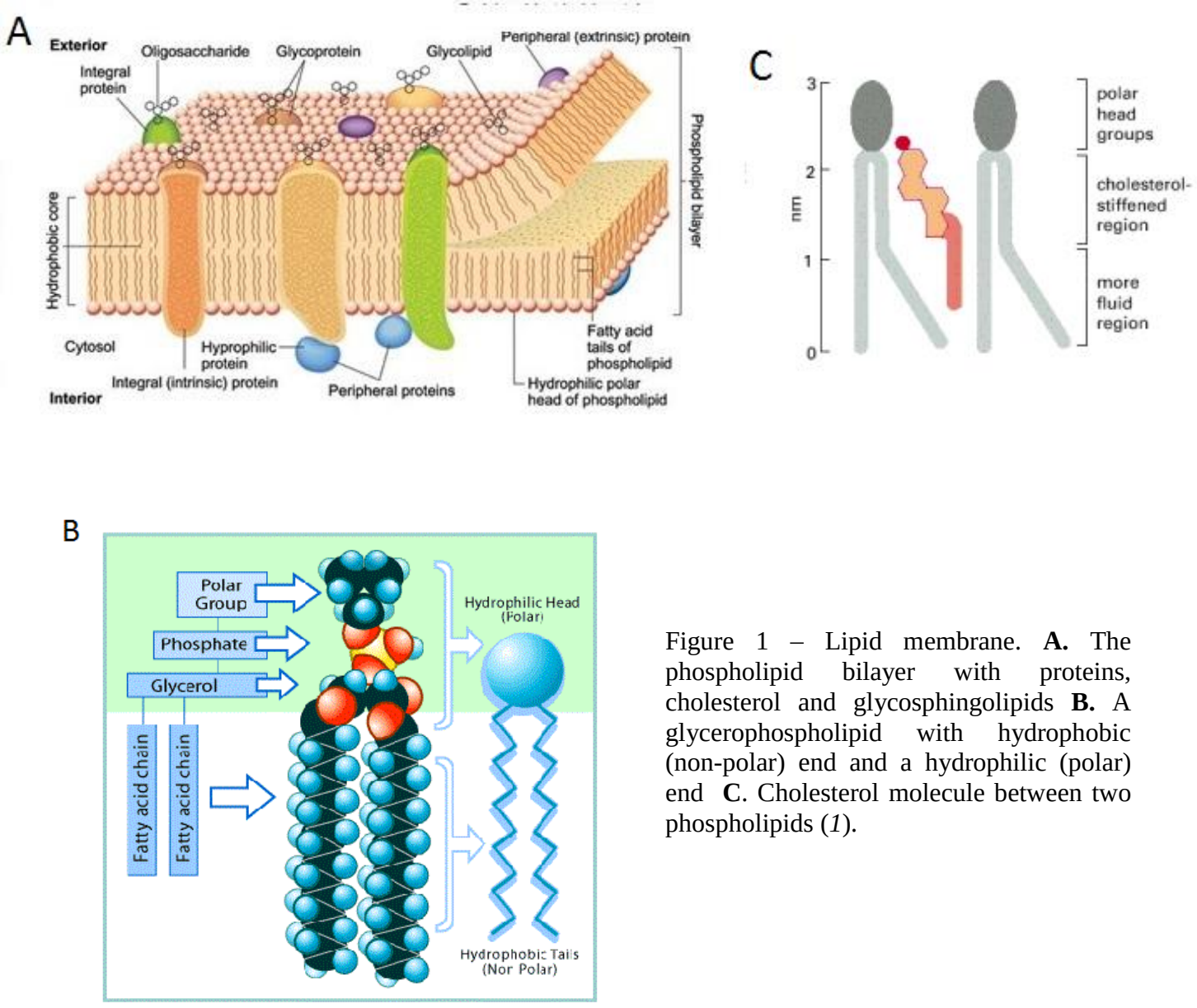

Figure 1 – Lipid membrane. **A.** The phospholipid bilayer with proteins, cholesterol and glycosphingolipids **B.** A glycerophospholipid with hydrophobic (non-polar) end and a hydrophilic (polar) end **C**. Cholesterol molecule between two phospholipids (*1*).

There are five major phospholipid classes; the glycerophospholipids include phosphatidylethanolamines, phosphatidylserines, phosphatidylcholines and phosphatidylinositols (Figure 2), and the fifth phospholipid class are the sphingomyelins, which have a sphingosine backbone instead of glyceride in the glycerophospholipids. Phospholipids constitute more than half of most eukaryotic cell membranes. Other important lipids in biomembranes are cholesterol (Figure 1.C) and glycosphingolipids. The fluidity of the lipid bilayer depends on the length and degree of unsaturation of the FAs of phospholipids in the membrane, and the cholesterol content. The rigid structure of cholesterol enhances the permeability-barrier properties of the lipid membrane and makes

the membrane less fluid. Cholesterols feature a hydroxyl group and a non-polar region that orient themselves in the bilayer with the hydroxyl group near the polar end of the phospholipids.

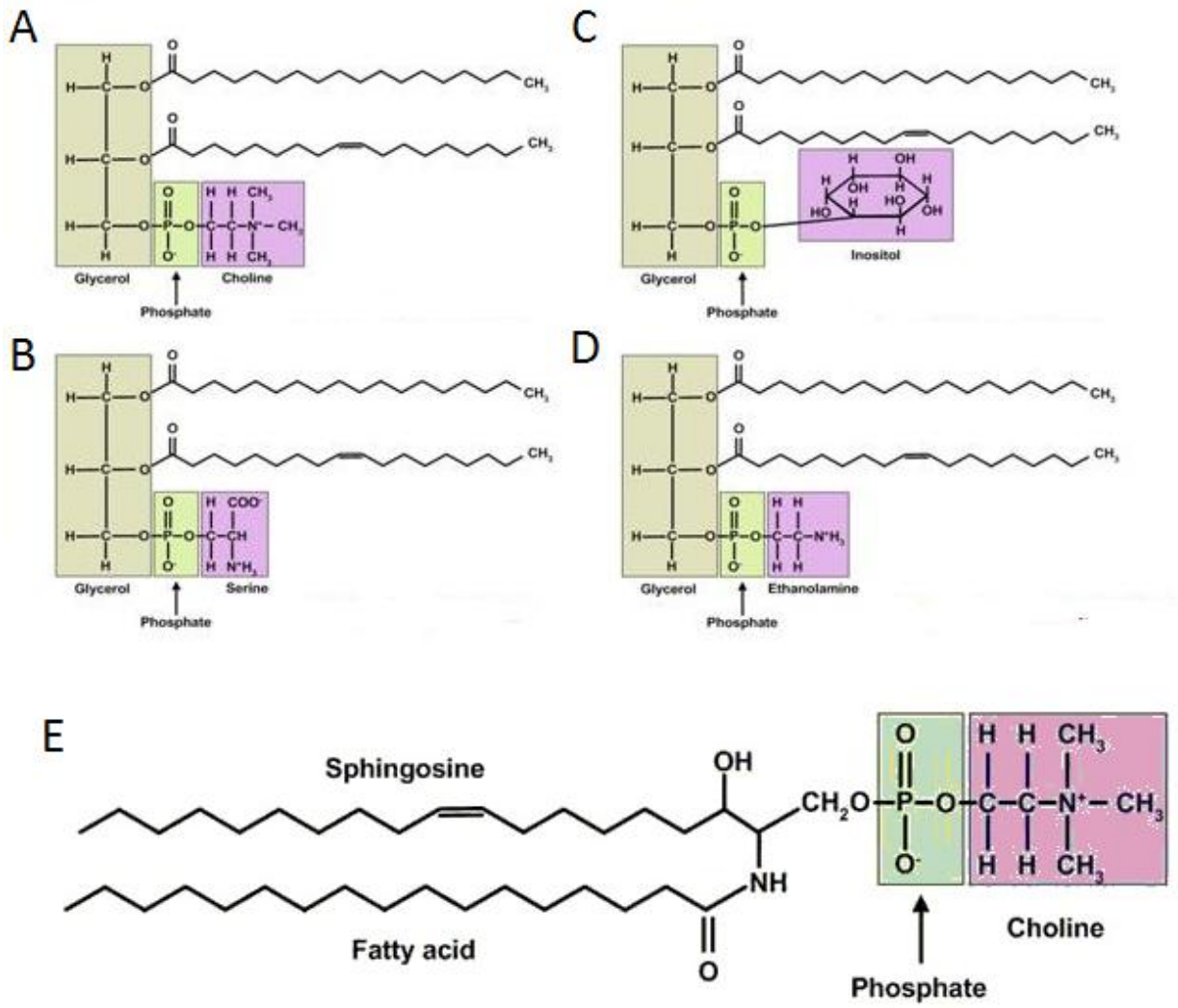

Figure 2 – Strucure of five major phospholipid classes. **A.** Phosphatidylcholine **B.** Phosphatidylserine **C.** Phosphatidylinositol **D.** Phosphatidylethanolamine **E.** Spinghomyelin (*2*).

1.2 Lipid rafts

Lipid rafts are microdomains in the cell membrane that are rich in sphingolipids and cholesterol (Figure 3). In 1988, Simons and van Meer, and others published the first articles that proposed the presence about these microdomains and were soon followed by

others (*20-22*). However, it was not until 2006 that scientists on an international conference accepted this hypothesis and agreed on a definition for lipid rafts (*23*). Lipid rafts work as a platform for signal transduction across the cell membrane. Components of certain signal transduction systems segregate together in the lipid rafts and are either activated or deactivated there (*24*, *25*). Lipid rafts can be considered to provide a concentrating platform for individual receptors which are activated by binding ligands. If a receptor is activated in a lipid raft the signaling complex is protected from non-raft enzymes such as membrane phosphatases. The phosphorylation state can then be modified by specific local kinasees or phosphatases inside the lipid rafts, resulting in downstream signaling (*26*).

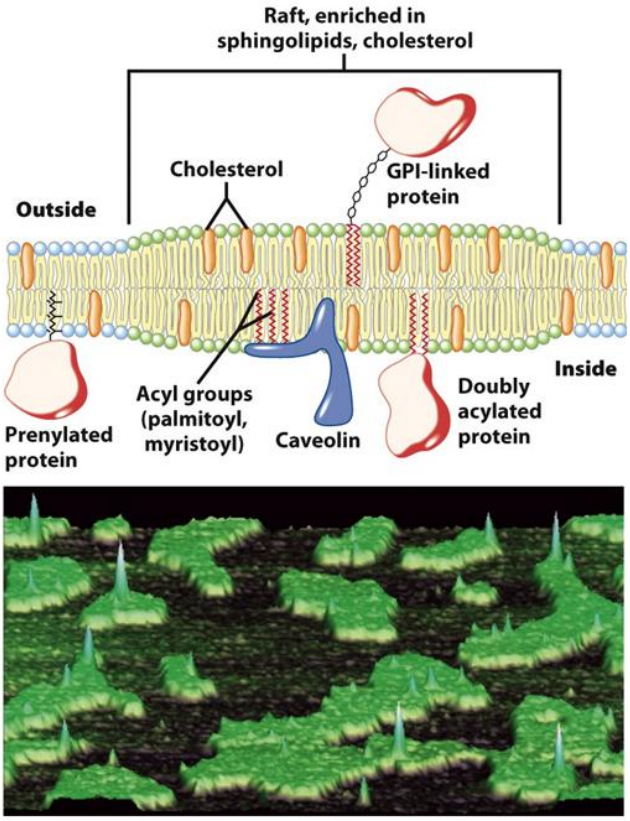

Figure 4 – Lipid rafts, microdomains in the cell membrane, rich in saturated fatty acids, sphingolipids, cholesterol and proteins (*1*).

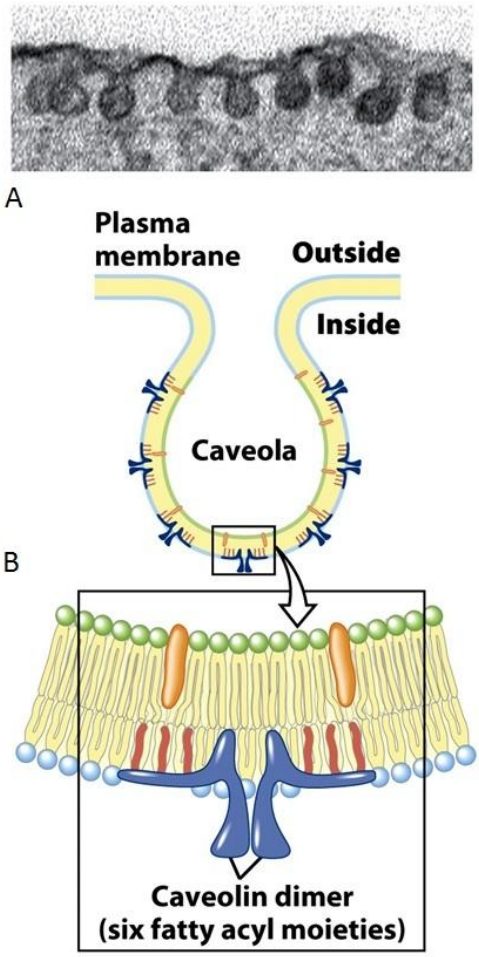

Figure 3 – Structure of caveolae **A.** An electron micrograph of caveolae **B.** The caveolin dimer supports the shape of the caveolae (*1*).

1.2.1 Caveolae

Caveolae are plasma membrane domains that have the same characteristics as ordinary lipid rafts. They are highly enriched in cholesterol and sphingolipids, but have a different shape. They form 50 to 100-nm invaginations in the membrane and are found in many different cell types (*27*). They were discovered in 1953 by Palade et al. using an electron microscope (Figure 4.A) (*28*). Besides their role as a site of signal transduction, caveolae have been suggested to be involved in transportation of albumin (*29*), iron-transferrin (*30*), insulin (*31*), low-density lipoproteins (*29*), chemokines, and regulation of endothelial nitric oxide synthase (eNOS) (*32*). Lipid rafts in the plasma membrane of cardiomyocytes have subset domains, which are caveolar forms (*33*). They were identified decades before the discovery of lipid rafts as they could be seen in electron microscopes. Caveolin is an integral membrane structure protein in the caveolae. It binds to cholesterol in the membrane and the presence of caveolin forces the lipid membrane to curve inward, forming a little "cave" or the caveolae (Figure 4.B). It has also been shown that caveolins interact with a variety of downstream signaling molecules, such as the Src-family tyrosine kinase and p42/44 mitogen-activated protein kinase (MAPK), and they are believed to keep the signal transduction molecules in their inactive form until activated with the right stimulus (*34*). Caveolin is found in three isoforms (caveolin-1 -2 and -3). Caveolin-1 and -2 are found in most cell types while caveolin-3 is only found in muscle cells (*35*).

Flotillin-1 is also an integral membrane protein found in lipid rafts. Flotillin-1 was discovered in the axon regeneration of retinal ganglion cells in goldfish and has since then been used as a marker protein for lipid rafts (*36, 37*). A recently published investigation has revealed that flotillin-1 directly activates epidermal growth factor receptor (EGFR) and also plays a direct role in the late phase of growth factor signaling, that is, the activation of MAPK. This proves its role as a novel scaffolding protein for MAPK signaling in lipid rafts. The authors also suggested that flotillin-1 may play a general role as a novel factor in tyrosine kinase receptor/MAPK signaling (*38*).

1.3 Essential fatty acids

In 1929, Burr and Burr revealed the nutritional importance of specific lipid molecules. They fed rats a fat-free diet and observed retarded growth, scaly skin, tail necrosis and finally death. The effects were reversible by feeding the rats with a diet that included fats. It was then discovered that linoleic- (LA) and α-linolenic (ALA) acids were active agents and later referred to as essential FAs (Figure 5) (*39*).

FAs are hydrocarbon chains with a carboxyl acid group on one end, the alpha end (α-end), and a methyl group on the other end, the omega end (ω- or n-end), alpha being the first letter and omega being the last latter in the Greek alphabet. FAs differ in length and saturation. A saturated FA does not contain a double bond, a monounsaturated FA (MUFA) contains one double bond and polyunsaturated fatty acids (PUFA) have two or more double bonds. The subclass of the PUFA is determined by the position of the double bond from the n-end. In a n-3 PUFA the first double bond is at the third carbon counted from the n-end. In n-6 PUFA the first double bond is at the sixth carbon counted from n-end (*40*).

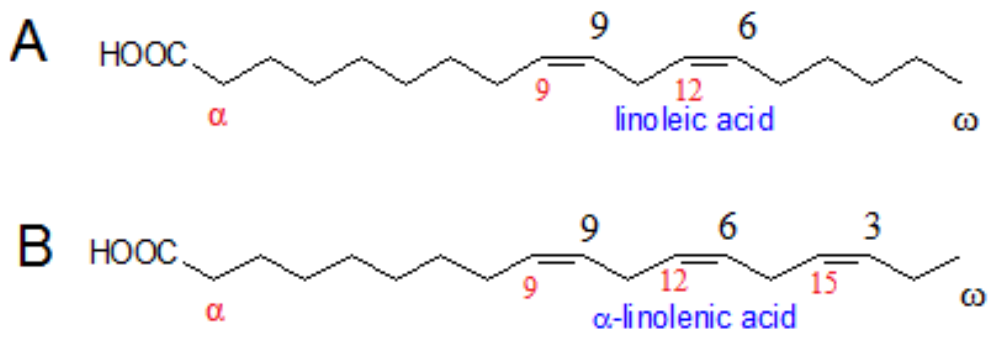

Figure 5 - The essential fatty acids **A.** Linoleic acid (18:2n-6) and **B.** α-Linolenic acid (18:3n-3). Hydrocarbon chain with a carboxyl group on the left side (the α-end) and a methyl group on the right end (the ω-end). PUFA subclass is determined by the position of double bond (*3*).

