Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics

Health benefits of seafood consumption

- with special focus on household preparations and bioactivity in animal models

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Ida-Johanne Jensen

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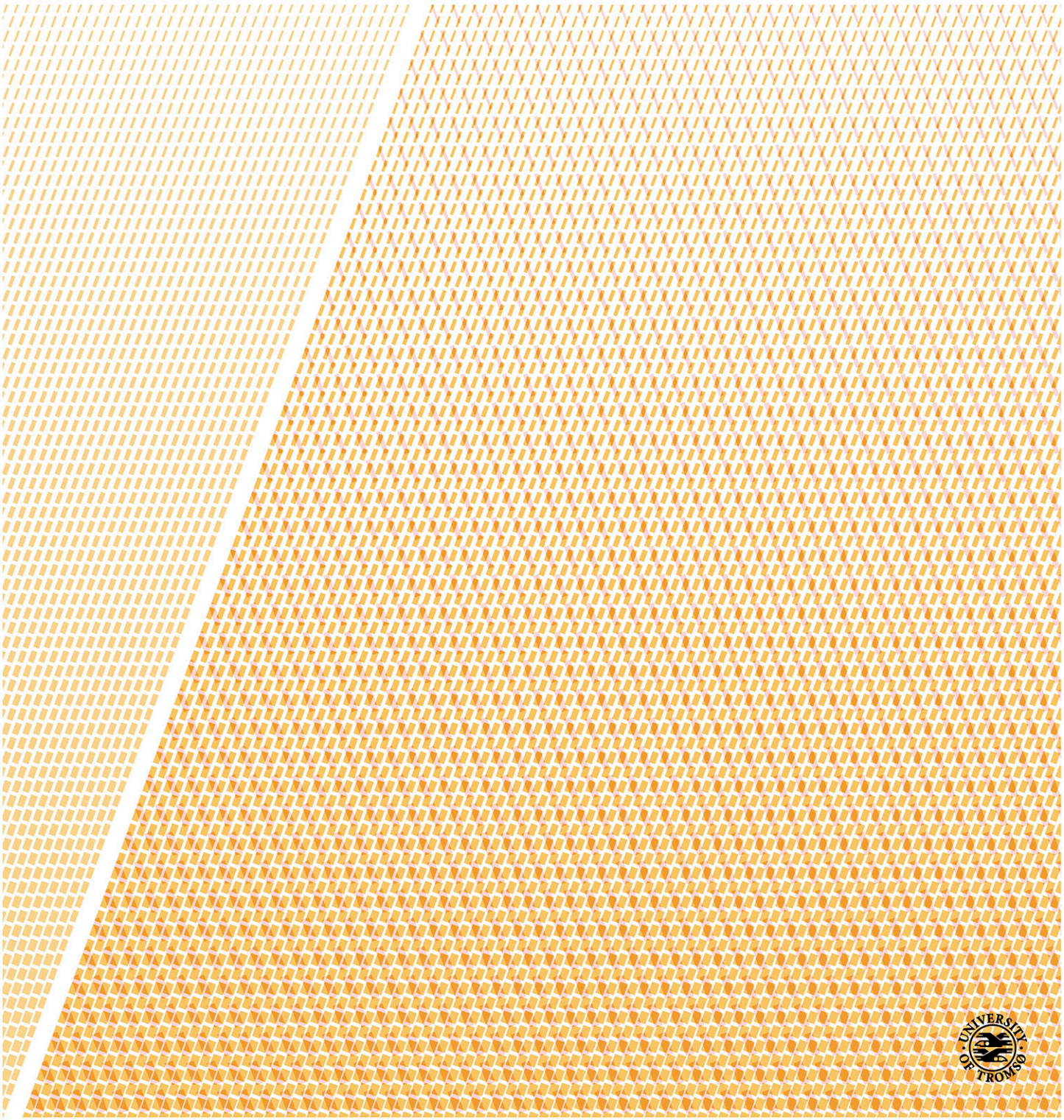

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Ida-Johanne Jensen

Summary

Fish consumption has for decades been acknowledged to reduce risk of cardiovascular disease, the health benefits mainly being associated with the long-chained omega-3 polyunsaturated fatty acids EPA (eicosapentaenoic acid, 20:5n3) and DHA (docosahexaenoic acid, 22:6n3). Increasing evidence, however, demonstrate that the beneficial effects are not limited to these fatty acids, but that the proteins and other bioactive compounds may be of relevance to human health. This project was initiated in order to examine the nutritional composition and bioactivity related to cardiovascular disease parameters in fish and terrestrial meat protein sources. The aims were limited to i) document differences in nutritional composition and *in vitro* bioactivity of wild and farmed Atlantic cod, pork and beef, ii) examine the impact of cooking and digestion on the nutrients and *in vitro* bioactive components and iii) evaluate the effects of different protein sources and bioactive peptides using animal models of hypertension and atherosclerosis.

The nutritional contents of cod and terrestrial meats were comparable, the main differences being lower fat content and three times higher taurine content in cod compared to the meats. Traditional household preparations mainly influenced the water content and thus the relative amount of lipids and protein. The composition of amino acids in proteins did not change after cooking, but the exclusively free amino acid taurine was lost correspondingly to the water loss. No significant difference in angiotensin converting enzyme (ACE) inhibitory activity was documented in the raw materials, but cooked cod provided higher ACE inhibitory activity compared to cooked meats. Raw pork exhibited higher antioxidative capacity compared to raw beef, while the cooked meats exhibited similar effects after a simulated digestion.

The antihypertensive effect of cod hydrolyzate was evaluated in spontaneously hypertensive rats by administering rats with 200 mg cod hydrolyzate/kg body weight for 21 days followed by 500 mg/kg for 7 days. After 7 days, the blood pressure increase leveled off and stabilized, suggesting some inhibitory activity of the cod hydrolyzate, although not proven significant in this study.

The cardioprotective effects of seafood proteins were compared to those of chicken protein in female apolipoprotein-E-deficient mice. Scallops were included together with cod, to enhance the taurine and glycine content in the marine protein diet. The total plaque burden in the aortas of mice fed cod-scallop protein was significantly lower compared to the plaque burden of mice fed chicken protein. It was demonstrated that seafood proteins exhibited a beneficial effect on overall atherosclerotic burden, probably due to a combination of factors.

Sammendrag

Sjømatkonsum har i tiår vært anerkjent for å redusere risikoen for hjerte- og karsykdommer, og helseeffektene har i hovedsak vært tilegnet langkjedete omega-3 fettsyrene EPA (eikosapentaensyre, 20:5n3) og DHA (dokosaheksaensyre, 22:6n3). Økende dokumentasjon viser nå at de positive helseeffektene ikke er begrenset til disse fettsyrene, men at proteiner samt andre bioaktive forbindelser også kan være relevante for helsen vår.

Dette prosjektet ble igangsatt for å undersøke næringsinnhold og bioaktivitet relatert til hjerte- og karsykdomsparametre i fisk og animalske proteinkilder. Målene ble begrenset til i) å dokumentere forskjeller i næringsinnhold og *in vitro* bioaktivitet i vill- og oppdrettet Atlantisk torsk, svin- og storfekjøtt, ii) undersøke effekten av tilberedning og fordøyelse på næringsstoffene og *in vitro* bioaktive komponenter og iii) evaluere effekten av forskjellige proteinkilder og bioaktive peptider ved hjelp av dyremodeller for hypertensjon og atherosklerose (åreforkalning).

Næringsinnholdet i torsk og animalsk kjøtt var sammenlignbart, hovedforskjellene var lavere fettinnhold og et tre ganger høyere innhold av taurin i torsk sammenlignet med kjøtt. Tradisjonelle tilberedningsmetoder påvirket i hovedsak vanninnholdet og dermed det relative innholdet av fett og protein. Aminosyresammensetningen endret seg ikke etter varmebehandling, men den frie aminosyren taurin ble redusert tilsvarende vanntapet. Ingen betydelig forskjell i hemming av Angiotensin converting enzyme (ACE) ble dokumentert i råmaterialene, men varmebehandlet torsk ga høyere ACE hemming sammenlignet med stekt kjøtt. Rått svinekjøtt hadde høyere antioksidativ kapasitet sammenlignet med storfekjøtt, mens etter steking ga svinekjøtt og storfekjøtt like effekter etter en simulert fordøyelse.

Den blodtrykksreduserende effekten av fordøyd torsk ble evaluert i spontant hypertensive rotter ved å gi rottene 200 mg torskehydrolysat/kg kroppsvekt i 21 dager og deretter 500 mg torskehydrolysat/kg kroppsvekt i 7 dager. Etter 7 dager, flatet den naturlige blodtrykksøkningen ut og stabiliserte seg, noe som kan indikere blodtrykksreduserende effekt av torsk. Resultatet var dog ikke signifikant i dette studiet.

Hjerte- og karbeskyttende effekt av sjømatprotein og kyllingprotein ble sammenlignet i et fôringsforsøk på hunnmus med genetisk apolipoprotein-E-mangel. Kamskjell ble inkludert sammen med torsk for å øke innholdet av taurin og glycin i fôret med sjømatprotein. Den totale plakkdannelsen i aorta hos mus fôret med torsk-kamskjell var signifikant redusert sammenlignet med plakkdannelsen i mus fôret med kylling. Det ble dermed vist at sjømatproteiner har en gunstig effekt på utviklingen av åreforkalkning, sannsynligvis påvirket av flere faktorer.