Mammalian cells lack the converting enzymes, Δ3- and Δ6-desaturases, and they can neither convert FAs n-3 to n-6 PUFA nor convert n-6 to n-3 LC-PUFA (*41*). Mammalian cells cannot introduce a double bond further than on carbon 9 from the α-end (Δ9-desaturase). Therefore, mammalian cells need to get LA and ALA from their diet. LA and ALA are the precursors for the longer chain n-6 and n-3 LC-PUFA, respectively, and are therefore listed as essential FAs. LA is found in most vegetable oils (corn- and safflower oil) and ALA is found in some vegetable oils (rapeseed-, flaxseed-, and soybean oil), green vegetables, and nuts. LA and ALA are elongated and desaturated with the corresponding enzymes to form the longer and more desaturated FAs that are needed. LA is the precursor for arachidonic acid (AA, (20:4n-6)) while ALA is the precursor for eicosapentaenoic acid (EPA, (20:5n-3)) and consequently docosahexaenoic acid (DHA, (22:6n-3)) (Figure 6) (*42*).

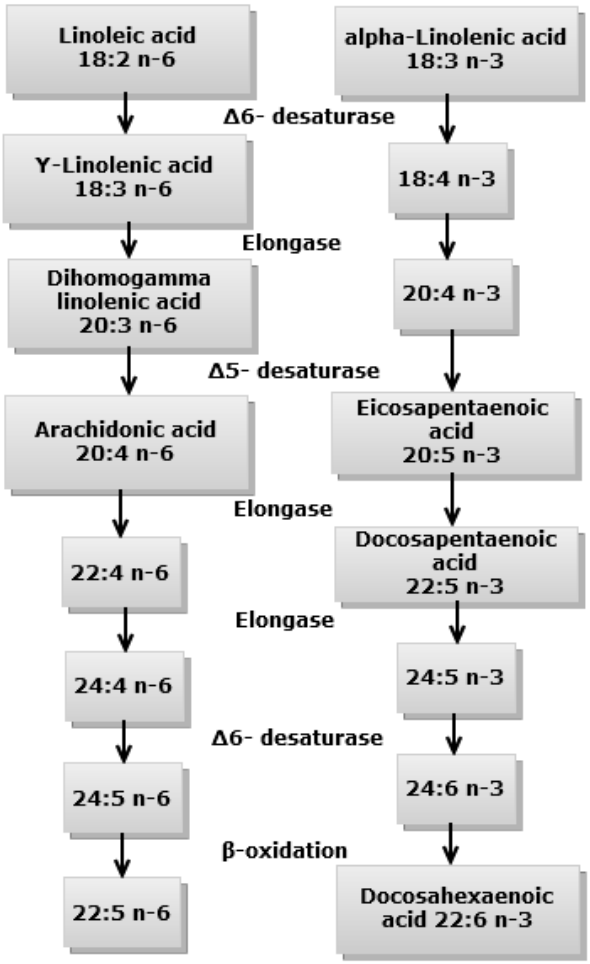

Figure 6 – The metabolic pathway of polyunsaturated fatty acids where n-3 PUFA and n-6 PUFA share the same pathway and therefore compete for the same enzymes.

The two pathways, LA to AA and ALA to EPA, use the same enzymes for elongation and desaturation, so the two pathways compete. The pathway from ALA to EPA and DHA in humans is limited and therefore humans have to get their EPA and DHA from diet, e.g. from fish and/or other marine products (*43*).

During the last 150 years, the composition of lipids in food consumed by humans, at least in the western countries, has changed drastically. Studies have shown that the human race evolved on a diet with the ratio of n-6 PUFA / n-3 PUFA that was approximately 1. Today this ratio is believed to be around 15/1, which has promoted the pathogenesis of cardiovascular diseases, cancer, osteoporosis and inflammatory- and autoimmune diseases (*41*). Increasing intake of n-3 LC-PUFA may have beneficial effects in health and disease (*44*) and it has been shown that starting a daily intake of n-3 LC-PUFA (1 g/day) at young ages reduces mortality (*45*). For cardiovascular health, the World Health Organization and an increasing number of organizations worldwide now recommend regular fish consumption of one to two servings per week, *i.e.* each serving should provide the equivalent of 200–500 mg of EPA+DHA (*46*).

1.4 Adrenergic receptors

Adrenergic receptors belong to the G-protein-coupled receptor (GPCR) family and are stimulated by catecholamine compounds (Figure 7A), noradrenaline and adrenaline (Figure 7B and 7C). When adrenergic receptors are activated by noradrenaline or adrenaline, they cause a sympathetic response that increases heart rate and energy availability expenditures. Blood flow

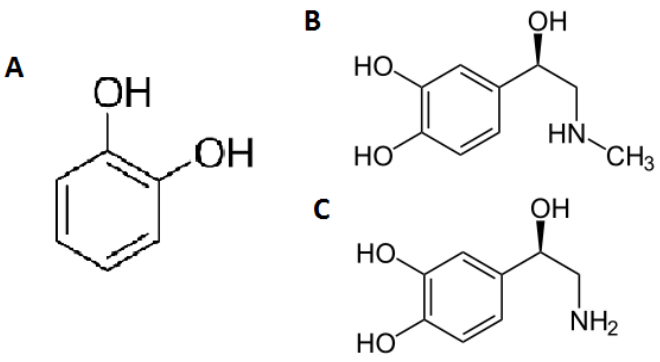

Figure 7 – Structure of **A.** Catecholamine, **B.** Adrenaline, and **C.** Noradrenaline.

is mobilized from organs such as gastrointestinal system, kidney and skin to the skeletal muscle. These responses are often called "fight-or-flight" response (*47*). Noradrenaline acts primarily as a neurotransmitter released from sympathetic-nerve terminals, and adrenaline functions as a circulatory hormone released from the adrenal medulla.

Adrenergic receptors are divided into two main groups, *i.e.* α- and β-adrenergic receptors. The α-adrenergic receptors have the subtypes α_1 and α_2. β-adrenergic receptors have the subtypes β_1, β_2 and β_3. The different adrenergic receptor types are linked to different types of G-proteins, which are a family of proteins involved in signal transduction across the cell membrane. The adrenergic receptor and G-protein activation is followed by stimulation of one of two types of second messenger systems.

Agonist binding to the β- and α_2-adrenergic receptors causes a rise or fall in the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP) (Figure 8). Increased cAMP concentration will promote relaxation in smooth muscle, while promoting increased contractility and pulse rate in cardiac muscle. The α_1-adrenergic receptor uses the inositol phosphate second messenger system where the binding of G-protein to the receptor activates phospholipase C, which cleaves inositol mono-, di-, or tri inositolphosphates from phosphorylated phosphatidylinositol molecules in the membranes. This gives two types of messenger molecules, inositolphosphates and diacylglycerol. To turn off a signaling adrenergic receptor, the receptor can be uncoupled from its signal- transducing G-protein. That is done by a protein kinase which phosphorylates the receptor. Activation of protein kinase A (PKA) or protein kinase C (PKC) can also phosphorylate GPCRs at a different site which causes desensitization, which is the waning of receptor responsiveness despite continuing agonist stimulation. (*48*). Hereafter, we will only focus on the α_1- and β_1-adrenergic receptors.

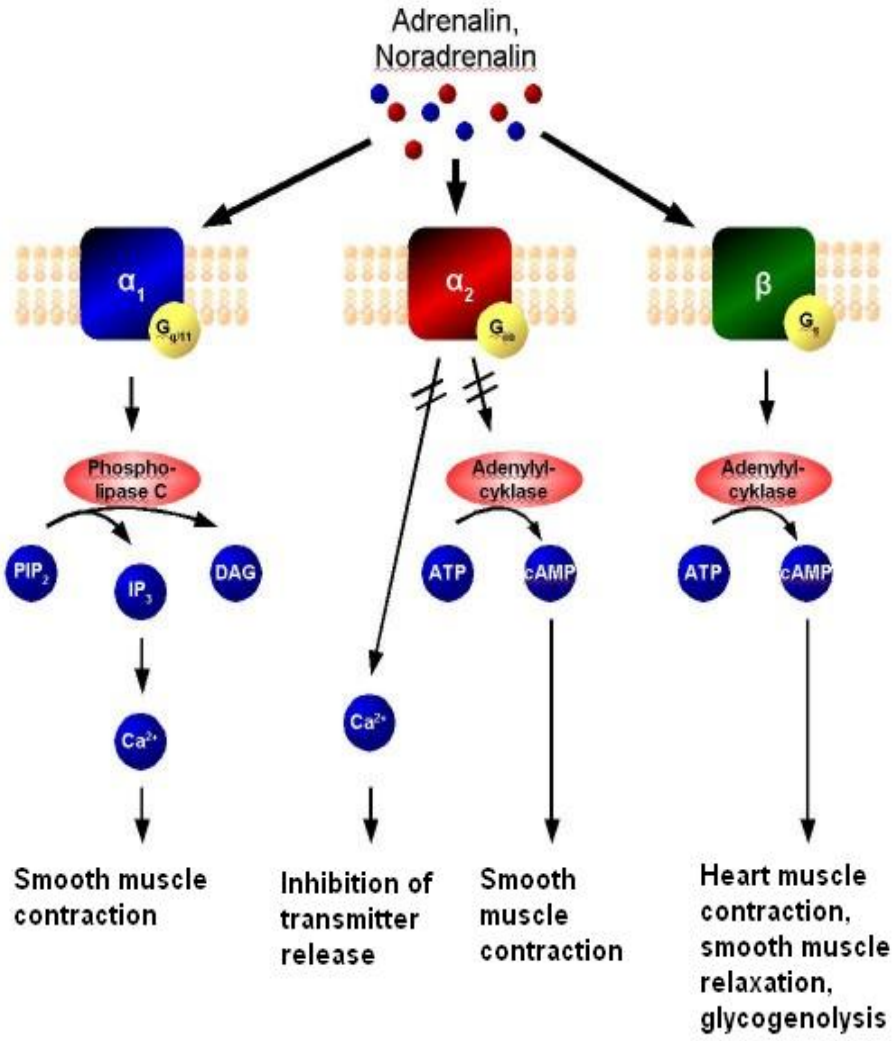

Figure 8 – Signaling pathway for α1, α2 and β adrenergic receptors and their effects.

1.4.1 α_1-adrenergic receptor

Hormones and neurotransmitters regulate cellular activity via the intermediate role of the regulatory G-protein. The α_1-adrenergic receptor consists of three highly homologous subtypes, α_{1A}, α_{1B}, and α_{1D}. In animals α_1- and α_2-adrenergic receptors mediate vasoconstriction of coronary arteries (*49*) and less motility of smooth muscle in rat gastrointestinal tract (*50*). Blood vessels with α_1-adrenergic receptors are present in skin, gastrointestinal system, kidney, and brain of rats. During the "fight-or-flight" responses, the α_1-adrenergic receptors mediate vasoconstriction and less blood flow to these organs. PKC activated by the α_1-adrenergic receptor stimulation produces chronotropic and inotropic responses in the heart by stimulation of the L-type Ca^{2+} channels. Stimulation of α_1-adrenergic receptors in cardiomyocytes can also produce hypertrophy, caused by

activation of MAPK by PKC in myocytes. Hypertrophy is defined as increased surface area and cell protein content (*51*). Cardiac hypertrophy is a physiological response of the heart to increased work load (increased inotropic effects (*51*)). It is a compensatory mechanism in humans to normalize wall stress in the heart, thereby preventing development of heart failure (*52, 53*), and this is the case during excessive exercise. There has also been indication of an association between ventricular hypertrophy and increased cardiac mortality which casts a shadow on the "wall stress" hypothesis (*54*).

1.4.2 β_1-adrenergic receptor

The stimulation of β_1-adrenergic receptors increases cardiac output. β_1-adrenergic receptors couple primarily to the stimulatory G-protein G_s, which activates adenylate cyclase; increasing intracellular cAMP levels that activate PKA which leads to phosphorylation of its substrates troponin I and the L-type Ca^{2+}-channels thus enhancing contractility (*55*). Stimulation of β_1-adrenergic receptor in heart leads to a chronotropic effect (increase in heart rate), increased contractility and automaticity of the ventricular cardiac muscle and atrioventricular node (*56*). β_1-adrenergic receptor is the most predominant subtype in the heart and is 75-80% of total β-adrenergic receptors in humans. The ratio of β- to α-adrenergic receptors is 10:1 in a healthy human myocardium (*57*). It has been shown that DHA selectively increased number of β-adrenergic receptors in astrocytes from rat cerebra, possibly suggesting a role for DHA in relation to β-adrenergic receptors in the membranes in those cells (*58*). It has also been suggested that DHA binds specifically to rhodopsin, which is a GPCR, and has a role in control of the motility and activation of the receptor (*59*).

In a failing human heart the β-adrenergic receptor pathway becomes abnormal. Increased catecholamine stimulation for a long period leads to β_1-adrenergic receptor downregulation (*60*) and desensitization (*61*). β_1 and β_2 are both uncoupled from their G-proteins in failing hearts (*60*). In a failing heart, these two pathways (uncoupling of the G-protein from the adrenergic receptor and the desensitizing of the adrenergic receptor) attribute to increased levels and increased activity of myocardial β–adrenergic receptor kinase (β-ARK1) (*62*). Patients that are experiencing a failing heart condition are sometimes given β-adrenergic receptor blocking agents (β-blockers). β-Blockers are a class of drugs used to manage

cardiac arrhythmias (decrease chronotrophy) for cardioprotection after heart attack and for hypertension. It has been shown that β- blockers are cardioprotective. They are used to resensitize the receptor system and thereby improve cardiac function (*63-65*) and recently it was discovered that two specific β-blockers (alprenolol and carvedilol) could stimulate a pathway to protect heart tissue (*66*). Their β-blocker effects are thought to be maximized after around 18 months (*67*) but some studies have shown that a long term use (~5 years) of β-blockers can be risky (*68*).

1.5 Lipid rafts and signaling components

Caveolin has, besides it role as a membrane scaffolding protein, been shown to take a part in the exocytic pathway. Wyse et al. (*69*) showed that a direct interaction with caveolin is required to traffic angiotensin II type 1 receptor (AT_1-R) through the exocytic pathway. Angiotensin is a peptide hormone that increases blood pressure and it is mediated through AT_1-R which is a GPCR. They also noticed that there was no accumulation of AT_1-R in the caveolae. Therefore, they explained that cavolin acted not only as a membrane scaffolding protein but also as a chaperone for the AT_1-R.

Signal proteins are gathered in lipid rafts and due to proximity to downstream effectors, lipid rafts, specially caveolae, are believed to be optimal for initiating signal transduction (*70*).

The downstream effectors are believed to localize within the caveolae and directly interact with cavolin protein. It has also been shown that palmitoylation enhances caveolar localization of signaling proteins (*71*). Palmitoylation is a covalent attachment of palmitic acid to a cysteine residue of a membrane proteins, which is a unique lipid modification of proteins in the sense that it is reversible and it can be regulated (*72*).

Caveolin negatively regulates eNOS and adenylyl cyclase (AC) activity. Caveolin inhibits the enzyme activity of eNOS and AC and loss of caveolin upregulates eNOS and AC activity (*73, 74*). Caveolins have been linked to numerous signaling protein pathways such as PKA/PKC (*75*), phosphatidylinositol-3-kinase/ protein kinase B (PI3K/PKB, Akt) (*76*), as well as tyrosine receptor kinase (*e.g.* EGF) and their downstream effectors, such as MAPK (*77, 78*).

A large number of GPCR, among them α_1- and β_1-adrenergic receptors, have been reported to co-localize with lipid rafts. Studies on signaling factors in caveolae of heart muscle cells from newborn rats revealed the location of the α_1-adrenergic receptors signaling pathway in those cells (*79*). The signaling precursor PIP_2 (Phosphatidylinositol 4.5-bisphosphate) was found both in caveolae and elsewhere in membranes, but decreased only in caveolae after stimulation of the α_1-adrenergic receptors. The amount of PIP_2 outside caveolae remained constant which suggested that signal transduction took place in the caveolae. This also indicated that active α_1-adrenergic receptors are found in the caveolae but an inactive portion of the receptor is outside of the caveolae. Another study on cardiomyocytes cultured from neonatal rats showed that β_1-adrenergic receptors were located in caveolae. Ostrom et al. (*80*) studied the localization and the activity of the β_1-adrenergic receptors and AC-6 and they found that these proteins were reduced in concentration if the caveolae were destroyed. The same group studied the location of GPCR in cardiomyocytes isolated from adult rats and found that caveolin-3 proteins were both inside and outside of caveolae and that the β_1-adrenergic receptors were also located inside and outside of the caveolae (*25*).

1.6 Effect of n-3 LC-PUFA on lipid rafts and signal transduction

Studies in cell cultures or animals have shown that n-3 LC-PUFA can alter location of proteins in lipid rafts and their lipid composition. The location of certain signal transduction proteins in lipid rafts regulates their function because the incorporation of those protein into lipid rafts activates or deactivates their signal transduction process. n-3 LC-PUFA in diet have been shown to lower the risk of cardiovascular diseases, probably by affecting signal transduction across the cardiomyocyte membrane (*8, 9*).
Studies have shown that increased EPA in culture medium changed the FA composition of lipid rafts in Jurkat T-cells by increasing n-3 LC-PUFA content (*81*).This altered the distribution of key molecules for the interleukin-2 receptor (IL-2R) signaling pathway in inflammatory responses, as those proteins were transferred out of the lipid rafts (*18, 19*).

In splenic T-cells of mice, dietary n-3 LC-PUFA were incorporated into lipid rafts, lowered the sphingomyelin content of lipid rafts by 30% and had a suppressing effect on PKC θ signaling transduction (*17*).

Increased EPA and DHA in cell growth medium decreased proliferation and induced apoptosis in MD-MB-231 human breast cancer cells (*16*). EPA and DHA were incorporated into the lipid rafts which led to a decrease in the amounts of sphingomyelin and cholesterols. This also decreased the levels of EGFR in lipid rafts while the levels of activated EGFR and p38 MAPK increased in the lipid membrane outside the lipid rafts. This has been associated with apoptosis in human breast cancer cells.

A study using cells from a mouse colon, showed that after adding n-3 LC-PUFA to their diet, the levels of n-3 LC-PUFA in phospholipids of caveolae in the lipid membrane were increased (*82*). The amount of cholesterol, caveolin, eNOS and H-Ras were reduced in caveolae. It was also noticed that feeding n-3 LC-PUFA to the mice led to reduced activation of EGF stimulated H-Ras but not K-Ras. The effect of n-3 LC-PUFA on location of proteins in lipid rafts in cardiomyocytes has not been investigated.

2. Objectives

It is not known exactly how increased consumption of fish or fish oil reduces mortality from cardiovascular disease, but n-3 LC-PUFA have been shown to have beneficial effects on the heart muscle. We wanted to investigate if the location of proteins in lipid rafts of the cardiac cell membrane was of importance. It has repeatedly been shown in animal studies that n-3 LC-PUFA from fish enter the cardiac muscle cell membranes and reduce their response to stimuli, with consequent lowering of heart rate and the risk of arrhythmias, but the underlying process is not fully understood.

Lipid rafts are a part of the cell membrane. These microdomains can move freely within the cell membrane and they have been shown to take part in the signaling pathways across the cell membrane by increasing speed and coordination of signal transferring. Certain signaling systems have been found to function in lipid rafts and, therefore, the rafts may have an important role in the control of transmembrane signaling. We considered it relevant to study lipid raft and location in the essential signaling of adrenergic receptors across cardiac cell membranes.

Main aims of the study:
The aim of this project was to study the effect of dietary n-3 LC-PUFA on the FA composition of lipid rafts in rat hearts and to investigate the location of raft markers and adrenergic receptors in lipid rafts of rat heart.

Specific aims of the study:

- To isolate lipid rafts from hearts of rats fed either fish oil or safflower oil (control) diet.
- To quantify and localize the lipid raft marker proteins (caveolin-3 and flotillin-1), cholesterol and GM1 in rat hearts, and compare these factors between the two diet groups.
- To quantify and localize the α_1- and β_1-receptors in rat heart and compare these factors between the two diet groups.
- To investigate the FA composition of phospholipids in lipid rafts and dissolved membranes of a rat heart and compare between the two diet groups.

3. Materials and Methods

3.1 Animals and diets

All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland (License number: 0309-0402). Twelve two months old Sprague-Dawley rats (Tacoma, Lille Skenved, Denmark) were divided into two groups of six rats each and housed two per cage in a stable humidity (30-40%) and temperature (23-25°C) environment with light and dark alternating every 12 hours. After acclimatization for two days they were randomly assigned to two types of diets, a control diet and a fish oil diet which they received for four weeks. The composition of the diets (Table 1) was based on a typical Western diet, i.e. the „US17" diet formulated by Monsanto (St. Louis, MO), from Research Diets Inc.(New Brunswick, NY).

The fish oil diet was made by adding 28 g of menhaden fish oil / kg of basal diet (Omega Protein, Reedville, VA) at the expense of safflower oil (Welch, Holme & Clark CO Inc., Newark, NJ). AA ethyl ester (Nu-Check-Prep, Elysian, MN) (0.515 g/kg) was added to the control diet to adjust for the AA content in the fish oil diet (*83*). n-6 PUFA/n-3 PUFA ratio is ~10/1 in the control diet but the n-6 PUFA/n-3 PUFA ratio is ~1 in the fish oil diet. The FA composition of the diets is shown in Table 2. The diets were divided into daily portions and were stored under an atmosphere of nitrogen at -20°C to prevent oxidation.

The rats were weighed before and after the experiment and the amount of food consumption was monitored. All rats were provided fresh food daily and consumed water and food *ad libitum*. At the end of the four week period with dietary administration all 12 rats were injected with heparin (1000-2000 units) in the abdomen 5 minutes before slaughter and then anesthetized with Isoba inhalation vapor (MSD). The heart was excised and rinsed by retrograde perfusion with phosphate buffered saline (PBS) at 4°C, frozen in liquid nitrogen (N_2) and stored at -80°C until isolation of lipid rafts.

Table 1 - Composition of the control and fish oil diets as provided by the manufacturer.

	Control/Safflower Diet	Fish Oil Diet
Ingredients	g/kg diet	
Casein	229	229
L-Cystine	3	3
Sucrose	114	114
Cornstarch	274	274
Maltodextrin 10	86	86
Cellulose	57	57
Cocoa butter	43	43
Linseed oil	5	5
Palm oil	60	60
Safflower oil	32.5	4.5
Sunflower, Trisun	31	31
Ethyl esters (AA)	0.515	0
Fish oil	0	28
Mineral Mix S10026/Salt mix RD-96	11	11
Dicalcium phosphate	15	15
Calcium carbonate	6	6
Potassium citrate	19	19
Vitamin Mix V13401	11	11
Choline Bitartrate	2	2
Vitamin E	0.15	0.15
t-BHQ	0.03	0.03
TOTAL (g)	999.705	999.18
TOTAL (kcal)	4421	4416

Table 2 – FA composition and cholesterol level of the control and fish oil diets provided by manufacturer.

	Control diet	Fish oil diet
Fatty acid	g/kg	
C12	0.2	0.2
C14,	0.4	2.1
C14:1	0.0	0.0
C15	0.0	0.1
C16	32.0	34.1
C16:1	0.2	2.6
C16:2	0.0	0.4
C16:3	0.0	0.4
C16:4	0.0	0.4
C17	0.0	0.1
C18,	17.2	17.3
C18:1	61.4	60.8
C18:2 (linoleic)	31.2	12.5
C18:3 (α-linolenic)	3.1	3.4
C18:4	0.0	0.8
C20	0.6	0.6
C20:1	0.0	0.4
C20:2	0.0	0.0
C20:3	0.0	0.1
C20:4 (AA)	0.515	0.5
C20:5 (EPA)	0.0	3.5
C21:5	0.0	0.2
C22	0.0	0.0
C22:1	0.0	0.0
C22:4	0.0	0.0
C22:5	0.0	0.7
C22:6 (DHA)	0.0	2.5
C24	0.0	0.1
C24:1	0.0	0.0
Cholesterol	74-98mg	

3.2 Lipid raft isolation and sample preparation

Lipid rafts were isolated with a detergent free method described by Song and Li (*84*) and modified by Cavalli et al. (*85*) using Na_2CO_3 at pH 11. The Na_2CO_3 detergent free buffer solution contained 25 mM 2-morpholinoethanesulfonic acid (MES), 150 mM NaCl , 250 mM Na_2CO_3 and pH was adjusted to 11. Approximately 1.1 -1.2 g of heart muscle were diced to small pieces in 7 mL of Na_2CO_3 buffer with a protease inhibitor cocktail from SIGMA Aldrich, 10 µL per mL of Na_2CO_3 buffer.

The heart muscle was homogenized in three steps:
1. With Polytron type PTA 10-35 (setting 5), for 3 x 20 seconds and cooled on ice for 30 seconds between rounds, and kept for 1 hour on ice.
2. With chilled dounce homogenizer (10 strokes).
3. Sonicated with Sonics Ultrasonic Processor sonicator (Amplitude 60), 3 x 20 seconds, on ice, and cooled on ice for 30 seconds between rounds.

The homogenatate was centrifuged at 1000xg for 10 min at 4°C in a Sorvall RC-5C rotor. Then 2 mL of supernatant was mixed with 2 mL of 80% (w/v) sucrose solution and adjusted to 40% (w/v) sucrose concentration placed on the bottom of a 12 mL ultracentrifuge tube. Then 4 mL of 30% sucrose were added slowly on top of the supernatant followed by 4 mL of 5% sucrose to complete the gradient. The gradient was then centrifuged at 38000 rpm (280.000xg) for 18.5 hours in a SW 41.Ti rotor. Twelve fractions of 1 mL each were collected from the top of the gradient and numbered from 1 to 12, fraction number 1 being the at top and fraction number 12 being on the bottom of the sucrose gradient.

The total protein amount in each fraction was measured with the method of Zaman and Verwilghen (*86*) on microplates. On each microplate, protein measurements of 12 fractions of sucrose gradients from heart preparations of one rat from each diet group were done in triplicate. At the same time measurements of a protein standard (bovine serum albumin, Sigma-Aldrich) in seven different concentrations, 0 -300 mg/mL, were obtained. Fractions with low concentration of protein (fractions 4-6) were precipitated with 10% TCA on ice for 30 minutes. Precipitated fractions were centrifuged for 15 minutes at 13.000xg at 4°C and washed two times with chilled acetone and centrifuged in the same way. Pellets were dissolved in SDS (sodium dodecyl sulfate)-DTT (dithiothreitol) buffer giving a final

concentration of 60 mM Tris-HCl, 1% SDS, 2% sucrose, 2 mM DTT and 0.004% bromophenyl blue and sonicated until pellets had dissolved. Samples with high concentration of protein (fractions 7-12) were diluted and SDS-DTT buffer was added as described above. The samples were then boiled for 10 minutes and analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) and Western blotting.
Lipid rafts were isolated from two separate hearts on each occasion, from each of the two diet groups. This was done to enable comparison of measured factors between the diet groups in pairs, aiming at minimizing the variation due to preparation.

3.3 SDS-PAGE and Western blotting

Fraction samples were separated by molecular weight on 10-15% polyacrylamide minigels according to the method of Laemmli (*87*). After separation the gels were equilibrated in a transfer buffer solution, containing 39 mM glycine, 48 mM Tris-HCl, 0.037 % SDS, and 20% methanol, pH 8.3 for 10 minutes at room temperature. The proteins in the gels were transferred to a polyvinyldifluoride (PVDF) membrane that had been soaked in methanol for 15 minutes, in a semi dry transfer apparatus using the same transfer buffer. After the transfer of the protein bands to the PVDF membranes, they were blocked in 12.5 mM HEPES , 75 mM NaCl, 1% Tween 20 at pH 7.1 (block buffer) containing 5% non-fat milk, for three hours and washed with 20x diluted block buffer (blot buffer). Then the membranes were incubated overnight at 4°C with primary antibodies diluted with blot buffer containing 1.5% non-fat milk. Rabbit polyclonal antibody dilutions in blot buffer with 1.5% non-fat milk were as follows: Anti-caveolin-3 (Thermo Scientific, PA1-066), 1:40.000, anti-flotilin1 (affinity isolated, Sigma-Aldrich F1180), 1:20.000, anti α_1-adrenergic receptor (Santa Cruz Biotech., sc-28982), 1:1.000 and anti-β_1-adrenergic receptor (Santa Cruz Biotech., sc-568), 1:1.500. After washing thoroughly in blot buffer, the membranes were incubated in secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (Santa Cruz Biotech. sc-2004), diluted 1:5.000 in blot buffer with 1.5% non-fat milk for three hours. All antibody incubation steps were performed at 4°C. At last, the PVDF membranes were washed with blot buffer and processed for chemiluminescence digital imaging analysis using ECL-plus reagent from GE Healthcare.

3.4 Dot blots

PVDF membranes were cut into suitable sized strips and covered in methanol for activation and equilibrated in transfer buffer for 15 minutes. During sample application, the PVDF membranes were kept wet by placing them on a paper soaked in transfer buffer while the surface of the membrane remained dry. Then 2 µL of each 12 fraction samples, straight from the sucrose gradient, one from each diet group were applied to the dry surface of the membrane. The membranes were blocked in blocking buffer containing 5% non-fat milk, for three hours at 4°C and then incubated with a HRP conjugated ganglioside GM 1-antibody in blot buffer containing 1.5% non-fat milk. The antibody (cholera toxin subunit B, C-3741, Sigma-Aldrich) was diluted 1:5000. The PVDF membrane strips were then washed with blot buffer and processed for chemiluminescence digital imaging analysis.

3.5 Image analysis

The immunoblotted proteins and ganglioside GM1(monosialotetrahexosyl ganglioside) on the PVDF membranes were visualized with the ECL+, chemiluminescence western blot detecting system (GE Healthcare) and pictures were taken with a CCD camera. CCD camera captured the light that was emitted from the western- and dot blots. The images were further analyzed and the proteins or lipid quantified, through light emission, with densitometry using ImageQuant 5.2 software.

3.6 Lipid extraction

Lipids were extracted with the method of Folch (*88*). About one mL each of fraction 4, 5 and 6 from the sucrose gradient (about 1 mg protein in total), which were considered to be the lipid raft fractions, were combined for lipid extraction. Equal samples of fractions 9, 10, and 11 (3 mg protein in total), were extracted as the dissolved lipid fraction. Both samples were extracted in $CHCl_3/CH_3OH$ /H_2O, (2:1:0.8).

5 mg butylated hydroxytoluene (BHT) were used per 100 mL extraction medium, and 35 µL diheptadecanoyl PC standard (2 mg/mL) was added as an internal standard to each sample before extraction to enable measurement of the loss of lipids during the extraction and separation procedure.

The extraction medium with the lipid raft or dissolved lipid samples were shaken vigorously and incubated for 1 hour at room temperature and centrifuged for 10 minutes at 1000xg to make the solution biphasic. Both the upper and lower phases were transferred to a new pyrex tube and the solid protein precipitate was discarded.

Methanol and 0.9% NaCl in water were added to give the ratios, $CHCl_3/CH_3OH/H_2O$, 1:1:0.8, the samples were shaken vigorously and centrifuged as before. The lower phase, $CHCl_3$, which included the total lipids was transferred to a new pyrex tube. $CHCl_3$ was evaporated under a stream of nitrogen (N_2) and dissolved in 80 µL of chloroform with BHT.

Samples were separated on a 20x20 TLC (thin layer chromatography) plate (Adsorbosil H, soft layer, Alltech) which was kept overnight in a 50:50 chloroform/ methanol (v/v) solution in a glass container. Before the separation, the plate was incubated for 40 minutes at 110°C and put in a glass container with drying crystals until they reached room temperature. Lipid raft and dissolved lipid samples were then put on the TLC plate and phospholipids separated from neutral lipids by using a mobilic phase of petroleum benzine/diethyl ether/acetic acid (glacial) (80:20:1) (v/v). The total phospholipids band was scraped off the TLC plate into a pyrex tube with a screw cap. The phospholipids FAs were methylated with 1 mL of 14 % boron trifluoride in methanol, and incubated at 100°C for 30 minutes. Then 15 µL of C21 methylated FA standard (2 mg/mL) was added to each sample. The fatty acid methyl esters (FAME) were then extracted three times with 1.5 mL of hexane. The combined hexane phases from each sample were evaporated under a stream of N_2 and dissolved in 80 µL of iso-octane. The analysis of FAME was done by 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector and a Varian CP-WAX 52CB capillary column (25 m x 0.32 mm i.d. x 0.2 µm film thickness) CP 7743 with H2 as a carrier gas. The oven was programmed to provide an initial temperature of 90°C for 2 min, then was increased to 165°C by 30°C/min and to 225°C by 3°C/min, and finally held isothermal for 4 min. The FAME peaks were

identified and calibrated against commercial standards (Sigma Chemical Co., St Louis, MO, USA; Nu-Check- Prep, Elysian, MN, USA). The results are expressed as proportion (%) of total FA in plasma PL.

3.7 Cholesterol measurements

The cholesterol amount was measured with a kit from Randox Laboratories LTD. A Randox R1 cholesterol calibration solution (197mg/dL) was used to make standard solutions of six different concentrations. Samples from one fish oil fed rat and one control rat were measured in duplicate as well as the cholesterol standard solutions in triplicate on a 96 well microplate from Nunc in a microplate reader from ThermoMax.