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Abbreviations

ACE Angiotensin Converting Enzyme

ALA α-linolenic acid

AOAC Association of Official Analytical Chemists

AOC Antioxidative capacity

Apo E Apolipoprotein E

ARA Arachidonic acid

BP Blood pressure

CHD Coronary heart disease

CVD Cardiovascular disease

DHA Docosahexaenoic acid

EDTA Ethylene-diamine-tetra acetic acid

EPA Eicosapentaenoic acid

FAO Food and Agricultural Organization of the United Nations

HA Hippuric acid

HDL High density lipoprotein

HHL Hippuryl-histidyl-leucine

HPLC High performance liquid chromatography

IC Inhibition coefficient

ICAM-1 Intercellular adhesion molecule-1

LA Linoleic acid

LDL Low density lipoprotein

LDLR Low density lipoprotein receptor

MUFA Monounsaturated fatty acids

ORAC Oxygen radical absorbance capacity

PON2 Paraoxonase2

PCB Polychlorinated biphenyls

POPs Persistent organic pollutants

PUFA Polyunsaturated fatty acid

RAAS Renin-angiotensin-aldosterone system

ROS Reactive oxygen species

SFA Saturated fatty acids

SHR Spontaneously hypertensive rats

SMC Smooth muscle cell

SRB1 Scavenger receptor B1

VCAM-1 Vascular cell adhesion molecule-1

VLDL Very low density lipoprotein

1 Introduction

Our life-style and food habits have changed considerably over the last decades due to economic and technological developments in our society. Diets in both developed and in many developing countries are highly caloric, rich in refined sugar and saturated fat. In both developed and developing countries the diets are often low in dietary fibre, protein, marine lipids, vitamins and minerals. In addition, physical activity is often limited to work related movement – if not completely absent. Life-style related disorders such as type 2 diabetes, hypertension, obesity, metabolic syndrome and cardiovascular disease (CVD) have increased and now pose a massive health burden worldwide [1]. A large amount of research has been performed in the area of diet and health and it is well established that diet has a major impact on the health status of the individual. There is also a broad consensus on nutritional intervention as an effective and safe approach to health maintenance and in particular CVD prevention, where a change in nutritional pattern may reduce CVD-related deaths by 60 % [2].

Fish consumption has for decades been acknowledged to reduce risk of CVD, particularly coronary heart disease (CHD), and mortality [3-8]. The health benefits have mainly been associated with the long-chained omega-3 polyunsaturated fatty acids generally referred to as LC n-3 PUFA, in particular eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Increasing evidence, however, demonstrate that the beneficial effects are not limited to PUFAs. The proteins and other bioactive compounds may also be relevant to human health by lowering blood pressure (BP) [9, 10], increasing plasma antioxidants [11], improving insulin sensitivity [12], reducing very low density lipoprotein (VLDL) cholesterol [13] and increasing high density lipoprotein (HDL) cholesterol [14, 15].

Despite general nutritional guidelines encouraging increased seafood consumption, the relative intake of fish proteins has declined over the last four decades and account for less than 17 % of animal protein intake in the human population [1]. Several publications have documented adverse health effects associated with red meat consumption, such as colorectal cancer [16], CHD [17] and type 2 diabetes mellitus [18]. These findings have later been questioned and a recent review confirmed detrimental health associations only with meat preserved by addition of salt and preservatives such as nitrate [19]. Since no distinctions have been made between raw meat and meat with preservations, lean and unprocessed meat may

possibly be healthy nutritional sources that contain bioactive compounds in addition to the high content of proteins.

1.1 Problem outline

Most research and documentation on nutritional value and bioactive properties of food protein sources have been conducted on raw material. However, to achieve palatable, safe and more easily digestible proteins, heat treatment is essential. Although heat treatment improve the quality by inactivation of pathogenic microorganisms and in most cases enhancement of flavor and tenderness, the nutritional composition of meats may be altered after heat treatment with consequent nutrient losses.

Further, while some *in vitro* studies have been performed to investigate cardiovascular parameters, there is a lack of *in vivo* studies documenting these mechanisms and potential health effects.

1.2 Project aims

The overall aim of this project was to examine the nutritional composition and bioactivity related to CVD parameters in fish and terrestrial meat protein sources. The specific goals were limited to the following:

- Document differences in nutritional composition and *in vitro* bioactivity of wild and farmed Atlantic cod, pork and beef

- Examine the impact of cooking and digestion on nutrients and *in vitro* bioactive components

- Evaluate the effects of different protein sources and bioactive peptides using animal models of hypertension and atherosclerosis

1.3 Research Design

The design of the *in vitro* research is schematically pictured in **figure 1**. Twenty samples from wild and farmed cod, pork and beef were prepared according to traditional household preparations or kept raw for control. After preparation, all samples were evaluated for proximate composition, fatty acid composition and amino acid composition. They were subjected to a simulated gastrointestinal digestion before analysis of antioxidative capacity (AOC) and angiotensin converting enzyme (ACE) inhibitory activity.

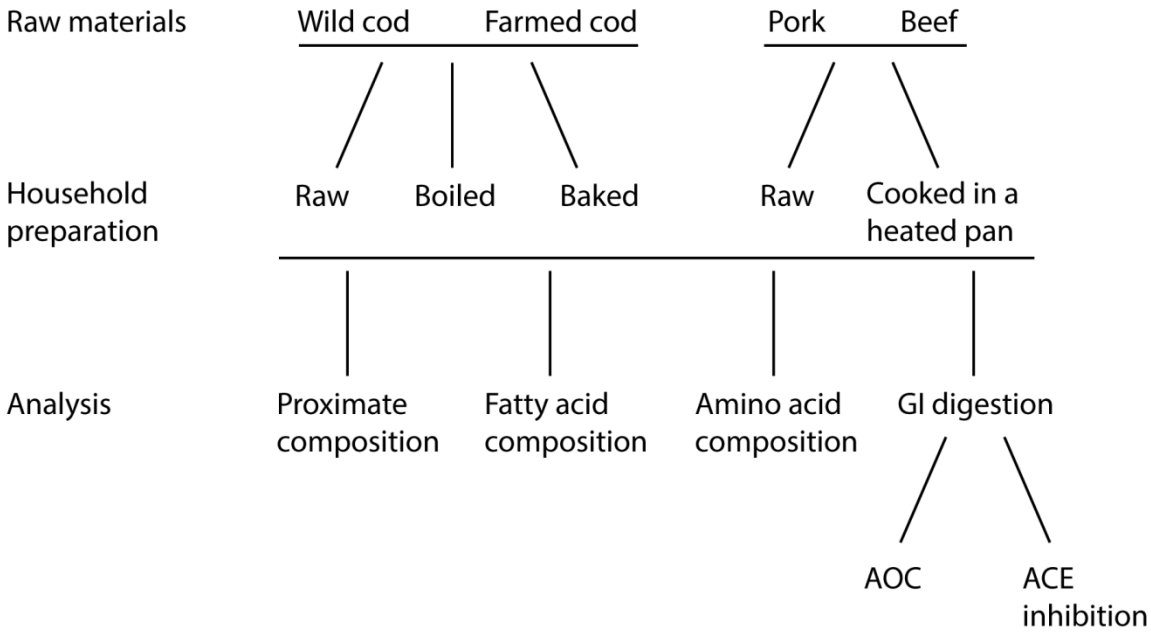

Figure 1 Overview of the *in vitro* research design. FA, fatty acid; AA, amino acid; GI, gastrointestinal; AOC, antioxidative capacity; ACE, angiotensin converting enzyme (ill. Ida-Johanne Jensen/Mari Walquist).

2 List of papers

This thesis is based on the following papers, referred to by their roman numerals in the text.

Paper I

Jensen, I-J., Larsen, R., Rustad, T. and Eilertsen, K.E. (2013) Nutritional content and bioactive properties of wild and farmed cod (*Gadus morhua* L.). *Journal of Food Composition and Analysis*, **31**, 212-216.

Paper II

Jensen, I-J. Dort, J. and Eilertsen, K.E. (2014) Proximate composition, antihypertensive and antioxidative properties of the semimembranosus muscle from pork and beef after cooking and in vitro digestion. *Meat Science*, **96**, 916-921.

Paper III

Jensen, I-J., Eystuskard, J., Madetoja, M. and Eilertsen, K.E. (2014) The potential of cod hydrolyzate to inhibit blood pressure in spontaneously hypertensive rats. *Nutrition Research*, **34**, 168-173.

Paper IV

Jensen, I-J., Walquist, M., Liaset, B., Elvevoll, E.O. and Eilertsen, K.E. (2014) Seafood protein reduce atherosclerotic burden in female apolipoprotein E deficient (*apoE*$^{-/-}$) mice fed a Western type high fat diet for 13 weeks (*Manuscript*).

3 Background

3.1 Nutritional aspects of fish and meat

Fish can be roughly classified based on its muscular fat content. Lean fish contains less than 3 % fat, medium fat fish contains 3-7 % fat and fatty fish contains more than 7 % fat. In meat, fat content differs substantially between species and cuts [20], but meat cuts where skin and visible fat is removed are commonly acknowledged as lean meats. The part of beef and pork with the lowest fat content is the loin, while the breast is the leanest part of poultry meat. Because of the vast species variations, nutritional characteristics valid for all fish and meats are complex to outline. Some nutrients are nonetheless particularly associated with fish or meat consumption.

3.1.1 Lipids

Lipids and fatty acids are the main energy sources for metabolic use and storage in the human body. Fatty acids function as important constituents of all cellular membranes. The two PUFAs linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are essential for humans. These PUFAs may be converted through a series of chain elongation and desaturation to the very long arachidonic acid (ARA, 20:4n6), EPA and DHA, where LA is the precursor of ARA and ALA is the precursor of EPA and DHA. These PUFAs are seminal constituents of the membrane phospholipids and important for maintaining normal cell functions. The two metabolic patterns use and compete for the same enzyme systems (containing elongases and desaturases) and thus the ratio of LA and ALA in our diet is reflected in the ratio between ARA and EPA, DHA. The 20-carbon fatty acids ARA and EPA may be metabolized via three pathways, termed the cyclooxygenase, lipoxygenase and epoxygenase pathways, into a series of different hormone-like compounds known as eicosanoids with very different biological properties depending on if they are synthesized from ARA or EPA (see **figures 2 and 3** for details).