Due to shortage of sucrose fraction sample, only two measurements were done for each point instead of three. For the microplate reader, 50 µL of sample and 200 µL of assay medium were applied to each well. The microplate was incubated at 37°C for 20 minutes and absorbance was then measured at 490 nm at room temperature. Data was calculated with a Softmax Pro program (Molecular Devices).

3.8 Statistical analysis

Results were expressed as the average with ± standard deviation (SD) or standard error of the mean (SEM). Paired t-test was used for light emission analysis from Western blots with $p < 0.05$ being considered as significant. Unpaired t-test was used for analysis of FA levels and $p < 0.02$ was considered significant to exclude false positives.

3.9 Overview

The aim of this project was to study the effect of dietary n-3 LC-PUFA on the lipid composition and the location of adrenergic receptors in lipid rafts in rat heart.
Lipid rafts were isolated on a sucrose gradient from hearts of adult rats that had been fed a controlled diet enriched with fish oil (n-3 LC-PUFA) or safflower oil (n-6 PUFA) (control). Proteins and the GM1 ganglioside were analyzed in 12 fractions of the sucrose gradient with western blot and dot blot techniques, respectively. Cholesterol was measured with a spectrophotometric assay kit. Phospholipids were isolated from lipid rafts and their FA composition was analyzed with a gas chromatograph (Figure 9).

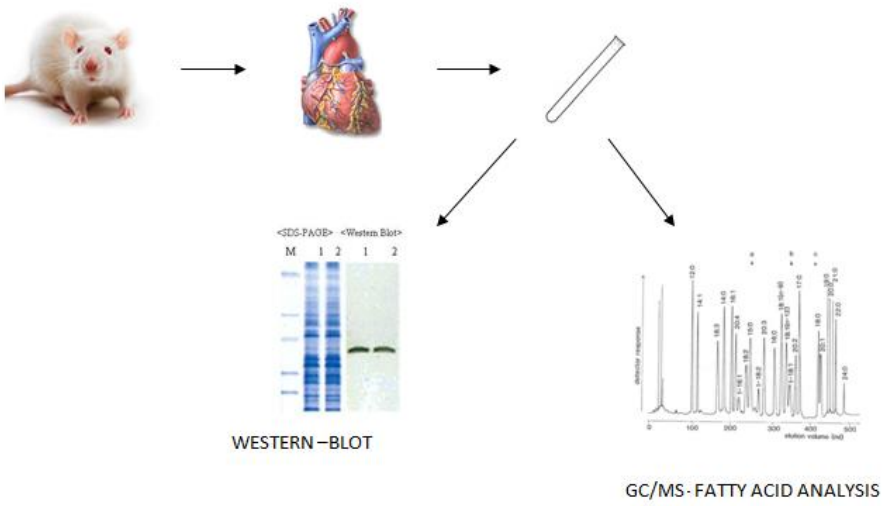

Figure 9 – Schematic flow of the experiment.

4 Results

4.1 Animals

The food intake of two rats housed together in each cage was measured during four weeks of the feeding period. Figure 10 shows the average of feed consumed/day/animal in the two diet groups for the first three weeks. There was no significant difference in feed consumption between the diet groups. The average weight of the control rats at the start of the feeding period was 246.7 ± 4.6 g (mean ± SD) and 448.3 ± 31.7 g after the four weeks of feeding. The weight and weight gain of the FO fed rats was similar.

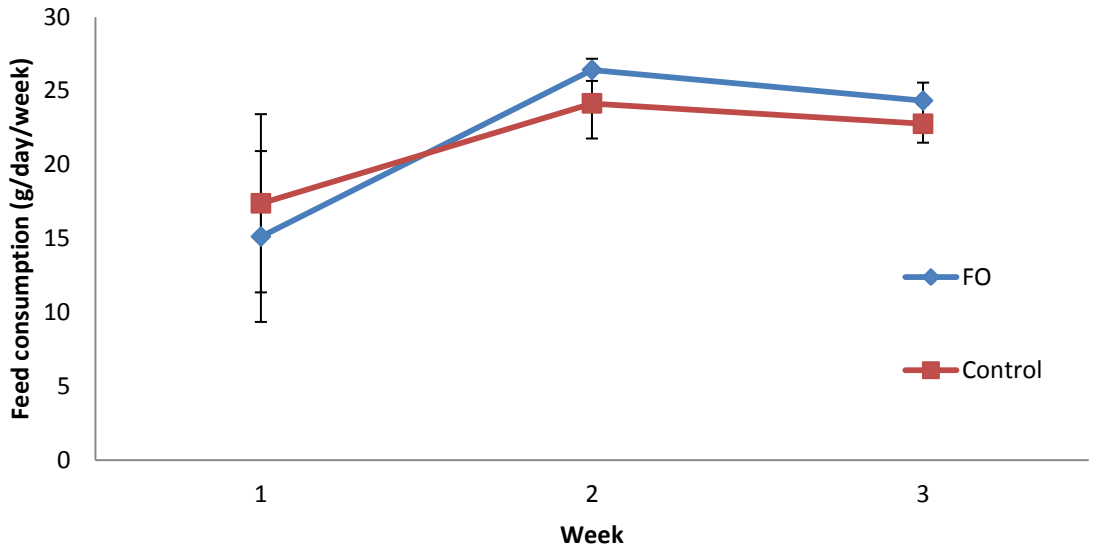

Figure 10 – Average daily amount of rat feed consumed (g/day) by each animal in the fish oil group (FO) and the control group for each week. Values are expressed as mean ± SD, n=6.

4.2 Isolation of lipid rafts

In order to demonstrate successful isolation of lipid rafts, total protein and cholesterol levels were measured and presence or absence of GM1 and two proteins considered to be lipid raft markers, caveolin-3 and flotillin-1, were established and quantified in all 12 fractions of the sucrose gradients used for isolation of lipid rafts.

4.2.1 Lipid raft markers measurements

The protein concentration (mg/mL) in the 12 sucrose gradient fractions from the two diet groups is shown in Figure 11. A closer view of the protein measurements of fractions 1-7 is shown in Figure 12. As shown in Figure 11, the bulk of proteins was in the lower half of the sucrose gradient, but in Figure 12 a small increase in total protein concentration can be seen in fractions 4, 5 and 6, probably indicating an isolation of lipid rafts in those fractions. There was no significant difference in total protein concentration between the diet groups.

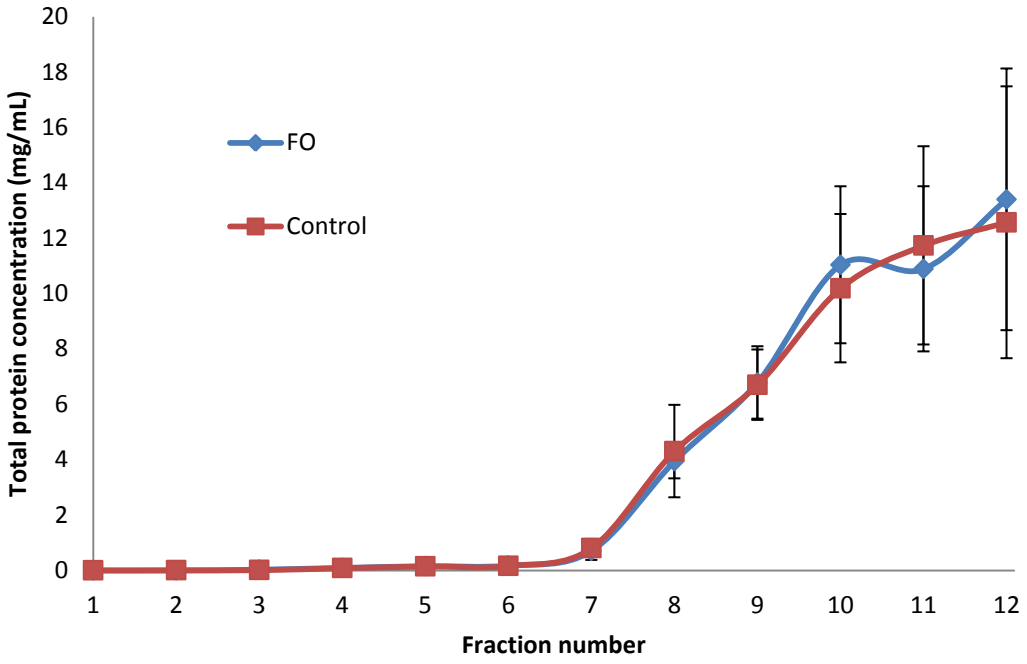

Figure 11 – Total protein concentration (mg/mL) in sucrose gradient fractions 1-12 from the fish oil group (FO) and the control group. Values are expressed as mean ± SD, n=6.

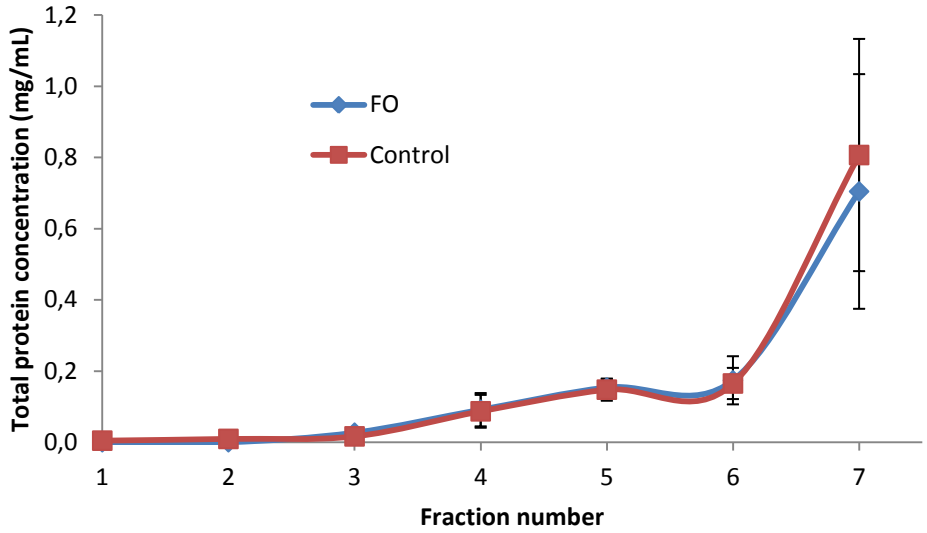

Figure 12 - Total protein concentration (mg/mL) in sucrose gradient fractions 1-7 from the fish oil group (FO) and the control group. Values are expressed as mean ± SD, n=6.

4.2.2 Cholesterol

The cholesterol amount (mg/mg protein) in the 12 sucrose gradient fractions from the two diet groups is shown Figure 13. The highest cholesterol/protein amount was observed in fractions 4-6, indicating an isolation of lipid rafts. There was no significant difference in cholesterol amount in the sucrose gradient fractions between the diet groups.

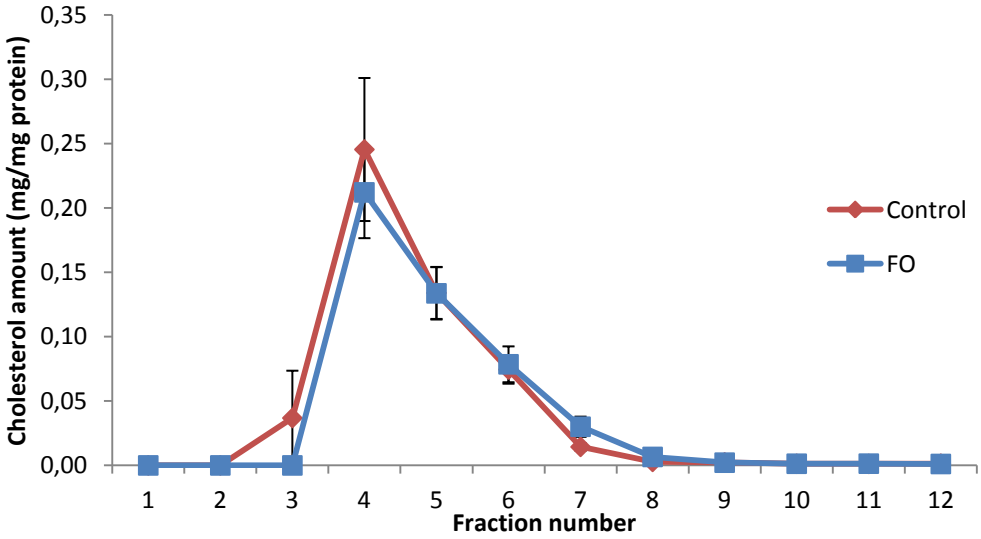

Figure 13 – Cholesterol amount (mg/mg protein) in sucrose gradient fractions 1-12 from the fish oil group (FO) and the control group. Values are expressed as mean ± SD, n=6.

4.2.3 Ganglioside GM1

The presence of GM1 is an indicator of lipid raft isolation as it is known to be localized in lipid rafts. Dot blot was used to determine whether GM1 was present in any of the sucrose gradient fractions 1 to 12. Figure 14 shows dot blots for GM1 in two batches of analyzes (A and B). Each batch contained of samples from both diet groups and they were analyzed in parallel.

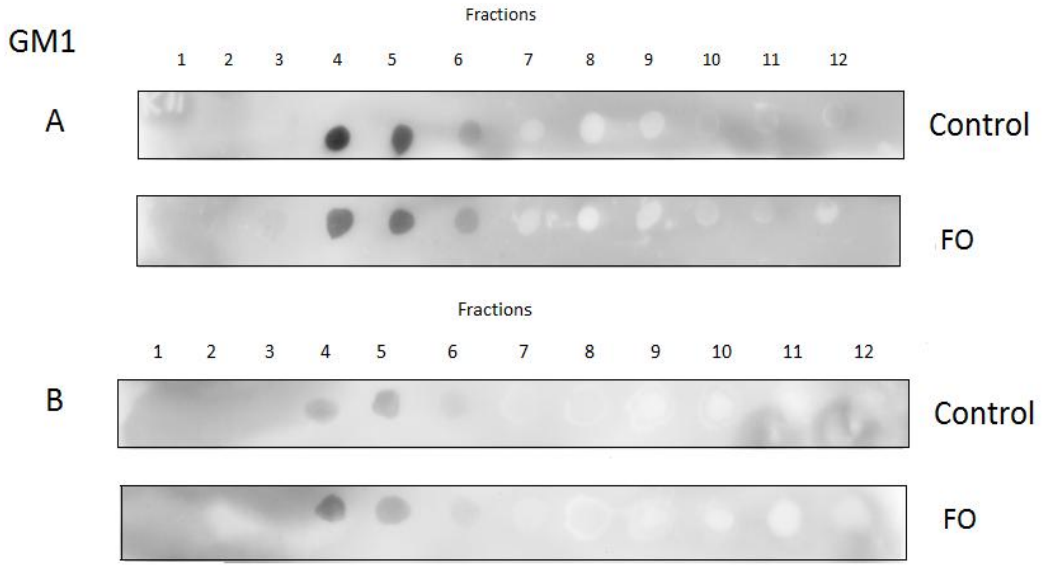

Figure 14 – Dot blot light emission of GM1 in 12 sucrose gradient fractions, from two different batches of lipid raft isolation (A and B) analyzed in parallel from the fish oil group (FO) and the control group.

As shown in Figure 14 GM1 was detected exclusively in fractions 4, 5, and 6 from the sucrose gradient, which indicated a successful isolation of lipid rafts in those fractions. Statistical analysis of light emission from the dot blots, did not show a significant difference in quantity of GM1 in lipid rafts between the diet groups.

4.2.4 Caveolin-3 and flotillin-1

Caveolin-3

Western blotting was used to visualize the caveolin-3 protein in fractions 1 to 12 from the sucrose gradient. Figure 15 shows light emission from two batches of caveolin-3 Western blot analysis from the fish oil group (FO) and the control group. An equal amount of protein was applied to each lane of the gels for both diet groups.

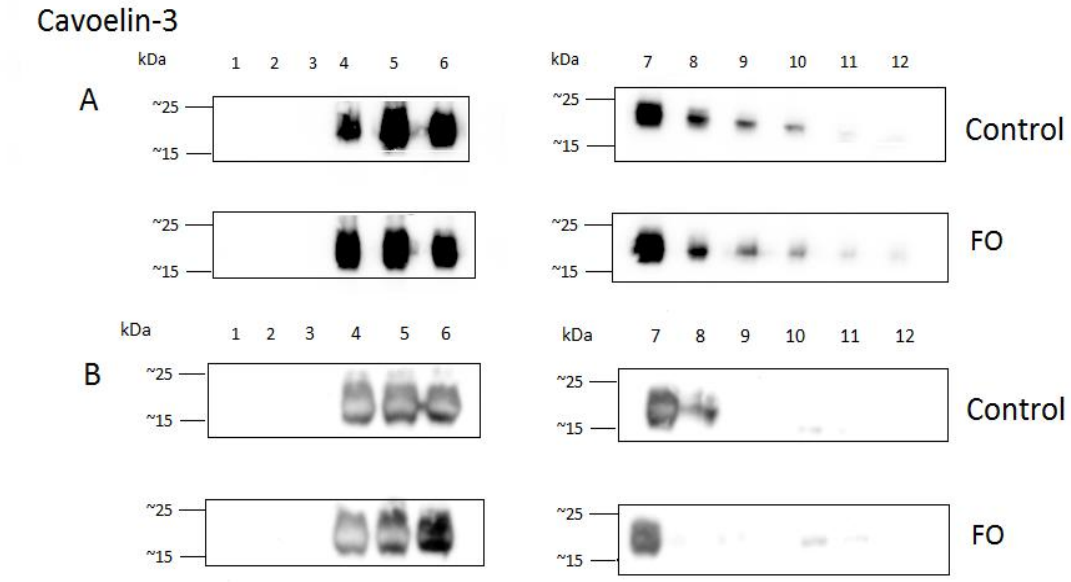

Figure 15 – Western blot light emission from caveolin-3 in 12 sucrose gradient fractions, from two different batches of lipid raft isolation (A and B) analyzed in parallel from the fish oil group (FO) and the control group.