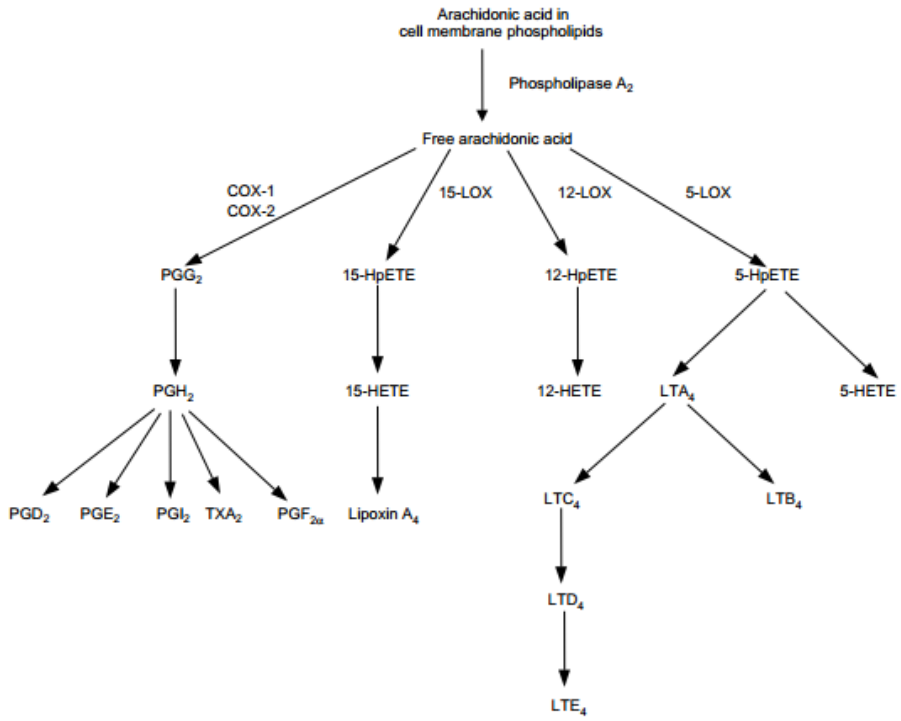

Figure 2 Schematic overview of the eicosanoid synthesis from arachidonic acid (ARA). COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane [21].

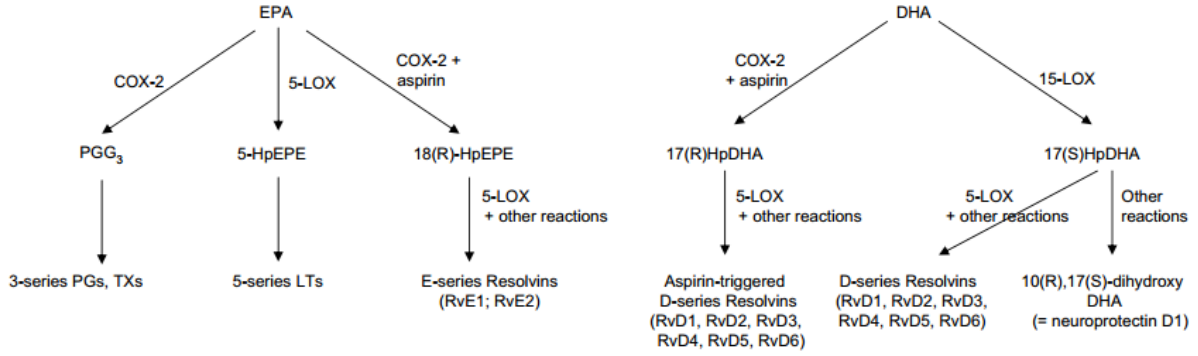

Figure 3 Schematic overview of the eicosanoid synthesis from eicosaheptaenoic acid (EPA) and docosahexaenoic acid (DHA). COX, cyclooxygenase; HpEPE, hydroperoxyeicosapentaenoic acid; HpDHA, hydroperoxydocosahexaenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane; RV, resolvin [21].

Eicosanoids derived from ARA are generally pro-inflammatory and pro-thrombotic, whereas eicosanoids derived from EPA are less inflammatory or even anti-inflammatory [21]. In addition, the EPA-derived eicosanoids are less potent than those derived from ARA. Also DHA is a precursor for anti-inflammatory compounds, called resolvins and protectins (**figure 3**). An optimal ratio between n-6 and n-3 fatty acid in the diet is anticipated to be 2-5:1,

depending on the particular diseases [22]. The change in diet towards more processed foods with higher amounts of LA, has shifted the n-6:n-3 ratio of the current Western diet to 15-17:1 [23]. As the previously mentioned enzymatic conversions of LA into ARA and ALA into EPA and DHA depend on the same cascade of enzymes, an imbalance between n-6 and n-3 fatty acids may favor the production of ARA over that of EPA and DHA. Very simplified this shift may have contributed to the increased incidence of inflammatory conditions such as rheumatoid arthritis, psoriasis, atherosclerosis, Crohn´s disease and ulcerative colitis [21]. Fish and seafood contain high amounts of EPA and DHA due to direct transfer up the food chain from phytoplankton which produce them *de novo.* For example, the content of EPA and DHA in wild salmon was 800 mg/100 g fillet and the n6/n3 ratio was 0.08 [24]. General recommendations are a daily intake between 200 and 500 mg EPA and DHA. This can be achieved by eating fish, preferably oily fish, twice a week [25, 26]. The fats in meat is generally composed of a higher percentage of monounsaturated fatty acids (MUFAs) and n-6 fatty acids than fish and seafood [27]. The concentration of LA has been determined to be 14 % and 2 % of total fatty acids in retail pork and beef, respectively, being the major contribution to the n6:n3 ratio of 7 and 2 [28]. It is, however, important to emphasize that ALA accounts for the major part of n-3 fatty acids in meats, whereas the LC n-3 PUFA EPA and DHA account for the n-3 fatty acids in fish.

3.1.2 Proteins and amino acids

Proteins are large molecules built up of chains of amino acids, and are essential for growth and maintenance of our body. The amount of protein needed to achieve the desired structure and function, is defined as the protein requirement [29]. The quality of a protein is determined as to what extent the protein meets our needs of essential amino acids, regarding both amount of essential amino acid and their absorption and utilization in the body [29]. This may vary according to amino acid composition, hydrolysis and effects of processing. The protein value of different food sources have been thoroughly reviewed by Friedman [30]. Raw fish consist of 12-24 % protein, while terrestrial meats consist of approximately 22 % and both contain all the essential amino acids in adequate amounts to cover our daily requirement. Fish is low in connective tissue compared to terrestrial meat, which may make it more easily digestible.

Taurine (2-aminoethanesulfonic acid) is generally referred to as an exclusively free amino acid not incorporated into proteins. It is involved in several physiological processes and

mounting evidence suggests that an increased dietary intake may be beneficial [31]. Taurine is present in most meats for human consumption, but seafood in general, and invertebrates in particular, are recognized as rich sources of taurine [32].

3.1.3 Vitamins and minerals

Vitamins and minerals are low molecular nutrients essential for human metabolism. Both fish and meat are good sources of several vitamins and minerals. Fish is high in vitamin B_{12} (cobalamin) and fatty fish is high in lipid soluble vitamins; A and D in particular. Meat is a good source of the vitamins in the B-group; riboflavin, niacin, pyridoxine, panthotenic acid and cobalamin. Fish is considered the best source of iodine, while meat is a good source of zinc and iron. Protein rich foods in general are recognized as good sources of selenium and hence, both fish and meat are good sources.

3.2 Risks and benefits of seafood consumption

Foods accumulate environmental pollutants that may reduce the overall nutritional value and negatively affect the consumer's health. Of the contaminants typically present in foods, the main focus has been on heavy metals and persistent organic pollutants (POPs). Among these, methylmercury is the most relevant of the heavy metals, while dioxins and polychlorinated biphenyls (PCB) are the most relevant POPs. Methylmercury is known to be neurotoxic and may have detrimental effects on development of the neurosystem [33, 34]. Documented adverse health effects of dioxins and PCB are skin reactions, damage of nervous, immune and endocrine systems, impaired reproductive function, and cancer [35]. All of these contaminants are present in most foods and the total intake is dependent on dietary patterns. Seafood is, however, recognized as the most significant source of dietary methylmercury, dioxins and PCB and has been the group most extensively evaluated for risks related to consumption. Especially in countries with high *per capita* seafood consumption such as Iceland, Japan, the Maldives and Portugal, balancing the risks and benefits associated with seafood consumption are of utmost importance. Several risk-benefit analysis have been performed, and the conclusions are unanimous, the benefits of seafood consumption outweighs the risks [6, 36, 37].

3.3 Cardiovascular disease

The strongest evidence from studies concerning fish consumption and chronic diseases are related to CVD. Cardiovascular disease is a collective term including disorders of the heart and blood vessels and are the largest cause of morbidity and premature deaths worldwide, the most recent numbers being 47% of all deaths in Europe [38] and 30.5% of all deaths worldwide [37]. The fact that 80 % of CVD deaths in the world occurred in low and middle-income countries, demonstrates that life-style related diseases are not limited to the Western world. The two most frequent disorders among the CVDs are CHD and cerebrovascular disease (stroke), affecting blood vessels of the heart and brain, respectively. Several risk factors are associated with the development of CVD. Some of the risk factors are non-modifiable such as age, gender and heredity. Other factors are modifiable, e.g. smoking, excessive alcohol consumption, insufficient intake of fruit and vegetables, lack of regular physical activity and as abdominal obesity [39]. Risk factors such as type 2 diabetes mellitus, hypertension and dyslipidemia are acknowledged as life-style related and are hence defined as modifiable. Despite the many risk factors associated with the development of CVD, only two have been recognized as independent causes of CVD, namely hypertension and atherosclerosis.

3.3.1 Regulation of blood pressure and hypertension

The regulation of BP is complex and involves several mechanisms. Some of them are purely mechanic, such as change of arteries diameter, regulation of blood volume in the blood stream and addition or removal of fluids in the blood stream. Other mechanisms involve more complex regulatory systems. The renin-angiotensin-aldosterone-system (RAAS) is one of these (**figure 4**). When blood flow or volume through the kidneys decreases, the enzyme renin is excreted converting angiotensinogen to form angiotensin I. Angiotensin I is an inactive decapeptide with the amino acid sequence Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu. Angiotensin converting enzyme is a dipeptidyl carboxypeptidase (EC. 3.4.15.1) within the class of zinc proteases and acts as an exopeptidase cleaving dipeptides from the C-terminus of various oligopeptides [40, 41]. In the RAAS system, ACE cleaves off the dipeptide His-Leu from angiotensin I to form the octapeptide angiotensin II [42]. This is a potent vasoconstrictor and induces a rise in BP by increasing the systemic resistance. In addition it stimulates

secretion of aldosterone from the adrenal cortex resulting in increased sodium and water reabsorption in the kidneys and thus a rise in BP [43].

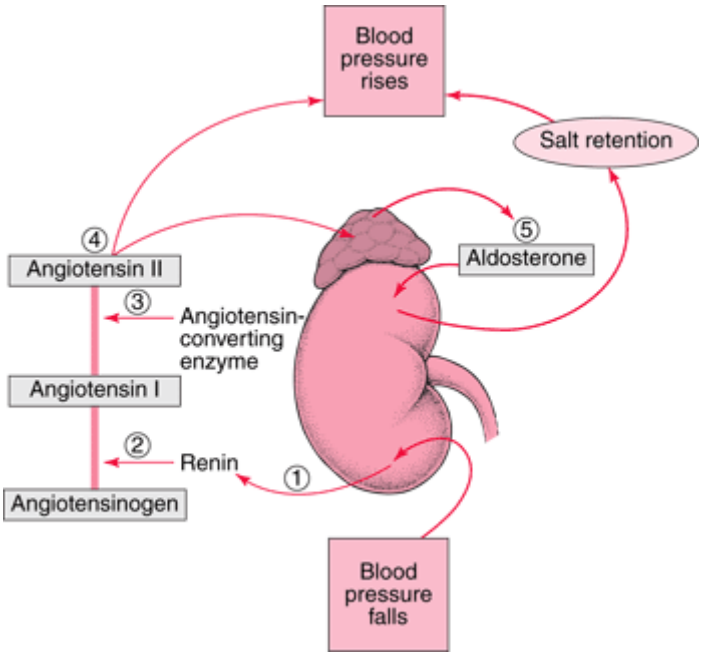

Figure 4 Schematic overview of the renin-angiotensin-aldosterone-system (RAAS) regulating an increase in blood pressure (BP). In the event of decreased BP, kidneys release the enzyme renin into the blood stream (1). Renin splits the protein angiotensinogen into the inactive decapeptide angiotensin I (2). Angiotensin converting enzyme (ACE) then cleaves off a dipeptide from angiotensin I, resulting in the active octapeptide angiotensin II (3). Angiotensin II is a vasoconstrictor increasing BP (4). In addition it triggers the release of the hormone aldosterone from the adrenal glands and antidiuretic hormone from the pituitary gland causing the kidneys to retain salt, thereby retaining water and thus increasing blood volume and BP (5) [44].

The kallikrein kinin system is another BP regulatory system where the enzyme kallikrein converts kininogen to the nonapeptide bradykinin, which has a vasodilating effect (**figure 4**). ACE inactivates bradykinin and thereby inhibits the vasodilating effect, contributing to an increase in BP [45].

A BP of 120/80 mmHg is considered normal and an increase of 20/10 mmHg has been reported to double the risk of fatal CVD among 40-49 year olds [46]. Elevated BP, defined as hypertension, is a condition where the heart´s workload is increased to maintain adequate blood circulation. The condition is associated with myocardial infarction, stroke and heart failure and is one of the most important precursors for CVD, estimated to affect 1.56 billion individuals worldwide in 2025 [47]. About 95 % of all cases of hypertension are classified as primary or essential hypertension with unknown causes. Still; obesity, stress, smoking, physical inactivity as well as high salt intake are recognized as high risk factors. The

remaining 5 % is classified as secondary hypertension and is typically an outcome of identifiable causes such as kidney disease or tumors [48].

3.3.2 Cholesterol and lipids metabolism

Triglycerides and cholesterol are the main lipid constituents of the human diet. Triglycerides are important for energy storage and metabolism and are, together with cholesterol, essential structural components of mammalian cell membranes, playing crucial roles in cell differentiation, nerve conduction, membrane fluidity as well as hormone and bile acid synthesis [49]. Homeostasis of cholesterol in our body is maintained by synthesis in the liver, absorption in the intestine followed by biliary and fecal excretion [50]. Too much cholesterol, due to increased intake/synthesis or decreased excretion may be detrimental. Triglycerides and cholesterol are insoluble in hydrophilic environment such as blood, so in order to enable blood transportation from the liver to peripheral tissues and back, these lipids are packed into water-soluble macromolecules called lipoproteins. The lipoproteins are made up of a membrane of protein, phospholipids and cholesterol and an inner core of triglycerides and cholesterylesters. The lipoproteins are divided based on density, into chylomicrons, VLDL, low density lipoprotein (LDL) and HDL. While chylomicrons and VLDL mainly transport triglycerides, LDL and HDL transport cholesterol to and from extrahepatic tissue. Chylomicrons are made in the gastrointestinal tract and ship dietary lipids and fat soluble vitamins to heart and skeletal muscle for energy, adipose tissue for storage, or liver. The remnants are taken up in the liver through an LDL receptor (LDLr) and are used to make VLDL which ships excess lipids to peripheral tissue. When the VLDL is depleted of triglycerides, it is converted to LDL rich in cholesterylesters. When cells need cholesterol, LDL is taken up from the blood stream through the LDLr. The HDL can take up excess cholesterol from various tissues and the acquired cholesterol is esterified to cholesterylester and stored in the core. The HDL derived cholesterylesters can be directly taken up in the liver via interaction with the scavenger receptor B1 (SRB1), where they can be stored, incorporated into new lipoproteins or excreted into bile in the form of bile acid or natural sterols [51]. High levels of LDL in the blood may lead to deposition in the vessel wall, where it may be modified and taken up by macrophages, contributing to the initiation process of atherosclerosis [52].

3.3.3 Atherosclerosis

Atherosclerosis is a complex, multifactorial and progressive inflammation condition affecting the arteries by accumulation of lipids, connective tissue, smooth muscle cells (SMC) and immune cells causing plaque formation and narrowing of the lumen [53] (**figure 5**).

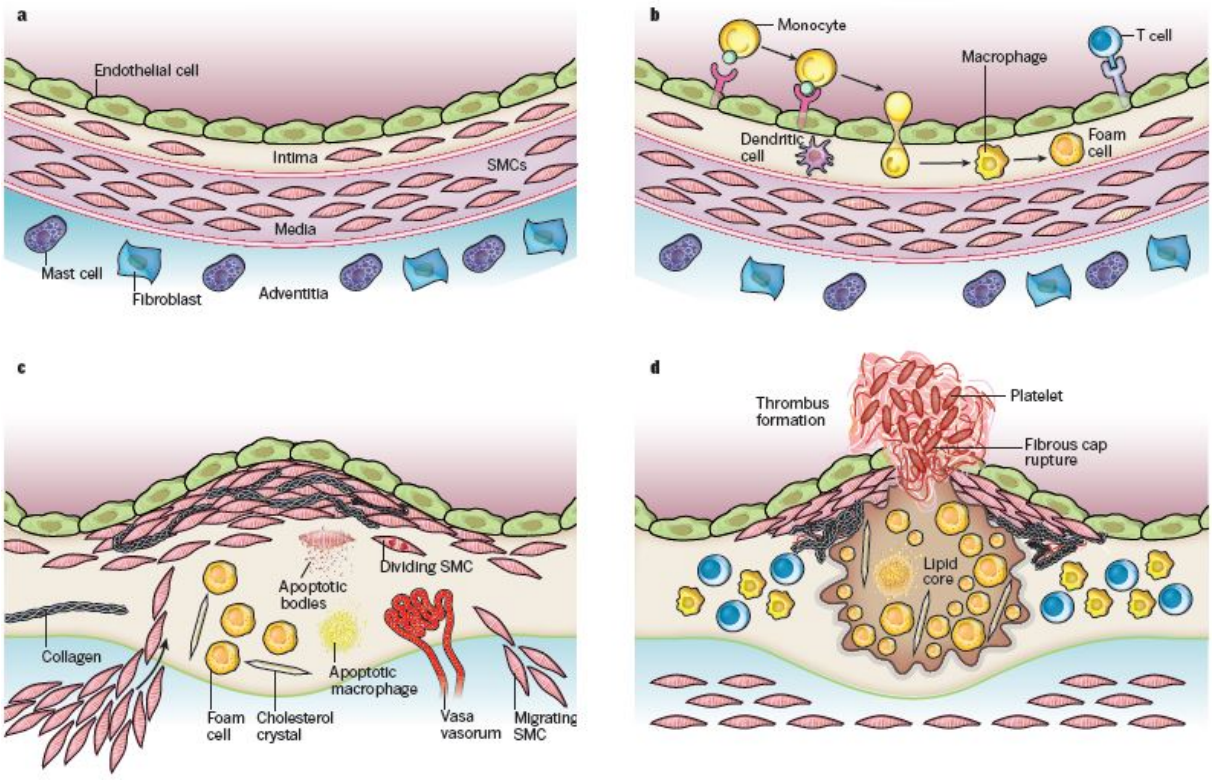

Figure 5 Development of atherosclerotic lesions in an artery and the cell changes occurring during disease progression to thrombosis. a) An artery consists of an outer layer, adventitia, containing mast cells, nerve endings and connective tissue, a middle layer, media, containing smooth muscle cells (SMC) embedded in an extracellular matrix, and an inner layer, intima, lined by a monolayer of endothelial cells. b) When the endothelium is stimulated by reactive oxygen species (ROS), lipid accumulation, hypertension or pro-inflammatory mediators, the early steps of atherosclerosis occur. This includes adhesion and migration of into the intima, maturation of monocytes into macrophages and their uptake of lipids resulting in foam cells. c) The lesion progression continues by migration of SMC from the media to the intima and proliferation of the SMC, together with an increased synthesis of extracellular matrix molecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMC that die in advancing lesions, and extracellular lipid derived from these dead and dying cells can accumulate in the central region, often denoted the lipid or necrotic core. d) A physical disruption of the atherosclerotic plaque including blood coagulation components, releases a thrombus that extends into the vessel lumen where it can impede blood flow [53].