The blots shown in Figure 15 indicate that caveolin-3 was present mainly in sucrose gradient fractions 4-7, and that no difference was evident between the two diet groups regarding the amounts of caveolin-3 in those fractions. To analyze this further the average light emission was calculated for caveolin-3 Western blots (Figure 16), in all sucrose gradient fractions from both the diet groups.

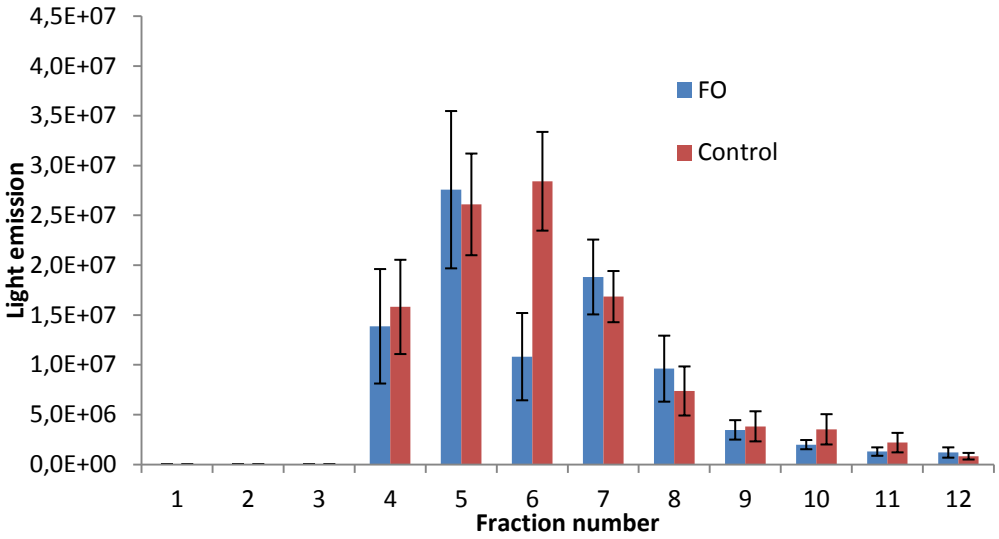

Figure 16 – Caveolin-3 Western blot light emission in 12 sucrose gradient fractions from the fish oil group (FO) and the control group. Values are expressed as mean ± SEM, n=4.

The caveolin-3 light emission ratio FO/control within each batch was calculated and the average FO/control ratios in the 12 sucrose gradient fractions are shown in Figure 17. The average ratio for each batch in caveolin-3 proteins indicates that there is neither an increase nor a decrease in caveolin-3 quantity in any of the fractions.

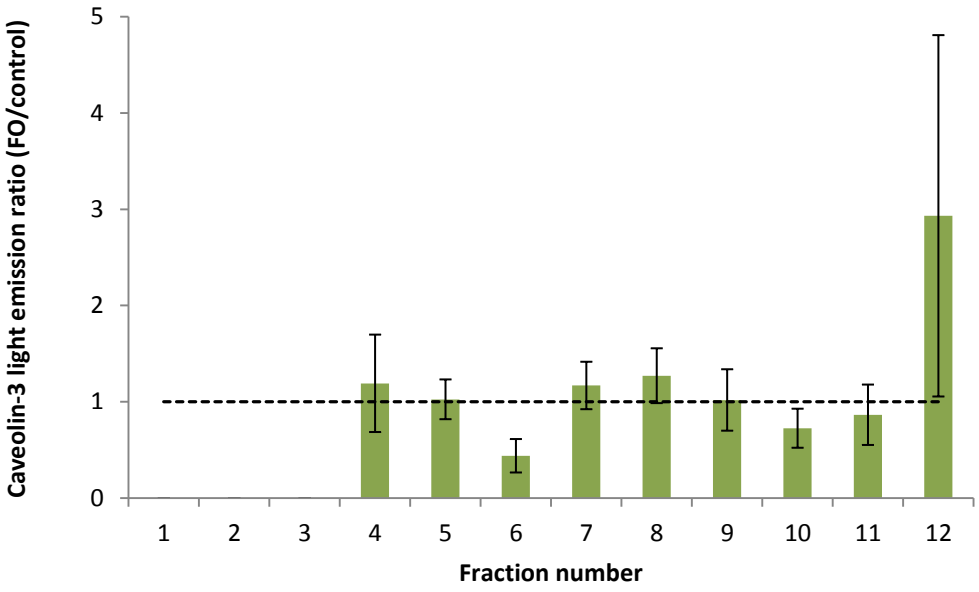

Figure 17 – Caveolin-3 light emission ratio (FO/control) between the diet groups from Western blots compared within each batch. Values are expressed as mean ± SEM, n=4.

A paired t-test was used for statistical analysis to determine whether there was a significant difference in caveolin-3 light emission between diet groups. The calculated p-values are shown in Table 3. There was no significant difference between caveolin-3 protein concentration between the FO group and the control group, neither in the lipid raft fractions (number 4, 5, and 6), nor in other fractions.

Table 3 – Paired t-test for light emission measurements from fractions 1 to 12 for caveolin-3 Western blots pictures, n=4.

	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12
Paired t-test p-value	0.85	0.81	0.09	0.62	0.23	0.80	0.35	0.32	0.61

Flotillin-1

Western blotting was used to visualize the flotillin-1 protein in fractions 1 to 12 from the sucrose gradient. Figure 18 shows light emission from two batches of flotillin-1 Western blot analysis. Equal protein amount was applied to each lane of the gels for both diet groups.

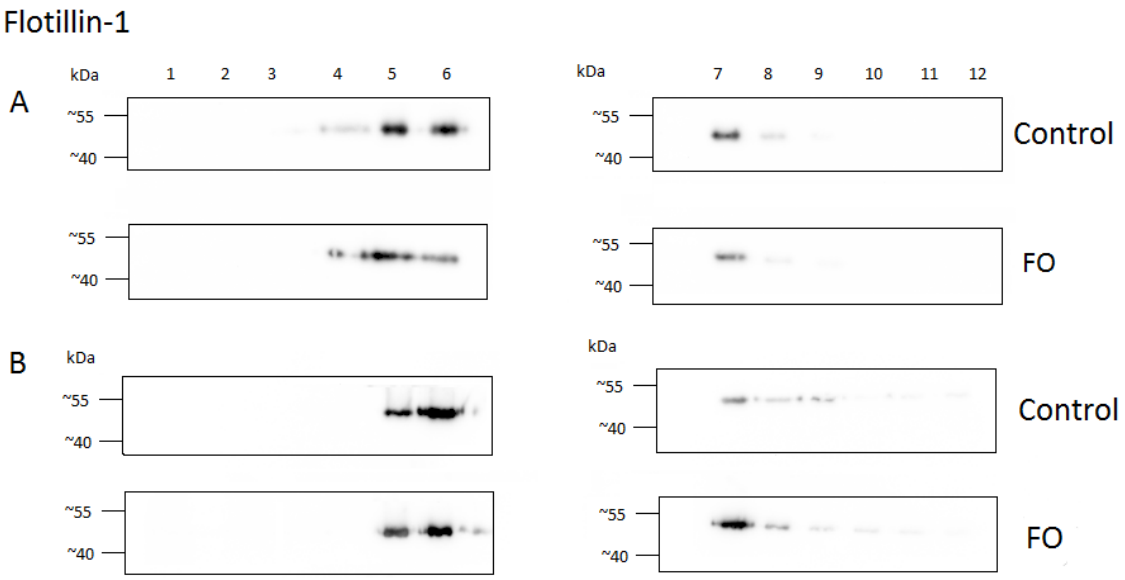

Figure 18 - Western blot light emission from flotilin-1 in 12 sucrose gradient fractions, from two different batches of lipid raft isolation (A and B) analyzed in parallel from the fish oil group (FO) and the control group.

The blots shown in Figure 18 indicated that flotillin-1 was isolated mainly in fractions 4-6, and also that no difference was evident between the two diet groups regarding the amounts of flotillin-1 in the sucrose gradient fractions. To analyze this further the average light emission was calculated for flotillin-1 Western blots (Figure 19) in all sucrose gradient fractions from both diet groups.

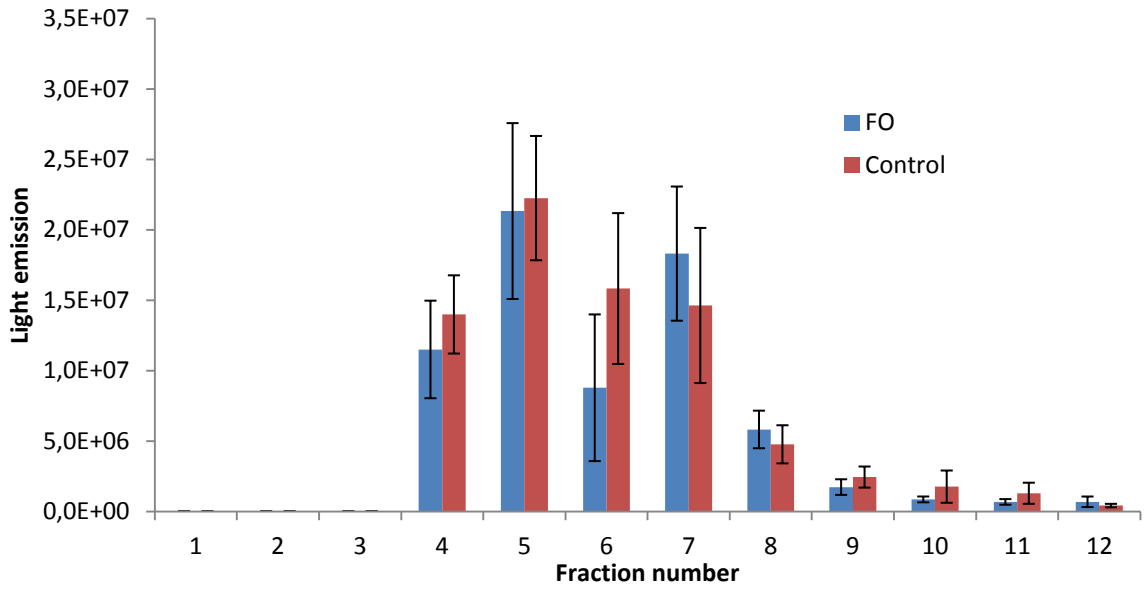

Figure 19 – Flotillin-1 Western blot light emission in 12 sucrose gradient fractions from the fish oil group (FO) and the control group. Values are expressed as mean ± SEM, n=4.

The flotillin-1 light emission ratio (FO/control) within each batch was calculated and the average FO/control ratios in the 12 sucrose gradient fractions are shown in Figure 20. The average ratio for each batch, in flotillin-1 proteins indicated that there is neither an increase nor a decrease in flotillin-1 quantity in any of the fractions.

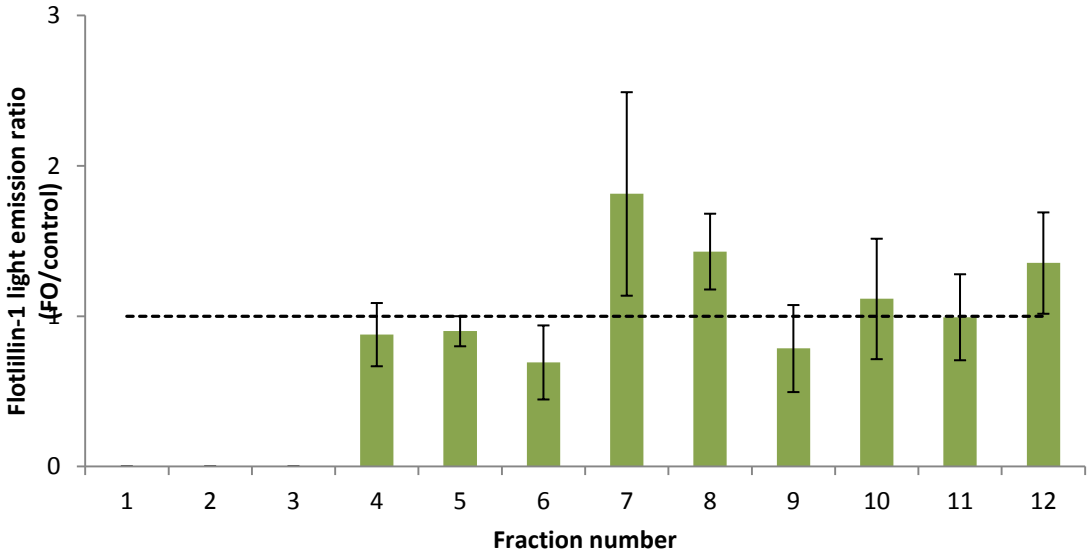

Figure 20 – Flotillin-1 light emission ratio (FO/control) between the diet groups from Western blots compared within each batch. Values are expressed as mean ± SEM, n=4.

A paired t-test was used for statistical analysis to determine whether there was a significant difference in flotillin-1 light emission between diet groups The calculated p-values are shown in Table 4. There was no significant difference between flotillin-1 protein amounts between the FO group and the control group, neither in the lipid raft fractions (number 4, 5, and 6) nor other fractions.

Table 4 – Paired t-test for light emission measurements from fractions 1 to 12 for flotillin-1 Western blots pictures, n=4.

	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12
Paired t-test p-value	0.60	0.39	0.30	0.32	0.19	0.51	0.79	0.98	0.37

4.3 Fatty acid composition of lipid rafts and dissolved fractions

Fractions 4, 5, and 6 were considered as lipid rafts membrane fractions as supported by the results of the measurements of lipid raft markers, caveolin-3, flotillin-1, ganglioside GM1 and cholesterol. Fractions 9, 10, and 11 were considered to contain dissolved membrane lipids. Table 5 shows the FA composition of total phospholipids in lipid rafts and the dissolved membrane fractions in both diet groups. Figures 21 and 22 show the percentage of saturated, monounsaturated, n-6 PUFA, and n-3 PUFA in lipid rafts and dissolved membrane fraction phospholipids in both diet groups.

Table 5 – FA (% of total fatty acids) composition of total phospholipids in lipid rafts and dissolved membrane fractions from rat heart of control and fish oil (FO) fed rats.
Mean ± SD.