The blood vessel consists of three layers (see **figure 5a**). The inner layer of the blood vessels, intima, is covered by a monolayer of endothelial cells, known as the endothelium. The second

layer, media, consists of SMCs and the third and outermost layer, adventitia consists of SMCs, fibroblasts, mast cells and connective tissue which stabilizes the vessel. The endothelium normally regulates vascular tone permeability and the flow of nutrients, biologically active molecules and blood cells [54]. It also produces a variety of regulation mediators, nitric oxide being one of the major factors for sustaining normal endothelial functions. Nitric oxide is also a potent vasodilator and has anti-inflammatory, anti-platelet, anti-proliferative and permeability-decreasing properties [55]. When the endothelial monolayer is stimulated by reactive oxygen species (ROS), dyslipidemia, hypertension or pro-inflammatory mediators [56], less nitric oxide is released. This leads to an increased expression of adhesion molecules such as vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1), which in turn leads to leukocytes being captured on the endothelial lining. Endothelial dysfunction is manifested as a reduction in the endothelial barrier properties and increased endothelial permeability [57]. These adhesion molecules recruit monocytes to the endothelium, followed by adherence and migration into the intima. The influx of monocytes is often accompanied by influx of other inflammation cells, such as T-cells, dendritic cells and mast cells. In the intima, monocytes are further activated by pro-inflammatory cytokines and differentiate into macrophages. Macrophages are phagocytic scavenger cells and may absorb modified and oxidized LDL forming foam cells in the arterial intima. Accumulation of such foam cells results in the formation of “fatty streak” [58] and plays a critical role in occurrence and development of atherosclerosis [59]. Vascular SMC migrate from the media into the intima and their proliferation contributes to the formation of plaque. Continuation of inflammatory responses accelerates the atherosclerotic process. Stimulation of proliferation and migration of SMC to the intima and release of intracellular contents (lipids, cholesterol) from both macrophages and SMC, build up a large plaque inside the intima. Protease secretion by macrophages degrade extracellular matrix, such as collagen, and a fibrous cap is formed around the excess lipids. The atherosclerotic process can develop silently for months and years. However, if the plaque´s surface is damaged, the intracellular content is released into the arteries, leading to formation of a thrombus that can occlude the blood vessel leading to stroke or myocardial infarction [53, 60].

3.4 Bioactive peptides

The definition “bioactive” refers to compounds that, in addition to its nutritional value, are able to regulate biological processes in the human body, and have an impact on body function and health. The term has been refined to include only compounds that have measurable effect at a physiologically realistic level and where the impact is positive [61]. Peptides may be such compounds. Like proteins they consist of chains of amino acids bound together by peptide bonds, the length of the chain determining whether it is a peptide or a protein. Over the last decades, an increased focus in nutritional science has been directed towards bioactive peptides [62]. Bioactivities depend on the composition of the amino acids and it is generally recognized that peptides consisting of 2 to 30 amino acids possess the highest bioactivity [63]. Although peptides may exist naturally as such in foods [64], they mainly occur as part of proteins. Here the amino acid side groups are buried and the bioactivity is compromised. The peptides are hence bioactive only after release through fermentation, food processing, proteolysis or gastrointestinal digestion. Most bioactive peptides reported are produced by *in vitro* enzymatic hydrolysis which enables manufacturing of bioactive peptides with desired properties [62, 65]. Peptides released by enzymes present in the stomach and intestines may be absorbed through the intestines and enter the circulatory system intact to exert various physiological effects, or they may exert local effects in the digestive tract and stomach [66]. These peptides cannot be controlled and are not relevant for commercial purposes, but may nevertheless influence physiological processes *in vivo* and exert a positive health effect on the respective individual. Several bioactive peptides have been documented from different fish, dairy and meats, the main activities by far being antihypertensive effect by inhibition of ACE [67-72] and antioxidative function [69].

3.4.1 Angiotensin converting enzyme and inhibitory peptides

Inhibition of ACE by synthetic ACE inhibitors such as captopril, enalapril, alcacepril and lisinopril has been a useful therapeutic approach to treat hypertension [73]. However, the use of synthetic drugs are often associated with side effects such as cough, taste alterations, skin rashes and renal dysfunction [74], which has led to an increased interest in finding natural inhibitors from different food sources [67, 75]. Whereas synthetic ACE inhibitors function by directly blocking the action of ACE, bioactive peptides react with ACE making it unavailable for cleaving Angiotensin I, both in a competitive and non-competitive manner (**figure 6**).

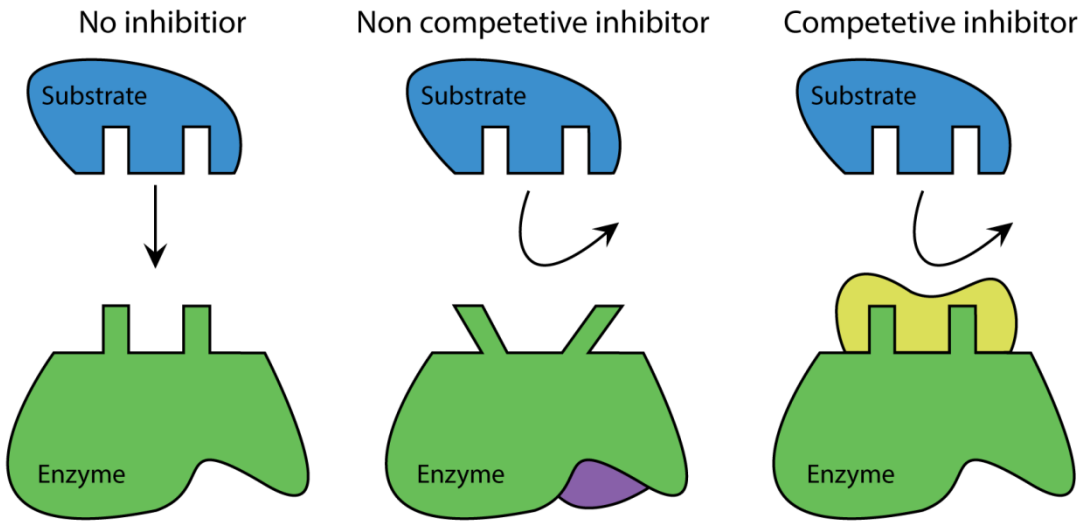

Figure 6 Mode of angiotensin converting enzyme inhibition. A non competitive inhibitor binds to the inhibitor site, remote from the active site to alter the enzyme conformation, so the substrate no longer binds to the active site. A competitive inhibitor binds to the active site of the enzyme, blocking it for substrates (ill. Mari Walquist).

Competitive ACE inhibitors block the ACE by binding to the active site of the enzyme [76, 77], while non-competitive ACE inhibitors bind to the inhibitor binding site, remote from the active site. [78-82]. Here they alter the enzyme conformations so the substrates no longer can bind to the active site [75]. Angiotensin converting enzyme inhibitors can also be classified according to their mode of action [67] into three groups. The first group comprises true inhibitors whose activity is not changed after pre-incubation with ACE. The second group acts as substrates for ACE which converts them to weaker or inactive peptides, and the third group includes the so-called pro-drug peptides that are converted to true inhibitors by ACE or gastrointestinal proteases. Only the ACE inhibitors in the first and third group exert antihypertensive activity after oral administration and may thus lower BP [67, 83]. Antihypertensive peptides, especially those with ACE inhibitory effect, are the most extensively researched bioactive peptides. Several studies have been performed on milk peptides [72], porcine peptides [70, 71], chicken peptides [67] and marine derived peptides including tuna frame, tuna muscle, yellowfin sole oyster, wakame, sea cucumber and oyster [76, 79-81, 84, 85].

3.4.2 Antioxidative peptides

To counter the production and harm of ROS the human body is equipped with several antioxidants and antioxidant systems. These defense systems involve mechanisms that prevent

free radicals from causing damage and mechanisms that repair or mitigate any occurred damage [86]. Antioxidants of both endogenous and exogenous origin can be of physiological relevance and are often classified as high-molecular (mostly proteins and enzymes) or low-molecular (smaller endogenous compounds and most exogenous compounds, such as vitamins and plant phenols) antioxidants. The AOC of proteins and peptides has been extensively reviewed previously [87]. They can act as multifunctional antioxidants e.g. by inactivating ROS [88], scavenging free radicals [89] and chelating pro-oxidative transition metals [90] in addition to reducing hydroperoxides to relatively non-reactive hydroxides [91, 92]. Some amino acids, in particular histidine, glutamic acid, aspartic acid, along with phosphorylated serine and threonine, have the ability to chelate prooxidative transition metals [93]. Both proteins and peptides can act as antioxidants through the mechanism of reducing hydroperoxides to relatively non-reactive hydroxides [91, 92]. Methionine is thought to be central in this process [91]. Peptides are usually considered to have higher AOC than free amino acids due to the stability of the resultant peptide radical that do not initiate or propagate further oxidative reactions [87]. However, the AOC depends on the amino acids being exposed and accessible to pro-oxidants and not buried in the core of the protein. An approach in order to increase the AOC is through disruption of the tertiary structure of proteins or peptides, and thus exposing amino acids. This can be attained by food processing, fermentation or gastrointestinal digestion.

Despite general acceptance that consumption of dietary antioxidants is an effective approach to increasing the body’s antioxidant load, and that evidence for association between oxidative stress and CVD is clear, experimental data on antioxidant intake and disease prevention are inconclusive. Some studies have demonstrated that dietary antioxidants in combination with n-3 PUFA reduced atherogenesis in animal atherosclerosis models [94-96]. However, while high natural antioxidant intake from foods have been proven beneficial [97], studies on antioxidant supplementation have failed to prove positive effects in preventing all-cause mortality and in some cases even demonstrated adverse effects [98, 99].

4 Summary of papers

Paper I. *Nutritional content and bioactive properties of wild and farmed cod (Gadus morhua L.)*

In this study the aims were to evaluate and compare the fatty acid and amino acid compositions and the ACE inhibitory activity of wild and farmed cod, as well as investigating how cooking affected these parameters. From twenty wild and farmed cod, three adjoining pieces were cut and randomly distributed in three groups; raw, poached and baked. The protein content was higher in farmed cod (19%) compared to its wild counterpart (16%), probably due to an intensive protein rich feeding regime. The relative distribution of amino acids, however, was quite similar. Cod is a lean species storing its lipids in the liver, and an intensive feeding regime would not affect muscular fat content significantly. Both counterparts had a fatty acid distribution of 30 % saturated fatty acids (SFA), 14 % MUFA and 50 % PUFA. The composition of the individual fatty acids, however, differed significantly, lipids of wild cod containing more DHA and less LA, EPA and docosapentaenoic acid. Poaching resulted in a significant loss of taurine, while the effect of baking was lower, corresponding to a lower water loss. The ACE inhibitory capacity did not differ significantly between wild and farmed cod (inhibitor coefficient $(IC)_{50}$ values of 0.063 and 0.060 mg/mL) and neither poaching nor baking affected the capacities to inhibit ACE. From this study we concluded that wild and farmed cod provides similar health-promoting effects that are maintained during cooking.

Paper II. *Proximate composition, antihypertensive and antioxidative properties of the semimembranosus muscle from pork and beef after cooking and in vitro digestion*

In this study we wanted to investigate the proximate composition of red meats; pork and beef. The AOC and ACE inhibitory activity were also measured and the impact of cooking on all parameters was evaluated. From 20 semimembranosus muscles from pork and beef, 100 g were cut and randomly distributed in two groups for cooking in a heated pan, or remaining raw as control. The fat content was significantly higher in pork (4.5 %) compared to beef (3.2 %) while the sum of amino acids was similar (approximately 19 %). The compositions of amino acids were also similar between pork and beef, providing sufficient amount of essential amino acids for our daily requirement. Taurine, however, was reduced significantly after cooking. The ACE inhibitory activity was measured after a simulated digestion and did not

differ significantly between pork and beef (IC_{50} values of 60 and 70 µg/ml, respectively), but was affected negatively by cooking (significant only for pork, however). The AOC was measured at three time points during a simulated digestion. At the end of digestion, raw samples of pork exhibited higher AOC than raw samples of beef, whereas the AOC of the cooked samples were equal. The conclusions from this study were that pork and beef are equally good nutritional sources and whereas the nutrients are not lost after cooking, the bioactive components may be reduced.

Paper III. *The potential of cod hydrolyzate to inhibit blood pressure in spontaneously hypertensive rats*

In this study we aimed to evaluate the *in vivo* antihypertensive effect of cod, haddock and salmon hydrolysates in SHR. Pieces of fish fillets were subjected to a simulated gastrointestinal digestion to obtain the hydrolysates. Male SHR, 10-weeks-old, were administered with 200 mg/kg body weight of cod, salmon or haddock hydrolysates for 21 days and thereafter 500 mg/kg body weight for seven days. Captopril was used as a positive control at a dosage of 20 mg/kg body weight and water as a negative control. Systolic BP increased on average 4.75 mm Hg/week in the negative control group. A significant BP decrease was seen in the group receiving captopril, while the hydrolysates failed to significantly inhibit the BP elevation during the 4 weeks. The group receiving cod hydrolyzate, however, experienced a lower BP rise compared to the other groups and after day 7 the BP leveled off and stabilized, not increasing further. The results obtained in this study indicate that cod may have a potential in inhibiting elevation of BP, although further studies are warranted to verify this.

Paper IV. S*eafood protein reduce atherosclerotic burden in female apolipoprotein E deficient (apoE$^{-/-}$) mice fed a Western type high fat diet for 13 weeks*

The last study was designed to evaluate the atherosclerotic burden and anti-atherogenic effects of different protein sources using female apolipoprotein E deficient (*apoE*$^{-/-}$) mice. Two groups (n=12) were fed *ad libitum* Western type diets containing chicken or a combination of cod and scallops (approximately 1:1 on nitrogen basis) as the protein source. After 13 weeks the mice were euthanized by carbon dioxide inhalation, the organs were harvested and the aorta dissected. The total aorta atherosclerotic plaque burden in mice fed cod-scallop was reduced 24% ($p<0.05$) compared to the chicken-fed group, whereas the reduction in the less lesion prone thoracic and abdominal parts of the descending aorta were

46% ($p<0.05$) and 56% ($p<0.05$), respectively. Mice fed cod-scallop gained less weight, and had lower serum levels of leptin and glucose, compared to the chicken-fed mice. The serum and hepatic concentrations of total cholesterol, triglycerides, glucagon, insulin and total proteins, did not differ significantly between the groups. Hepatic expression of the *Pon2* gene encoding for the endogenous antioxidant paraoxonase 2 was down regulated in mice fed cod-scallop compared to mice fed chicken, suggesting lower oxidative stress in these mice. The vascular cell adhesion molecule VCAM-1 encoding gene *Vcam1* was also down regulated. This study demonstrated a beneficial effect of marine proteins compared to chicken, as aorta atherosclerotic burden, serum glucose and leptin levels, were reduced in mice fed cod-scallop.

5 Methodological considerations

As mentioned in the introduction, it is generally accepted that diet has a major influence on human health and the development of chronic diseases, such as CVD. However, in order to investigate potential causal relationships between single dietary components and health effects, ultimately leading to establishing official dietary advice, several different studies are needed (**figure 7)**. Before the ultimate goal; documenting health effects in humans, preferably through randomized clinical trials, it is necessary to perform a series of initial basic experiments to form a fundament for further studies. In this thesis such experimental studies are performed, both *in vitro* and *in vivo*. *In vitro* studies are rapid and inexpensive to conduct, and although extremely simplified, such methods may function as indicators of mechanisms and modes of action. Further proofs of concepts must be completed by the use of animal studies. It is debatable to what degree results from animal models are of human relevance, but such models allow us to investigate mechanisms and disease development in a way intolerable in humans. In addition, animal models are demanded before clinical human trials can take place and further dietary advices and nutritional guidelines can be designed.

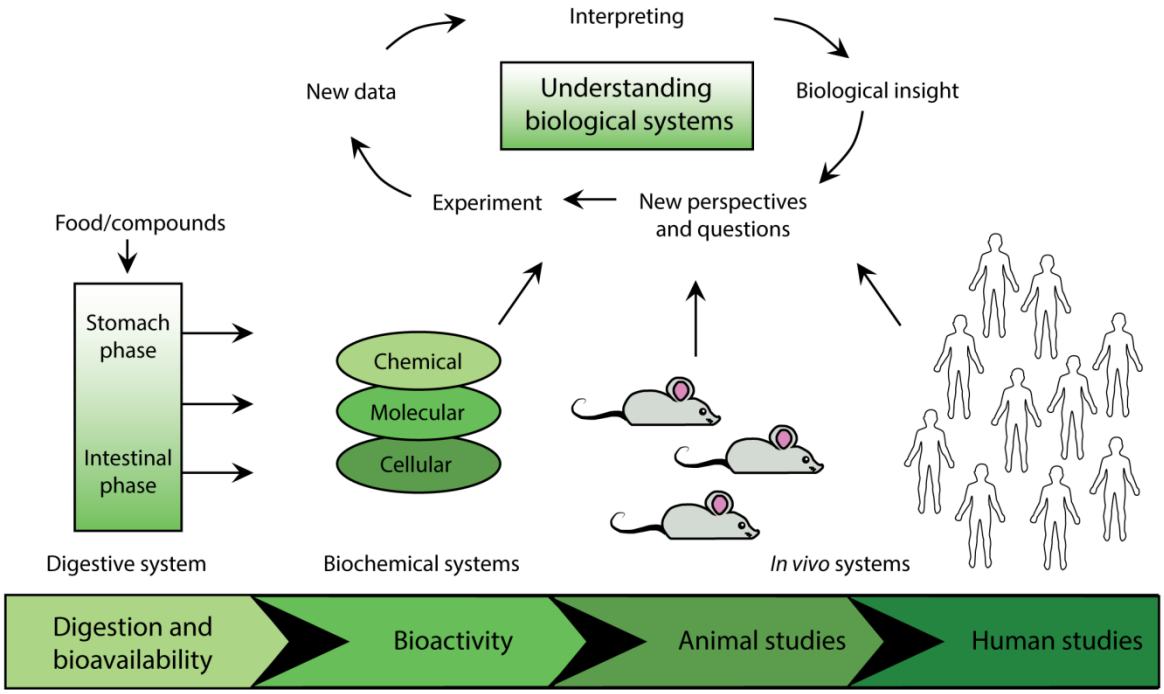

Figure 7 Schematic illustration of processes involved in documentation of health effects (ill. Ida-Johanne Jensen/Mari Walquist).

5.1 Raw material

5.1.1 Cod

Biochemical composition and nutritional quality of wild fish fillet may change during season. In fatty fish, it is mainly the fat content that is altered. The nutritional content of farmed fish, in particular the fatty acid composition and other lipids, may be influenced by the feed composition [100]. Nutritional changes due to seasonal variations or different feed compositions were considered to be outside the scope of this study. A limitation of the comparison made between wild and farmed cod (*Gadus morhua* L.) is the season in which they were obtained. The wild caught cod was caught in September, whereas the farmed cod was harvested in December.

5.1.2 Pork and beef

As pointed out in other publications, the nutritional composition, and in particular the fatty acid composition, of both pork [101] and beef [102] can also be influenced by the feed composition. Determination of the exact feed composition would require tracking of the specific animals from the farm to the slaughter house, a process logistically challenging and time consuming. As this was considered outside the scope of the project, it was not done in this study. Hence, flesh from pig (*Sus scrofa domesticus*) and beef cattle (*Bos taurus*) were obtained from a slaughter house and our results represent the nutritional value of pork and beef available for Norwegian consumers at the present time.

5.1.3 North Atlantic fish species

Paper III was a part of our cooperation with the Faroe Island Company Fiskaaling and includes several species and samples screened for ACE inhibitory activity. These species have, however, not been focused on in this thesis and the results will therefore not be discussed further.

5.2 Household preparations

Fish and meat are rarely consumed raw and in order to resemble the form in which they are ingested as part of the daily diet, all the raw materials were heat treated. Normally, fish and meat are exposed to different household preparations and the most common heat treatment

methods for each species were thus applied in these studies. However, although it is common to add oil or butter during preparation, this was omitted to exclude the bias of additional fat or altered fatty acid composition. Grilling, which is another common household preparation method, was deselected due to proteins being destructed at extremely high temperatures. Hence, cod was either poached or baked. Poaching was performed by soaking the pieces in 0.5 % NaCl boiling water (90-95 ºC) until reaching a core temperature of 64 ºC, after approximately 10 minutes. Baking was performed by wrapping the pieces in aluminum foil and heating in an oven at 175 ºC for 20 minutes. Meat was heated in a frying pan without oil until thoroughly cooked, reaching a core temperature of 75±3 ºC, after ten minutes on each side.

5.3 Proximate compositions

Water, ash, fat and protein content are important parameters in nutritional studies. For analysis of water and ash content, modified versions of the AOAC methods 925.04 and 938.08 [103] were used.