	Lipid rafts		Dissolved membranes	
	Control	FO	Control	FO
Fatty Acids	n=4	n=4	n=3	n=4
C14	0.29±0.14	0.49±0.23	1.13±0.72	n.d.
C16	12.07±1.14	14.26±1.78	8.94±0.53	11.14±1.33 *
C16:1n7	0.22±0.03	0.36±0.05 *	n.d.	n.d.
C18:0	26.54±0.51	25.33±1.62	25.53±1.75	27.03±2.54
C18:1n9	4.81±0.15	5.38±0.56	4.24±0.21	4.66±0.66
C18:1n7	2.41±0.10	2.63±0.15	2.36±0.12	3.02±0.09 *
C18:2n6	8.37±1.06	6.83±2.26	12.37±1.59	9.87±3,21
C20:0	0.68±0.15	0.72±0.19	n.d.	n.d.
C20:4n6	24.37±1.06	13.42±1.54 *	25.03±1.86	16.42±0.55 *
C20:5n3	n.d.	2.04±.041	n.d.	1.54±0.33
C22:0	0.66±0.12	0.70±0.17	n.d.	n.d.
C22:4n6	1.58±0.07	0.18±0.03 *	0.88±0.20	n.d
C22:5n6	0.95±0.33	0.25±0.01	0.90±0.47	n.d.
C22:5n3	1.77±0.21	3.93±0.52 *	1.81±0.21	4.02±0,47 *
C22:6n3	9.81±1.00	16.66±1.83 *	10.00±1.58	20.74±2,38 *
C24:0	0.50 ±0.07	0.50±0.23	n.d.	n.d.
Other FA:	5.41±0.62	6.33±1.43	7.19±7.68	n.d.
Saturated	40.58±1.13	42.00±3.81	35.22±2.48	38.16±3.82
Monounsat.	7.38±0.15	8.36±0.68	6.60±0.29	7.68±0.72 *
n-6 PUFA	35.28±1.67	20.68±3.76 *	39.18±3.8	26.29±3.72
n-3 LC-PUFA	11.58±0.82	22.63±1.36 *	11.80±1.55	25.92±1.95 *

** P<0.02 compared to control animals.*
n.d. (not detectable)

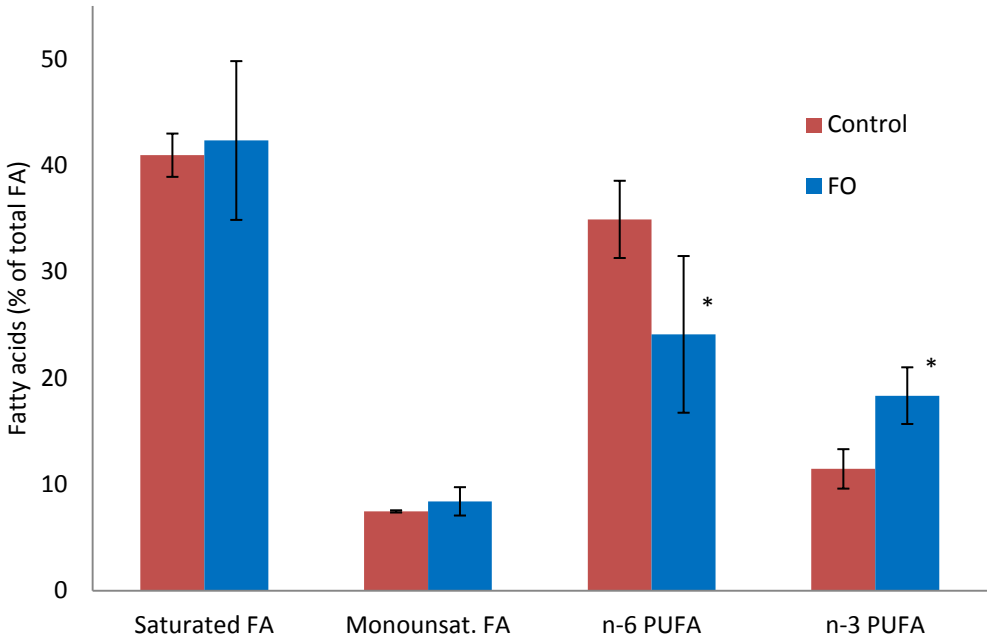

Figure 21 – Saturated FA, monounsaturated FA, n-6 PUFA and n-3 PUFA (% of total FA) in phospholipids of lipid rafts in fish oil (FO) and control groups. Values are expressed as mean ± SD, n=4. * P < 0.02 compared to control group

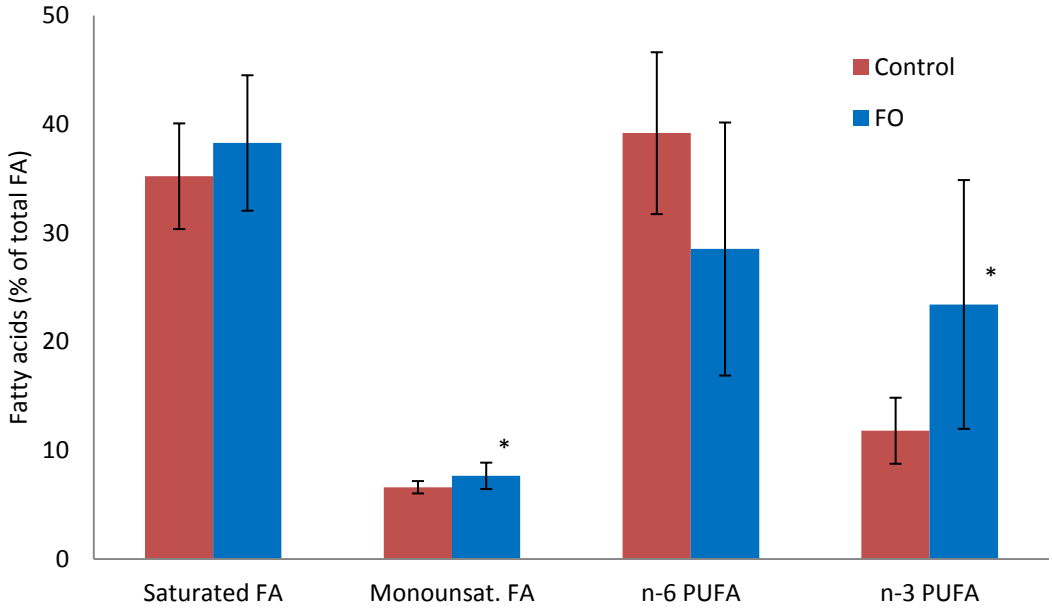

Figure 22 - Saturated FA, monounsaturated FA, n-6 PUFA and n-3 PUFA (% of total FA) in phospholipids of dissolved membrane fraction in fish oil (FO), n=4, and control groups, n=3. Values are expressed as mean ± SD, n=4. * P < 0.02 compared to control group

No significant difference between the diet groups was found for saturated FA levels in phospholipids of neither lipid rafts nor dissolved membrane fraction when using a 98% confidence limit proportions (% of total FA) (Figure 21 and 22). The monounsaturated FAs levels were significantly different between the phospholipids of the dissolved membrane fractions, but no difference was found in the levels of lipid rafts. There was a significant difference in the levels of monounsaturated FAs in the phospholipids of the dissolved fractions between the diet groups (Figure 22). Levels of both n-6 PUFA and n-3 PUFA were significantly different in the phospholipids of lipid rafts between the diet groups, as the fish oil diet caused a replacement of n-6 PUFA by n-3 PUFA(Table 5). Similarly, n-3 PUFA levels were significantly higher in phospholipids in the dissolved fraction whereas the decrease in n-6 PUFA levels was not significant (Figure 22). However, AA level were significantly decreased in the FO group compared to control group in both lipid rafts and dissolved membranes.

4.4 Adrenergic receptors

Western blot was used to visualize the α_1-and β_1-adrenergic receptor proteins from the sucrose gradient fractions 1 to 12. For every batch, samples were analyzed from both diet groups, isolated at the same time. Light emission from α_1- and β_1-adrenergic receptor proteins can be seen in Figure 23. Equal amount of protein was applied to each lane of the gels for both diet groups.

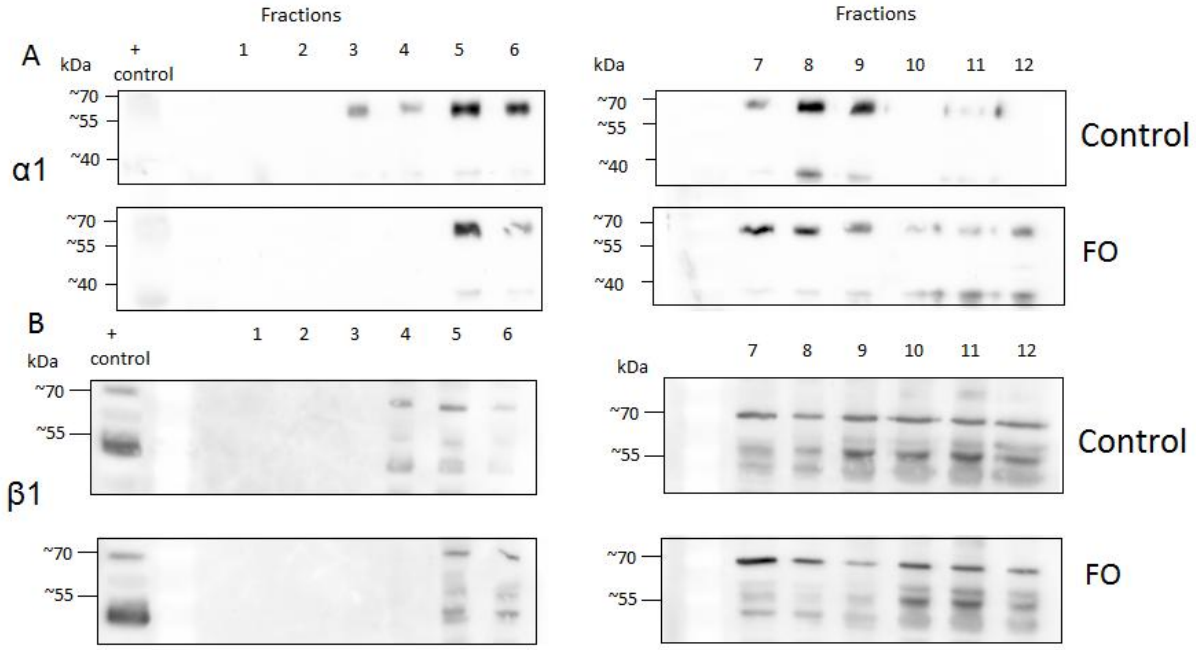

Figure 23 - Western blot light emission from α_1-(A) and β_1-adrenergic receptors (B) in 12 sucrose gradient fractions, each from one batch of lipid raft isolation analyzed in parallel from the fish oil group (FO) and the control group.

The mean light emission from each fraction in both diet groups was calculated and the average light emission amount for each fraction of the sucrose gradient is shown in Figure 23 for α_1-adrenergirc receptor and Figure 24 for β_1-adrenergirc receptor.

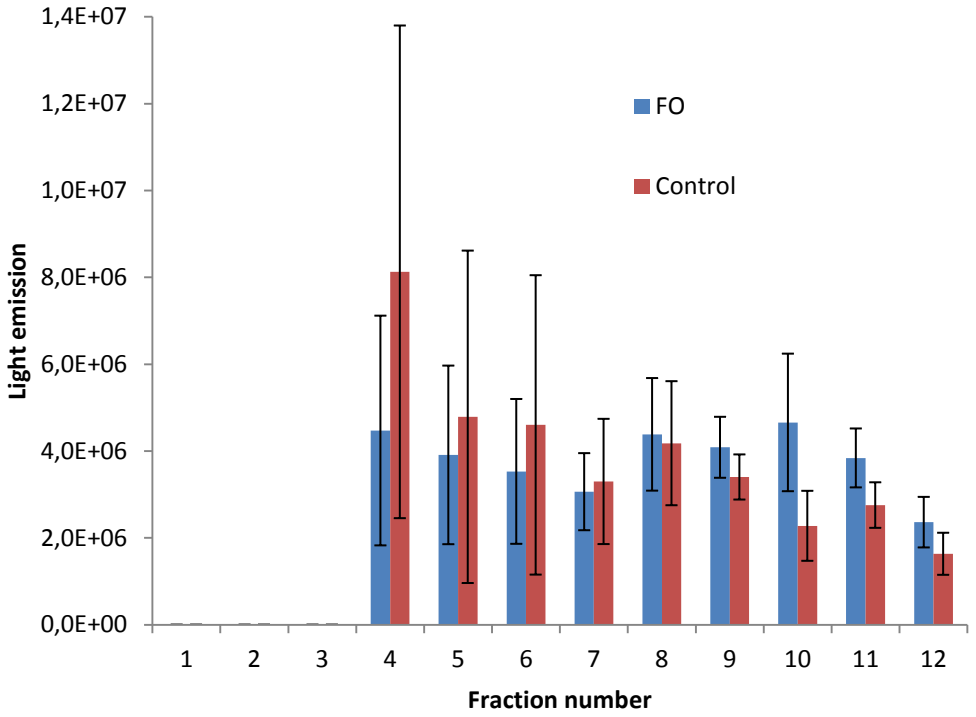

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Figure 24 – α_1-adrenergic receptor Western blot light emission in 12 sucrose gradient fractions from the fish oil group (FO) and the control group. Values are expressed as mean ± SEM, n=4.

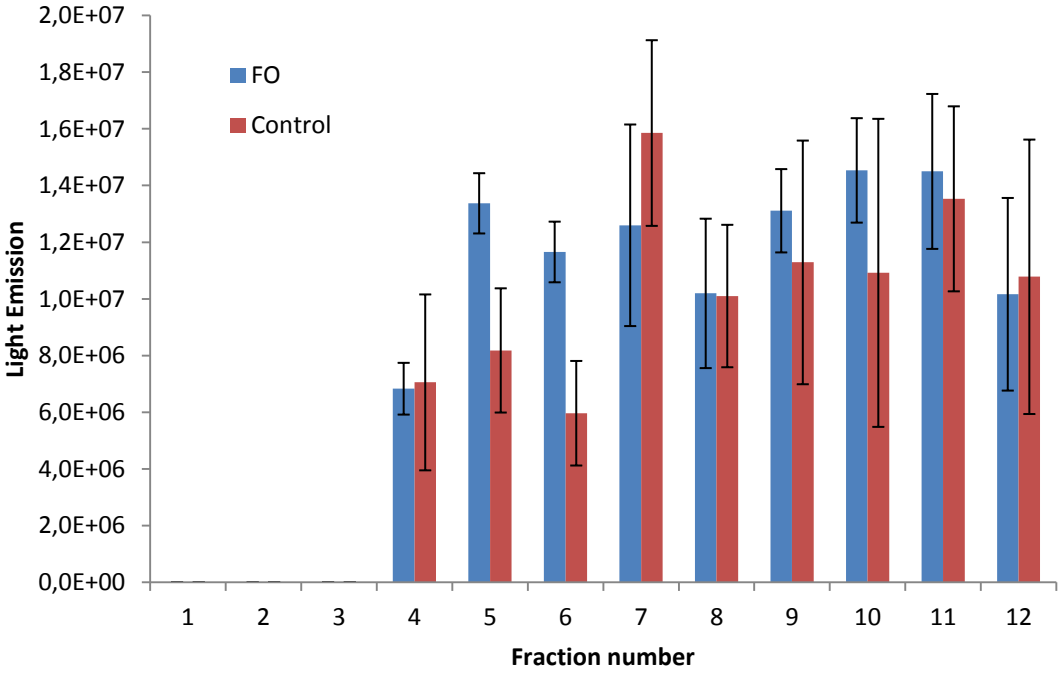

Figure 25 - β_1-adrenergic receptor Western blot light emission in 12 sucrose gradient fractions from the fish oil group (FO) and the control group. Values are expressed as mean ± SEM, n=3.

The α_1- and β_1-adrenergirc receptor light emission ratio FO/control within each batch was calculated and the average FO/control ratios in the 12 sucrose gradient fractions are shown in Figure 25 and 26, respectively. The average ratio for each batch, in flotillin-1 proteins indicates that there is neither an increase nor a decrease in flotillin-1 quantity in any of the fractions.

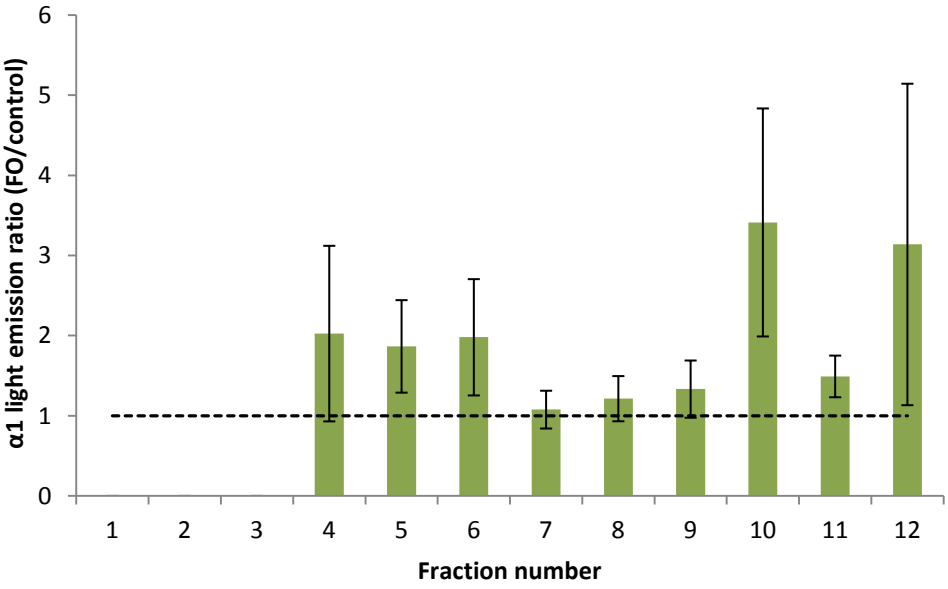

Figure 26 – α_1-adrenergic receptor light emission ratio (FO/control) between the diet groups from Western blots compared within each batch. Values are expressed as mean ± SEM, n=4.

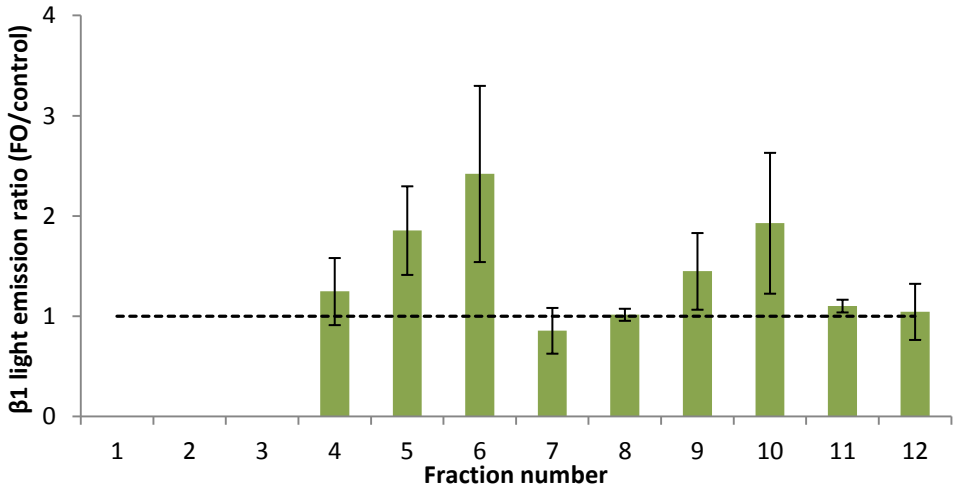

Figure 27 – β_1-adrenergic receptor light emission ratio (FO/control) between the diet groups from Western blots compared within each batch. Values are expressed as mean ± SEM, n=3.