Extraction and determination of lipid content in different tissues can be performed by various methods [104]. According to FAO [105], the preferred method to determine lipid content for energy purposes is to analyze fatty acids and express lipid content as triglyceride equivalents, as this approach excludes wax and the phosphate content of phospholipids. Determining the lipid content gravimetrically is, however, also acceptable. In this thesis the lipid extraction method of Folch *et al*. [106] was used and lipid content was determined gravimetrically. The advantages of this method compared to the official AOAC method 945.16 using ether as solvent [103], are that it is less time consuming, requires less solvent, and also extracts polar lipids. In addition, Folch´s method allows for further analysis of the fatty acid composition. For safety reasons, chloroform was replaced with dichloromethane, since the latter is known to be less toxic [107-109]. This replacement was done both in lipid extraction, fatty acid analysis and for triglycerides and cholesterol analysis. The impact of this replacement was thoroughly tested for all types of raw material used and no differences in results were observed, neither in the amount of fat nor fatty acid composition.

Analysis of protein content in biological materials can also be conducted by different methods. The most common way of determining protein content is by determining the total nitrogen content and multiplying with 6.25 (a factor based on proteins containing 16%

nitrogen [110]). However, the presence of non-protein-nitrogen, such as free amino acids, nucleotides and creatine, and the varying nitrogen content of specific amino acids, result in a nitrogen content in proteins varying between 13 and 19 %, equaling conversion factors between 5.26 and 7.69 [105]. FAO [105] recommends that protein content is given as the sums of the individual amino acids (molecular weight of each amino acid minus the molecular weight of water). This recommendation was followed in this thesis.

5.4 ACE inhibitory activity

As mentioned, ACE converts the inactive decapeptide angiotensin I into the potent vasoconstrictor angiotensin II by cleaving off the dipeptide His-Leu. Evaluation of ACE inhibitory activity *in vitro* would ideally involve the substrate angiotensin I. However, this decapeptide is unstable and the method commonly used [111] is based on hydrolysis of the synthetic compound hippuryl-His-Leu (HHL) by ACE, resulting in hippuric acid (HA) and His-Leu as products. The amount of HA is then measured and compared to a control reaction where no ACE inhibitor is added. Traditionally the HA was extracted into ethyl acetate and quantified from its absorbance at 228 nm. This extraction is time consuming and complicated and a later developed direct analysis of the ACE mixture in a high performance liquid chromatography (HPLC) [112] was performed in this study. Other methods may also be used to evaluate ACE inhibitory activity [113, 114] and results are presented in various ways. This makes it challenging to compare results across studies. The results obtained in this thesis were therefore compared only within the samples evaluated or to previously reported results from our research group.

5.5 Antioxidative capacity

The term AOC is often used for simplicity reasons. It is, however, important to emphasize that this expression is rather unspecific as the different assays measure different capacities, such as reducing power, peroxyl scavenging or superoxide scavenging capacity [115]. Different assays are based on different strategies in terms of substrates, probes, reaction condition and quantitation and will thus give different information about the oxidant-antioxidant interaction. In this thesis, the oxygen radical absorbance capacity (ORAC) was chosen as the AOC indicator. The strength of the ORAC assay compared to other assays is that it is a kinetic reaction and thus measures AOC over a given time span, the reaction is

taken to completion, and thus both degree and time of inhibition is considered. This is beneficial when evaluating whole foods containing different compounds acting as either slow or fast antioxidants with or without lag phases. The results are often presented as gallic acid or Trolox equivalents, making them comparable across studies. In addition, this assay is performed at physiological temperature and pH, making it relevant to *in vivo* processes. Nevertheless, the ORAC assay has several shortcomings. It does not consider the lipophilicity which is crucial for incorporation into cell membranes. Since the reaction is carried out in a homogenous solution with an artificial radical to initiate the oxidation, it does not reflect the stability *in vivo* where the composition is heterogeneous and oxidation reactions are initiated by ROS, metal ions, heat or light.

5.6 Simulated gastrointestinal digestions

As previously mentioned some bioactive peptides are present in foods *per ce*, but more commonly they are part of larger proteins and need to be released to exert any effect. *In vivo*, proteins are catabolized during gastrointestinal digestion by enzymes present in the stomach and intestines, and may be degraded to bioactive peptides [116]. These may either exert an effect directly in the stomach or digestive tract, or in the circulatory system following absorption through the intestines [66]. Peptides exerting AOC can exhibit their effect already in the stomach or digestive tract, whereas ACE inhibitory peptides perform their activity in the circulatory system. Hence, the latter need to be absorbed intact through the intestine and further transported to the vascular system. In order to evaluate the potential bioactivity of peptides, evaluation of the effect of gastrointestinal digestion on proteins is necessary. *In vivo* feeding methods, using either animals or humans, would give the most accurate results. However, these methods are time consuming, expensive and often associated with ethical issues. Therefore, *in vitro* methods are commonly used, although compromises between accuracy and ease of utilization must be made. A number of *in vitro* digestion models are used, differing from each other in several aspects. Number of steps included in the digestion sequence (mouth, stomach, small intestine, large intestine), composition of the digestive fluids used in each step (salts, buffers, enzymes) and the mechanical stresses and fluid flows utilized in each step (magnitude and direction of applied stresses, flow profiles) commonly differ between models [117]. In this thesis, AOC and ACE inhibitory activity of digested protein sources were evaluated and due to the differences in biological mode of action, different gastrointestinal digestion models were chosen for evaluation of the different bioactivities.

Peptides exhibiting AOC may operate both in the stomach and intestines and hence, it was decided to use a previously described digestion model for changes in AOC during digestion [118] where samples were collected at start, after the gastric digestion and after the total gastrointestinal digestion (**figure 8**). In contrast, ACE inhibitory peptides do not exhibit any activity until they are absorbed intact and transported into the circulatory system. Therefore, another, more simple digestion model was used in which samples were collected only after termination of the simulated digestion [119] (**figure 9**). Several publications have presented the amino acid sequence of purified peptides derived from digested food proteins. There are, however, several challenges associated with such purified bioactive peptides. The separation and purification steps are time consuming and costly and the amount of purified peptide is often too small to be economically viable. It is difficult to provide the required documentation for a potential nutraceutical product, and if a peptide is commercially interesting, it is often cheaper to chemically synthesize it [120]. In addition, some amino acids, such as valine, leucine, isoleucine, phenylalanine and tyrosine [121], and peptides whose hydrophobic amino acid side chains are exposed, may have a bitter taste [122], making them less attractive as food components. The aim of this thesis was not to create a purified peptide to be isolated and up-scaled, but to study the potential bioactive effect one might get from consuming a particular protein source. The digests therefore contained a wide range of peptides and it was the collective ability to exert AOC or inhibit ACE that was tested.

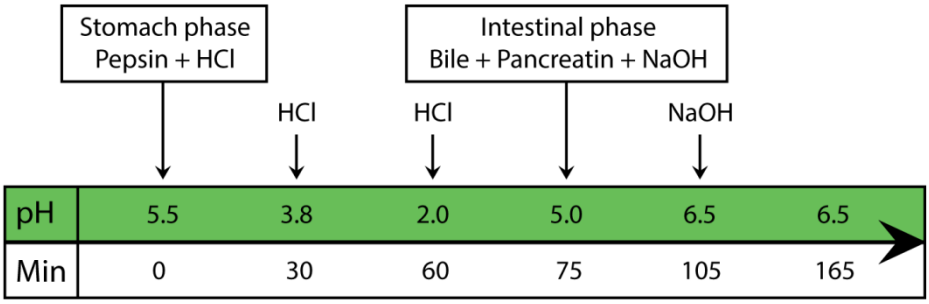

Figure 8 Illustration of the simulated *in vitro* digestion model used in evaluation of oxygen radical absorbance capacity (ORAC) (ill. Ida-Johanne Jensen/Mari Walquist).

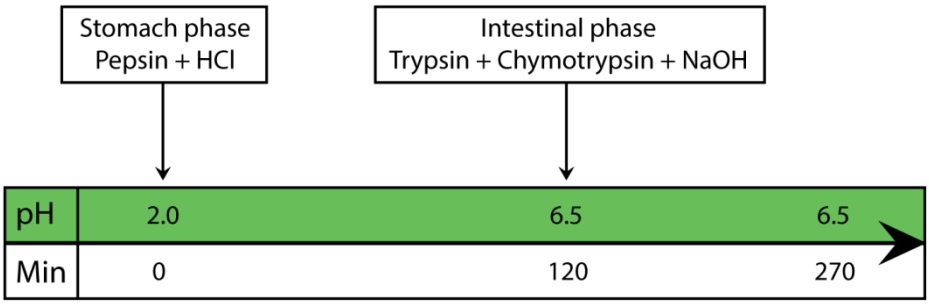

Figure 9 Illustration of the simulated *in vitro* digestion model used in evaluation of angiotensin converting enzyme (ACE) inhibitory activity (ill. Ida-Johanne Jensen/Mari Walquist).

5.7 Use of animals in laboratory experiments

The use of animals in science is of ethical concern. In addition, standardization is often difficult to achieve. Ethical guidelines are therefore established and a set of laws and regulations implemented to control animal experiments. Regulations state that one has to apply to the principles introduced by Russell and Burch [123] of humane experimental techniques to ensure animal welfare without comprising the research. These principles are known as the three R's: replacing animal experiments with non-animal alternatives whenever possible, reducing the number of animals to a minimum (however, a sufficient number to obtain significant results) and refining the experimental protocol, making sure animals suffers as little as possible. In this project we aimed to evaluate the effect of diet on hypertension and development of atherosclerosis. Mild hypertension can be studied in humans, but determining the connection between food consumption and inhibition of BP elevation is complex. For determination of mechanisms and progress of atherosclerosis, no *in vitro* models exist and there are obvious limitations in evaluating the risk of CVD in humans as invasive techniques cannot be performed. Therefore it was decided to use animal models proven to be appropriate for evaluating hypertension and development of atherosclerosis and risk factors associated with CVD.