A paired t-test was used for statistical analysis to determine whether there was a significant difference in the α_1- and β_1-adrenergirc receptor emission between diet groups and the calculated p-values are shown in Table 6 and 7, respectively. No significant difference was found using a p-value lower than 0.05 for α_1-adrenergic receptor, but a trend was observed in fraction 5 for the β_1-adrenergic receptors.

Table 6 – Paired t-test for light emission measurements from fractions 1 to 12 for α_1-adrenergic receptor for Western blot pictures (n=4).

	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12
Paired t-test p-value	0.65	0.68	0.80	0.78	0.81	0.54	0.24	0.14	0.53

Table 7 – Paired t-test for light emission measurements from fractions 1 to 12 for β_1-adrenergic receptor for Western blot pictures (n=3).

	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12
Paired t-test p-value	0.93	0.054	0.12	0.54	0.88	0.70	0.46	0.26	0.86

5 Discussion

The objective of this project was to isolate lipid rafts from rat hearts and study the effect of dietary n-3 LC-PUFA on the fatty acid composition and location of proteins in the lipid rafts. As expected, the diet containing n-3 LC-PUFA caused a decrease in the n-6 PUFA levels while n-3 LC-PUFA levels increased significantly in phospholipids of both lipid raft and dissolved fractions. In contrast to what has been found by others in various tissues (*16-18*) the present study showed that a diet containing n-3 LC-PUFA did not cause any increase or decrease in protein concentration (caveolin-3 and flotillin-1) in lipid rafts nor did it reduce the proportion of membrane lipids the lipid raft. There was no significant difference in the location of caveolin-3, flotillin-1, or GM1 lipid markers between the diet groups. Furthermore, there was no significant difference between diet groups in quantity or localization of α_1- and β_1-adrenergic receptors in the rat hearts from lipid rafts. However, a trend was observed towards a higher concentration of β_1-adrenergic receptors in lipid rafts between diet groups.

5.1 Isolation of lipid rafts

A detergent free method described by Song and Li (*84*) and modified by Cavalli (*85*) for isolation of lipid rafts from a whole rat heart was used in this study. Previously, an attempt had been made to use another method (*89*) in which a detergent (1% Triton X-100) was used to solubilize membranes other than lipid rafts (data not shown). This procedure gave practically no isolation of lipid rafts compared to the detergent free method in our hands. Head et al. (*25*) reported a similar experience regarding the use of detergent for the isolation of lipid rafts from isolated rat cardiomyocytes. In this study, detergent free method used to isolate lipid rafts from rat heart was demonstrated in several applications.

High cholesterol content indicates that lipid rafts were present. Figure 13 demonstrates that fractions 4, 5, and 6 from the sucrose gradient exhibited the highest amount of cholesterol/protein (mg/mg). The cholesterol content in lipid rafts in our study was 80% higher than Ma et al. found in caveolae in a mouse colon (*82*), but in this study and also in the study of Schley et al. (*16*) on breast cancer cells, incorporation and n-3 LC-PUFA into

lipid rafts decreased the cholesterol content by 40-50%. Conversely the study of Fan et al. (*15*), on mouse splenic T-cells, showed that a 4% fish oil diet did not alter cholesterol content significantly in lipid rafts which was in agreement with our results.
Dot blot results showed that GM1 segregated into fractions 4, 5, and 6 from the sucrose gradient.
The proteins caveolin-3 (*27*, *90*) and flotillin-1 (*36*, *37*) are also considered to be lipid raft markers. Western blot was used to detect caveolin-3 and flotillin-1 proteins in the sucrose gradient fractions 1 to 12. Western blot results showed isolation of caveolin-3 and flotillin-1 proteins segregated into fractions 4-6. These results show that isolation of the lipid rafts was achieved in fractions 4-6 and, therefore, those fractions were defined as the lipid raft fractions. Although caveolin-3 and flotillin-1 were both found as bands in fraction 7 as shown on the Western blots, we decided not to include fraction 7 in our lipid raft definition, because of the risk of protein contamination from the dissolved membrane section.

5.2 Fatty acid composition in lipid rafts and dissolved membrane fractions

Lipid extraction was performed with the Folch et al. method (*88*), which was considered more suitable than the also widely used Bligh and Dyer method (*91*) because of high protein amount in samples. Based on lipid raft marker measurements, fractions 4, 5, and 6 were identified as containing the lipid raft and fractions 9, 10, and 11 containing the dissolved membrane lipids. Fraction 8 was considered too close to the lipid raft section and fraction 12 was found to contain too much protein. Therefore, these fractions were excluded in the comparison. It was discovered during the fatty acid analysis that two pairs of samples did not qualify for further analysis. Therefore, they were taken out of any comparison measurements such as in the Western blot and dot blot measurements.
The results of fatty acids measurements showed a statistically significant difference (Table 5) between diet groups in phospholipids n-6 LC-PUFA and n-3 LC-PUFA content, both in the lipid raft and the dissolved membrane fractions. The n-3 LC-PUFA diet caused a statistically significant decrease in n-6 PUFA levels in phospholipids in lipid rafts. It also caused an increase in n-3 PUFA levels in phospholipids in both lipid rafts and dissolved

membrane fractions. In the dissolved membrane fraction there was also a statistically significant increase in monounsaturated fatty acids of phospholipids. These results are in agreement with the results of Fan et al. (*17*). They isolated T-cells from spleen of mice that had been fed n-3 LC-PUFA enriched diet, containing 4 % DHA of the total FA weight for 2 weeks, and their results also indicated that n-3 LC-PUFA from diet were incorporated into lipid rafts and dissolved membrane phospholipids. Schley et al. (*16*) also showed that EPA and DHA were incorporated into lipid rafts in MDA-MB-231 human breast cancer cells after treatment with culture medium enriched with EPA and DHA.

5.3 Protein localization in lipid rafts.

5.3.1 Lipid raft markers

Caveolin-3 and flotillin-1 were segregated mostly into fractions 4-7 and no difference could be observed in the localization of the proteins between the diet groups. There was no evidence of a significant increase or a decrease of either marker proteins between the groups but a borderline significant trend could be observed in fraction 6 for higher amount of caveolin-3 in the control group than in the FO group. It is noteworthy also that the flotillin-1 Western blot light emission FO/control ratio is below one in all the lipid raft fractions, 4, 5 and 6 although the difference was not significant. This deserves further investigation.

A decrease in sphingomyelin content by including 4% fish oil in diet was shown in mouse splenic T-cells, suggesting that lipid raft amount had decreased (*17*). In a study on MDA-MB-231 human breast cancer cells, enrichment of culture medium with EPA and DHA decreased raft cholesterol, sphingomyelin, and diacylglycerol levels (*16*). Those authors did not measure lipid raft protein markers. Ma et al. (*82*) showed that by including 4% fish oil in diet of mice, lipid raft marker proteins and cholesterol levels decreased markedly in mouse colon. Our results showed that there was no statistically significant difference in the quantity of caveolin-3 and flotillin-1 or cholesterol levels between the diet groups. That could be attributed to the relatively low fish oil content of 28 g menhaden oil/ kg diet in the rat feed in our study and different cell types and methods used by those authors.

5.3.2 α_1-adrenergic receptor

Results show that α_1-adrenergic receptors were localized in lipid rafts, but a considerable amount was also found in fractions 7, 8, and 9. There was no difference in the localization of the α_1-adrenergic receptors between the diet groups. This is also shown in the average light emission for each fraction and when shown as α_1-adrenergic receptor light emission ratios between the fish oil group and the control group. No statistically significant difference was found in the quantity of α_1-adrenergic receptors between the diet groups (Table 6). However, a noticeable trend was observed, indicating that there were more α_1-adrenergic receptors in the lipid raft fractions from hearts of fish oil fed rats than in the control rats. The mean ratio FO/control was above one in all three lipid raft fractions. Further studies are needed to support this finding. Studies from Morris et al. (*79*) showed that an α_1-adrenergic receptor signaling was localized to caveolae in neonatal rat cardiomyocytes. That is partly in agreement with our study and the findings of Lanzafame et al. (*92*) in rat heart fibroblasts, who also found the signaling system of α_1-adrenergic receptors in caveolae, although only about one third of the α_1-adrenergic receptors were localized in caveolae, and the rest outside the caveolae similar to this study. Fujita et al. (*93*) showed, however, that α_1-adrenergic receptor is mainly localized in caveolae and just a small portion outside them in the rat cardiomyocyte cell line, H9C2 cells which partly contradicts our research, but this could be due to a different cell type used. Besides, the lipid raft preparation in our study is from a mixture of all the cell types of the heart muscle, mainly fibroblasts, endothelial cells and muscle cells, although the last mentioned are the dominating type.

5.3.3 β_1-drenergic receptor

The results of this study showed that a small amount of the β_1-adrenergic receptors was localized in lipid raft fractions but they were mainly localized in the heavier fractions, number 7-12, representing dissolved membranes. Head et al. (*25*) showed similar results from a study on isolated cardiac myocytes from an adult rat heart. They showed that β_1-adrenergic receptors could be localized both in the lipid rafts and the dissolved membrane. They saw that AC 5/6 and $G\alpha_s$ was localized along with β_1-adrenergic receptors in the lipid raft. AC 5/6 was mainly localized in the lipid rafts while the $G\alpha_s$ was localized both in the

lipid rafts and the dissolved membrane.

Localization of the receptor proteins in the different sucrose gradient fractions was not statistically significantly different between the control and fish oil fed groups. The average light emission of β_1-adrenergic receptor bands from Western blots of each fraction and the β_1-adrenergic receptors FO/control light emission ratios were not significantly different between the diet groups. However, in fraction number 5 (lipid rafts) there was a trend suggesting an increase in β_1-adrenergic receptors in lipid rafts in hearts from the fish oil fed rats compared to the control. If this is the case it would agree with the results of Joerdar et al. (*58*). They showed that in cultured brain cells (astrocytes) a supplementation with DHA in the culture medium caused an increase in the number of β_1-adrenergic receptors in the lipid membrane.

In a failing heart, there are a number of molecular mechanisms that limit the β_1-adrenergic receptor function including down regulation and decreased responsiveness. The decrease in β_1-adrenergic receptor signaling limits energy expenditures in a heart that has little metabolic reserve and is thought to be an adaptive response (*55*). It has also been shown that age modifies the number of the β_1-adrenergic receptors, which decreases with age (*94*). Long term dietary n-3 LC-PUFA supplement during ageing into senescence diminished this loss of β_1-adrenergic receptors (*95*). Studies on rats have shown that downregulation of adrenergic receptors, induced by cathecolamine injections, was accompanied by a noticeable change in the fatty acid composition in phospholipids of the heart muscle, especially regarding the long chain PUFA (*96*).
It has been shown that the β_1-adrenergic receptors are downregulated in both heart failure and senescence. n-3 LC-PUFA has been demonstrated to have beneficial effects on cardiovascular diseases, including coronary artery disease, myocardial infarction and stroke. In our study we saw a trend toward an increase in β_1-adrenergic receptors in the heart in the fish oil diet group. Therefore, one could speculate that an increase of the β_1-adrenergic receptor in lipid rafts in heart muscle could be beneficial and could improve life expectancies.

Conclusion

The conclusion from this study is that there was no significant difference in the levels of lipid raft marker proteins (caveolin-3 and flotillin-1), GM1 and cholesterol between diet groups. The α_1- and β_1-adrenergic receptor did not show any significant difference in quantity and localization but an increasing trend was observed in the α_1- and β_1-adrenergic receptor in the lipid raft from the fish oil group. In our study we used a well-controlled diet which is supposed to be similar to a western diet of humans while the diets used in the comparative studies of others generally have higher n-3 LC-PUFA levels. This could play a part in explaining the discrepancies between our results and theirs.

Further studies should be conducted to validate the observed trend regarding the β_1-adrenergic receptor difference in lipid rafts between the diet groups using methods that give less variance and more statistical power. Measurements of mRNA β_1-adrenergic receptor levels expression and activity measurements would be the methods of choice to determine if the n-3 LC-PUFA has an effect on the β_1-adrenergic receptor activity.

6. References

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7. Appendix

Protein measurements

Example for sample number 1. Table A1 demonstrates protein measurements for fish oil sample number 1. The protein concentration of all the measured samples (mg/mL) are shown in table A2 and table A3.

Figure A1 shows a standard curve calculated from absorbance values (OD) in Table A1. The standard curve was then used for further calculations of protein concentration fraction 1 to 12, shown as sample (mg/mL) in Table A1.

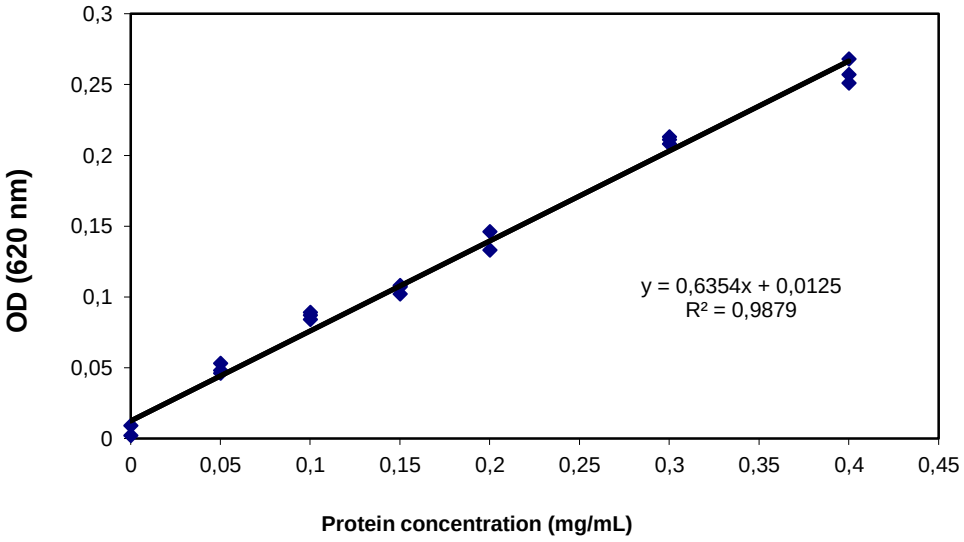

Figure A1 – Standard curve for protein measurements.

Table A1 – Example of how protein amount for sample number 1 for a fish oil rat, were calculated through measurements.

Standard (mg/mL)	OD (620 nm)	Grd smpl	OD (620 nm)	Sample (mg/mL)	Average (mg/mL)	Dilutand Factor	Corrected dilutant (mg/mL)
0	-0,011	1	0,006	-0,010	-0,008		0,00
0	0,002		0,012	-0,001			
0	0,009		0,005	-0,012			
0,05	0,046	2	0,012	-0,001	-0,007		0,00
0,05	0,048		0,009	-0,006			
0,05	0,053		0,003	-0,015			
0,1	0,084	3	0,014	0,002	0,004		0,00
0,1	0,089		0,015	0,004			
0,1	0,087		0,016	0,006			
0,15	0,102	4	0,04	0,043	0,041		0,04
0,15	0,107		0,036	0,037			
0,15	0,108		0,039	0,042			
0,2	0,146	5	0,101	0,139	0,152		0,15
0,2			0,116	0,163			
0,2	0,133		0,11	0,153			
0,3	0,208	6	0,083	0,111	0,111		0,11
0,3	0,211		0,085	0,114			
0,3	0,213		0,082	0,109			
0,4	0,251	7	0,058	0,072	0,071	5x	0,36
0,4	0,268		0,058	0,072			
0,4	0,257		0,057	0,070			
		8	0,132	0,188	0,211	20x	4,22
			0,16	0,232			
			0,148	0,213			
		9	0,226	0,336	0,328	20x	6,55
			0,235	0,350			
			0,201	0,297			
		10	0,338	0,512	0,499	20x	9,97
			0,341	0,517			
			0,309	0,467			
		11	0,338	0,512	0,502	20x	10,05
			0,348	0,528			
			0,309	0,467			
		12	0,319	0,482	0,490	20x	9,79
			0,361	0,548			
			0,291	0,438			

Table A2 – Total protein amount in samples from the fish oil group, mg/mL.

	Fish oil samples (mg/mL)							
Fractions	FO 1	FO 2	FO 3	FO 4	FO 5	FO 6	Mean	SD
1	0,00	0,00	0,00	0,00	0,02	0,02	0	0,00
2	0,00	0,01	0,00	0,01	0,01	0,02	0	0,01
3	0,00	0,03	0,03	0,04	0,02	0,03	0,03	0,00
4	0,04	0,17	0,09	0,08	0,06	0,11	0,09	0,05
5	0,15	0,18	0,17	0,16	0,11	0,16	0,15	0,02
6	0,11	0,24	0,27	0,14	0,12	0,15	0,17	0,07
7	0,36	1,07	1,15	0,66	0,57	0,43	0,70	0,33
8	4,22	4,48	4,61	2,97	3,51	3,87	3,94	0,62
9	6,55	5,26	7,15	5,44	8,66	7,63	6,78	1,31
10	9,97	11,10	8,80	12,11	8,20	16,02	11,03	2,83
11	10,05	8,12	9,00	12,95	9,25	15,95	10,89	2,98
12	9,79	11,30	9,46	19,63	10,97	19,23	13,40	4,72

Table A3 – Total protein amount in samples from the control group, mg/mL.

	Control samples (mg/mL)							
Fractions	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Mean	SD
1	0,00	0,00	0,00	0,00	0,01	0,01	0	0,01
2	0,00	0,02	0,00	0,00	0,02	0,02	0	0,01
3	0,01	0,00	0,00	0,04	0,01	0,03	0,02	0,02
4	0,09	0,14	0,05	0,14	0,03	0,07	0,09	0,05
5	0,18	0,18	0,16	0,14	0,10	0,13	0,15	0,03
6	0,19	0,15	0,24	0,14	0,12	0,16	0,17	0,04
7	1,12	0,68	1,25	0,77	0,37	0,65	0,81	0,33
8	6,06	2,52	5,06	6,19	2,71	3,29	4,31	1,67
9	7,28	8,43	5,70	5,71	5,38	7,72	6,70	1,27
10	9,72	13,48	7,70	7,45	9,36	13,42	10,19	2,68
11	11,62	9,91	8,88	7,96	16,94	15,09	11,73	3,58
12	7,78	17,58	9,17	10,51	10,55	19,82	12,57	4,91

Cholesterol measurements

Example for sample number 1. Table A4 demonstrates a cholesterol measurements for control sample number 1. The cholesterol concentration of all measured samples is shown in table A5 and table A6.

Figure A2 shows a standard curve that was calculated from values in Table A4. The standard curve was then used for further concentration calculations on fraction 1 to 12. Values shown as Cholesterol (mg/mL) in Table A4.

Table A4 - Example of how cholesterol amount for sample number 1 for a control rat, were calculated from absorbance (OD) measurements.

		Control1							
Standard			OD	Blank	Corrected for Blank	Cholesterol	Average	protein	Chol/ protein
mg chol	A460	Fraction	(460 nm)		(460 nm)	(mg/mL)	(mg/mL)	(mg/mL)	(mg/mg)
0	-0,002	1	-0,007						n. d.
0	0,008		-0,007						
0	-0,005	2	-0,005					0,002361	n. d.
0,000493	0,023		-0,004						
0,000493	0,017	3	-0,001					0,008131	n. d.
0,000493	0,019		-0,001						
		4	0,042		0,042	0,0192634	0,018805	0,094166	0,200
0,000985	0,036		0,040		0,040	0,0183461			
0,000985	0,036	5	0,035		0,035	0,0160528	0,015594	0,17548	0,089
0,00197	0,067		0,033		0,033	0,0151355			
0,00197	0,074	6	0,024		0,024	0,0110077	0,011008	0,185447	0,059
0,00197	0,076		0,024		0,024	0,0110077			
0,004925	0,21	7	0,120	0,099	0,021	0,0096317	0,01009	1,121341	0,009
0,004925	0,223		0,119	0,096	0,023	0,010549			
0,004925	0,242	8	0,107	0,08	0,027	0,0123836	0,01697	6,059175	0,003
0,00985	0,417		0,119	0,072	0,047	0,0215567			
0,00985	0,425	9	0,024	0,005	0,019	0,0087144	0,011237	7,276256	0,002
0,00985	0,441		0,033	0,003	0,030	0,0137596			
		10	0,016	-0,006	0,022	0,0100904	0,010549	9,720911	0,001
			0,018	-0,006	0,024	0,0110077			
		11	0,019	-0,005	0,024	0,0110077	0,011466	11,61998	0,001
			0,023	-0,003	0,026	0,011925			
		12	0,023	-0,001	0,024	0,0110077	0,012384	7,779876	0,002
			0,025	-0,005	0,030	0,0137596			

n.d. = not detected

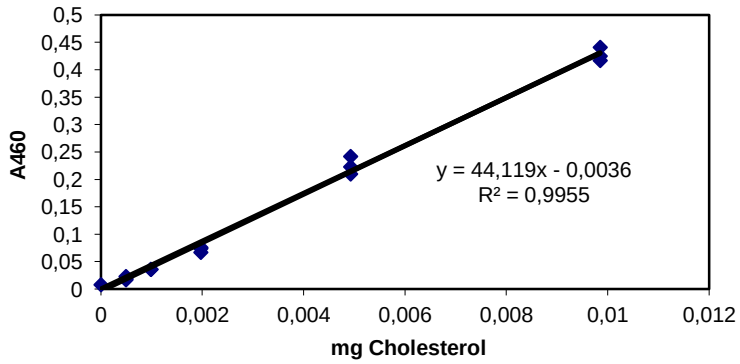

Figure A2 – Standard curve for cholesterol measurements.

Table A5 – Cholesterol amounts for control group, mg cholesterol/mg protein.

Fraction	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Mean	SD
1	0,000	0,0000	0,000	0,000	0,000	0,000	0,000	0,000
2	0,000	0,0000	0,000	0,000	0,000	0,000	0,000	0,000
3	0,000	0,0000	0,000	0,221	0,000	0,000	0,037	0,082
4	0,200	0,1228	0,337	0,133	0,475	0,205	0,245	0,124
5	0,089	0,0978	0,145	0,092	0,210	0,171	0,134	0,045
6	0,059	0,0326	0,075	0,077	0,111	0,092	0,074	0,025
7	0,009	0,0160	0,010	0,019	0,007	0,026	0,014	0,006
8	0,003	0,0032	0,003	0,003	0,000	0,005	0,003	0,001
9	0,002	0,0029	0,002	0,002	0,003	0,002	0,002	0,001
10	0,001	0,0010	0,001	0,002	0,001	0,001	0,001	0,000
11	0,001	0,0013	0,001	0,002	0,001	0,001	0,001	0,000
12	0,002	0,0013	0,001	0,002	0,002	0,001	0,001	0,000

Table A6 – Cholesterol amount for fish oil group, mg cholesterol/mg protein.

Fraction	FO 1	FO 2	FO 3	FO 4	FO 5	FO 6	Mean	SD
1	0,000	0,0000	0,000	0,000	0,000	0,000	0,000	0,000
2	0,000	0,0000	0,000	0,000	0,000	0,000	0,000	0,000
3	0,000	0,0000	0,000	0,000	0,000	0,000	0,000	0,000
4	0,259	0,1178	0,184	0,177	0,346	0,188	0,212	0,080
5	0,157	0,0730	0,100	0,114	0,163	0,195	0,134	0,045
6	n. d.	0,0407	0,062	0,081	0,125	0,085	0,079	0,031
7	0,045	0,0132	0,018	0,020	0,026	0,058	0,030	0,018
8	0,005	0,0080	0,003	0,006	0,006	0,011	0,007	0,003
9	0,002	0,0019	0,002	0,003	0,002	0,003	0,002	0,001
10	0,001	0,0009	0,001	0,001	0,002	0,001	0,001	0,000
11	0,001	0,0015	0,001	0,001	0,002	0,001	0,001	0,000
12	0,001	0,0010	0,001	0,001	0,001	0,001	0,001	0,000

Light emission measurements for Western blots

Tables A7-A10 show light emission values for caveolin-3, flotillin-1, α_1-adrenergic receptor and β_1-adrenergic receptor, respectively. These values were used for average light emission, and FO/control ratio measurements.

Table A7 – Light emission for caveolin-3 protein.

Fraction	FO 1	FO 3	FO 4	FO 5	Control 1	Control 3	Control 4	Control 5
4	6782918	23501417	1422674	23771356	8773352	15218938	29358795	9899220
5	9283707	22251483	32116643	46660285	12051365	27455071	36478592	28424175
6	9851056	23370218	3470842	6595550	14407409	29588684	32188373	37515045
7	17327902	24611922	24542659	8779636	10595007	16890204	23176223	16722967
8	11530606	7083385	17717955	2134617	5631647	6267310	14481293	3130716
9	4610763	4333912	4396867	549227	2547035	3439229	8143462	1182893
10	2694620	2293222	2350333	632658	2935766	1922552	7973597	1276791
11	2290179	725889	1691332	470427	2587478	416681	4785740	981091
12	1531135	554153	2503825	215412	1607610	256669	297124	1153348

Table A8 – Light emission for flotillin-1.

Fraction	FO 1	FO 3	FO 4	FO 5	Control 1	Control 3	Control 4	Control 5
4	8441846	8795929	6974018	21835491	10261825	8719759	20751359	16252382
5	9238421	12885782	27193371	36025938	13099874	17082772	26221484	32615654
6	7831416	1924976	1596233	23809298	13940662	1584609	21998633	25810940
7	9674449	10705990	28404553	24471921	8268837	2786857	26681986	20791648
8	7846585	1905763	6961170	6609123	5117340	913366	5927002	7143741
9	3096601	577225	2132336	1153382	1875016	1205988	4622313	2098560
10	1290547	441373	1141062	580030	975029	209353	5174349	717800
11	1243574	314728	526795	660302	918691	279245	3514063	491863
12	1803869	166928	401432	409968	787406	242937	319274	347796

Table A9 – Light emission for α_1-adrenergic receptor.

Fraction	FO1	FO3	FO4	FO5	Control 1	Control 3	Control 4	Control 5
4	12324041	2591453	799254	2178725	2520565	4107454	25027209	853691
5	4028922	743480	9681954	1193487	1837747	225597	16227744	864321
6	8142043	243412	3330912	2410772	2156157	120827	14868837	1265332
7	2582363	3403814	5268863	1004805	1683142	2374706	7592072	1551739
8	2184713	3106838	8079328	4169814	4087570	1744176	8215354	2678426
9	5237457	5151825	2269905	3689501	4408069	2262594	4150534	2791987
10	2735583	2034410	9074092	4794218	2660518	295328	1977450	4182317
11	2518143	3111602	5619346	4119806	3070601	1518305	4006409	2431202
12	2103076	3309036	3218115	823272	1945033	1566756	355487	2672344

Table A10 – Light emission for β_1-adrenergic receptor

Fraction	FO 1	FO 3	FO 4	Contrl 1	Contrl 3	Contrl 4
4	5710974	8640713	6143679	3148215	13185720	4833062
5	12561259	15480958	12077181	8096681	12020181	4430388
6	13191175	9597578	12180888	3177753	5270275	9452623
7	8082492	19615489	10097656	20249111	17861424	9448874
8	8962678	15249441	6372191	9984101	14512791	5809819
9	15452654	13478764	10400580	8496419	19726766	5643387
10	11457031	17828779	14318560	6612249	21715933	4431295
11	9513292	18934581	15048175	7896939	19203423	13493847
12	12768025	14299962	3425680	7979295	20207869	4154909

Fatty acids

Table A11 and A12 show fatty acid composition in lipid rafts and dissolved fractions, respectively.

Table A11 – Fatty acid composition (% of total FA) in lipid raft phospholipids in control and fish oil groups.

Fatty acids	Control 1	Control 3	Control 5	Control 6	Average	SD
C14	0,15	0,2	0,42	0,41	0,29	0,14
C16	10,62	11,84	13,32	12,51	12,07	1,14
C16:1n7	0,18	0,23	0,25		0,22	0,03
C18:0	26,58	26,85	26,93	25,81	26,54	0,51
C18:1n9	4,68	4,91	4,68	4,95	4,81	0,15
C18:1n7	2,3	2,35	2,46	2,52	2,41	0,1
C18:2n6	9,47	8,53	6,92	8,56	8,37	1,06
C20:0	0,73		0,52	0,8	0,68	0,15
C20:4n6	23,89	25,73	23,26	24,6	24,37	1,06
C20:5n3						
C22:0	0,8	0,71	0,52	0,61	0,66	0,12
C22:4n6	1,64	1,53	1,65	1,52	1,58	0,07
C22:5n6	1,36	0,77	1,08	0,61	0,95	0,33
C22:5n3	1,54	1,94	1,66	1,96	1,77	0,21
C22:6n3	10,43	9,03	10,88	8,88	9,81	1
C24:0	0,57	0,52	0,41	0,49	0,5	0,07
Other:	5,07	4,86	5,45	6,26	5,41	0,62
saturated	39,45529	40,12	42,12	40,63	40,58	1,13
monouns.	7,15915	7,49	7,39	7,47	7,38	0,15
n-6 PUFA	36,344	36,56	32,91	35,29	35,28	1,67
n-3 LC-PUFA	11,96977	10,97	12,54	10,84	11,58	0,82
Fatty acids	FO 1	FO 2	FO 3	FO 5	Average	SD
C14	0,37	0,56	0,25	0,77	0,49	0,23
C16	14,14	13,88	12,37	16,66	14,26	1,78
C16:1n7	0,38	0,4	0,28	0,37	0,36	0,05
C18:0	25,37	24,39	23,96	27,59	25,33	1,62
C18:1n9	5,05	4,93	5,37	6,17	5,38	0,56
C18:1n7	2,44	2,56	2,72	2,78	2,63	0,15
C18:2n6	7,21	7,15	9,2	3,76	6,83	2,26
C20:0	0,94	0,82	0,53	0,59	0,72	0,19
C20:4n6	14,18	13,82	14,53	11,15	13,42	1,54
C20:5n3	1,81	1,61	2,49	2,27	2,04	0,41
C22:0	0,87	0,73	0,47	0,73	0,7	0,17
C22:4n6	0,22	0,18	0,14	0,19	0,18	0,03
C22:5n6	0,26	0,26	0,24	0,23	0,25	0,01
C22:5n3	4,68	3,56	3,63	3,85	3,93	0,52
C22:6n3	16,3	19,35	15,52	15,48	16,66	1,83
C24:0	0,71	0,53	0,18	0,57	0,5	0,23
Other:	5,07	5,3	8,12	6,84	6,33	1,43
saturated	42,41	40,91	37,76	46,91	42	3,81
monouns.	7,86	7,89	8,37	9,32	8,36	0,68
n-6 PUFA	21,87	21,4	24,11	15,33	20,68	3,76
n-3 PUFA	22,79	24,51	21,64	21,6	22,63	1,36

Table A13 – Fatty acid composition (% of total FA) in dissolved membranes phospholipids in control and fish oil groups.

Fatty acids	Control 1	Control 3	Control 6	Average	SD
C14:0		1,64	0,62	1,13	0,72
C16:0	8,75	9,54	8,52	8,94	0,53
C16:1n7					
C18:0	26,66	26,42	23,52	25,53	1,75
C18:1n9	4,31	4,40	4,00	4,24	0,21
C18:1n7	2,49	2,33	2,27	2,36	0,12
C18:2n6	13,28	13,30	10,54	12,37	1,59
C20:0					
C20:4n6	25,07	26,87	23,15	25,03	1,86
C20:5n3					
C22:0					
C22:4n6	1,02	0,96	0,65	0,88	0,20
C22:5n6	1,42	0,79	0,50	0,90	0,47
C22:5n3	1,67	2,05	1,70	1,81	0,21
C22:6n3	11,67	9,80	8,53	10,00	1,58
C24:0					
Other	3,66	1,90	16,00	7,19	7,68
saturated	35,41	37,60	32,66	35,22	2,48
monouns.	6,81	6,73	6,27	6,60	0,29
n-6 PUFA	40,79	41,92	34,84	39,18	3,80
n-3 LC-PUFA	13,33	11,85	10,23	11,80	1,55

Fatty acids	FO 1	FO 2	FO 3	FO 5	Average	SD
C14:0						
C16:0	10,23	10,40	10,83	13,09	11,14	1,33
C16:1n7						
C18:0	25,64	26,47	25,23	30,76	27,03	2,54
C18:1n9	4,34	3,94	5,45	4,92	4,66	0,66
C18:1n7	2,96	2,95	3,02	3,14	3,02	0,09
C18:2n6	11,43	10,54	12,33	5,18	9,87	3,21
C20:0						
C20:4n6	16,95	16,52	16,55	15,65	16,42	0,55
C20:5n3	1,32	1,40	1,92		1,54	0,33
C22:0						
C22:4n6						
C22:5n6						
C22:5n3	4,29	3,59	3,67	4,54	4,02	0,47
C22:6n3	19,94	22,59	17,73	22,71	20,74	2,38
C24:0						
Other				0,01	0,01	
saturated	35,88	36,86	36,06	43,85	38,16	3,82
monouns.	7,30	6,89	8,47	8,06	7,68	0,72
n-6 PUFA	28,38	27,06	28,88	20,83	26,29	3,72
n-3 PUFA	25,55	27,57	23,32	27,25	25,92	1,95