5.8 Animal models used in this study

5.8.1 Spontaneously hypertensive rats

The first animal used to study hypertension was a dog in which the renal artery was clipped, creating secondary hypertension [124]. Today, the most frequently used model for studying

hypertension and inhibition of BP elevation is the one using spontaneously hypertensive rats (SHR) [125]. These rats are selectively bred Wistar rats which experience an elevation in BP already around 5-6 weeks of age. The SHR develop similar hypertension end-organ damages as humans such as cardiac hypertrophy [126], cardiac failure [126, 127], renal dysfunction [128, 129] and impaired endothelium-dependent relaxation [130]. They do, however, not develop stroke, atherosclerosis or thrombosis. Other rat models may be as accurate in order to evaluate inhibition of hypertension [125] but the SHR model is extensively used in the literature, thus ease comparison and this model was therefore elected in this study.

5.8.2 Apolipoprotein E-deficient mice

The use of animal models has been essential in the understanding of the atherosclerotic process. The study of pigs, rabbits, dogs and monkeys have been invaluable in defining cellular events in the initiation and development of atherosclerotic lesions [131]. In later years the use of rats and mice to study biomedical mechanisms of the atherosclerosis has increased tremendously due to them being readily available, having low maintenance costs, short generation time and reduced ethical concerns compared to larger animals. The lipid profile of wild type mice is markedly different from that of humans, having high levels of HDL and low levels of LDL and VLDL [132, 133]. Because of their lipid profile, mice do not usually develop atherosclerosis and feeding them an atherogenic diet would only lead to minimal lesion development, so-called fatty streak. Mice with a deleted gene (knock out) resulting in a defect lipid metabolism and consequent hypercholesterolemia, are therefore efficient animal models to study specific mechanisms related to atherosclerosis [134]. The two strains most extensively used are the LDLr deficient mouse (*ldlr*$^{-/-}$) and the *apoE*$^{-/-}$ mouse. While the *ldlr*$^{-/-}$ mice develop atherosclerotic lesions only after being fed cholesterol/high fat diets, the *apoE*$^{-/-}$ mice develop atherosclerosis spontaneously on normal diets. This advantage has made the *apoE*$^{-/-}$ mice particularly popular and the model is recognized as suitable for studying atherogenic mechanisms and effects of anti-atherogenic diets [132]. Apolipoprotein E$^{-/-}$ mice were therefore chosen for this project. There are several disadvantages and limitations in the use of *apoE*$^{-/-}$ mice and the transferability from mouse models to humans may be argued. While humans develop atherosclerosis most commonly in coronary arteries and aortic arch, the earliest lesions in mice are seen in the aortic sinus, the proximal part of the brachiocephalic artery and in the lesser curvature of the aortic arch. Mice do not develop plaque rupture and thrombosis, which is the most feared complication of atherosclerosis in

humans. In fact, most people do not recognize the atherosclerosis before thrombosis occurs. Despite these differences, it appears that atherosclerotic development is similar between mice and humans at early stages with initial formation of fatty streak that further progress into advanced lesions with a fibrous cap. The mouse model is therefore relevant for mechanisms that may be targeted by preventive approaches such as dietary guidelines.

5.9 Blood pressure measurement

Evaluation of BP is one of the basic procedures in biomedical research and a BP rise is a vital parameter to assess cardiovascular function. Measurements of BP in rats are usually conducted by three different methods. Operating an intra-arterial catheter into the artery allows for direct recording of BP real time. This method is invasive, requires anaesthesia and the animals must be euthanized after measuring. It is uncertain how the BP is affected by anaesthesia. The telemetric method is performed by implanting a transmitter in the abdominal cavity of the rats. Thereafter the BP can be monitored while the rats are awake and freely moving, thus eliminating or reducing handling and restraining stress [135]. This latter method is considered the gold standard of BP measurements [136] but is costly and implantation requires surgical skills. In this project we chose to use the tail cuff method. This method is a non-invasive method where the tail arterial blood flow is measured in the rats while being conscious. Despite acclimation of the animals to restraint, the situation is stressful and may affect BP. Another drawback of the method is that it is necessary to induce vasodilation in the tail in order to be able to measure BP. This is done by increasing the body temperature of the animal and may lead to a false elevation of BP.

5.10 Determination of plaque burden in aorta

The aorta and the proximal parts of its major branches, are the only vascular areas in mice that consistently develop quantifiable atherosclerotic lesions [134]. The extent of atherosclerotic plaque has usually been determined in cross-sections of the aortic origin close to the aortic valve. Cross-sections show to what extent the vessel lumen is occluded by lesion, but this is only a small area in the aortic origin and it is not practical for quantification of lesions involving major portions of the aorta. Since humans develop lesions most commonly in the coronary arteries and in the aortic arch [137], evaluating the lesion development in the mouse aortic arch, and entire aortic three, may seem more relevant. Such lesions can be quantified as

affected areas of the surface of the opened aorta. In this project, the aortas were removed in their entity and opened longitudinally. The luminal surface was then stained using oil red O which specifically binds to lipids (**figure 10**). The area of staining, covered by plaque and affected by atherosclerosis, was determined by photographic images and presented relative the total aortic area. The threshold levels were determined by the first aorta image and thereafter used as "default" threshold. This was, nevertheless, adjusted to the actual aorta image so that differences in background light did not affect the determination. The results from this method correlate well with those from cross-section determinations [138]. An advantage of this *en face* method is that is allows for a more accurate determination of distribution, number and shape of the lesions. However, determining the plaque burden *en face* has several shortcomings. The three-dimensional nature of the lesions is unclear and thus determining the developmental stage of the plaque is difficult. Lesions with the same area coverage might be severely different in progress, narrowing the lumen differently. Despite being genetically homogenous and kept in a highly controlled environment; there is a large degree of between-mice variability in lesion development. Histological and immunohistochemical evaluation of the lesions would provide important additional information as it would be possible to identify the content of the atheroma, individual cells and stained cellular markers and proteins, and thereby the developmental stage. The most thorough quantitative evaluation would be to do *en face* evaluation, cross sectional evaluation and immunohistological staining of the lesions. We did, however, not have the possibility to perform the two latter analyses.

Figure 10 Quantification of atherosclerotic plaque burden. The opened aorta was stained using oil red O which specifically bind to lipids. The area of staining and covered by plaque was presented as relative area of the total aortic area (Photo: Ida-Johanne Jensen).

5.11 Evaluation of gene expression

In the mouse atherosclerosis study we chose to study gene expression of a selection of genes involved in various phases of the atherosclerotic process. Some genes were chosen to evaluate endogenous antioxidants, some were chosen to study the production of cytokines and

chemokines, and others because they are important in fatty acid and cholesterol metabolism. We analyzed this using the liver, since this is the central organ for lipid and cholesterol metabolism/regulation. By measuring a battery of genes related to the function of the heart muscle we aimed to observe early indications of malfunction in this muscle. We also wanted to investigate the gene expression in the cardiac muscle as a surrogate for direct assessment of the cardiac function.

5.12 Serum or plasma for analysis

Blood volumes of the mice used in this project are rather limited as 50 to 500 µl of plasma or serum may be obtained. Apolipoprotein $E^{-/-}$ mice are characterized by accumulation of circulating lipids, and both plasma and serum samples have a high content of lipids. These samples can be used for various hematological and lipid analyses such as determination of cholesterol, triglycerides, insulin and glucose, in addition to measurement of inflammatory markers such as cytokines, chemokines, adipokines and adhesion molecules. In human studies, serum and plasma derived from blood samples containing different anticoagulants are taken for the different analyses. The limited sampling volumes available from these studies forced us to choose one separate sampling protocol for all tests. In previous studies using such mice, it was experienced that heparin-, citrate- and EDTA-plasma, often contained particles that interfered with the lipid analyses. Based on this knowledge serum was chosen, despite the serum not being the optimal sample for assessment of circulating cytokines, chemokines and other inflammatory markers. Plasma samples need to be centrifuged and frozen immediately after blood sampling to avoid bias from different incubation and handling times and by using serum samples these biases were limited.

6 Discussion of main results

The main objective of this thesis was to compare the proteins of seafood with proteins of terrestrial meats, emphasizing the cardio-protective effects. The effects of cooking and digestion on nutritional composition and bioactivity were studied *in vitro* whereas the the antihypertensive and anti-atherogenic effects were investigated *in vivo* in rats and mice, respectively. The research materials were farmed and wild cod, together with pork and beef. As scallops are known to be high in taurine, these were included together with cod to create a marine protein source high in taurine. Pork was initially selected to represent a source of white meat. However, from January 2011 the Norwegian health authorities adjusted their definition of white meat and redefined pork to be a red meat [139]. Hence, when the animal experiment started it was decided that chicken was to be used as a terrestrial white meat source.

6.1 Nutritional composition

The nutritional composition was evaluated in all raw materials (paper I and II). Water content was higher in cod (81 and 78 % for wild and farmed, respectively) than in both pork (74 %) and beef (75 %) and the fat content was lower (approximately 1 % compared to 4.5 % in pork and 3.2 % in beef). The protein contents, defined as the sum of amino acids were similar for pork, beef and farmed cod and slightly lower for wild cod. The distribution of the individual amino acids was relatively similar for all four protein sources; the relative content of cysteine was higher in cod than in the meats and that of proline, β-alanine and histidine was higher in pork. Taurine was present in cod in almost three times the amount of meat, whereas histidine was almost twice as high in meat compared to fish. All analyzed protein sources contained sufficient amounts of essential amino acids in order to cover our daily requirement in one portion of 100 g.

6.1.1 Losses of nutrients after cooking

The impact of cooking on the nutritional composition and bioactive components were evaluated (paper I and II) and it was shown that the main influence of all cooking methods was loss of water content and thus a relative increase in protein and fat contents. As mentioned, the heat treatments applied in this study were relatively mild and thus, using rougher methods could have resulted in larger alterations after cooking. When true retention

was calculated, no reductions in protein or lipid contents after cooking were observed, in fact the fat retention in pork and beef was higher than 100 %. This may be explained by the fact that proteins may be denatured under heat resulting in bound lipids being released and thus more easily extracted [140]. The heat treatments were designed to simulate the normal preparing techniques applied when preparing meals from these protein sources. Even if all heat treatments applied were considered mild, the treatment of pork and beef was by nature tougher than that of cod. This may explain why lipid retentions from pork and beef were higher compared to that of cod. The core temperature in the meat pieces reached 75°C, whereas the outside were subjected to direct contact with the heating element thus reaching higher temperatures. Thus, Maillard browning reaction and formation of insoluble protein aggregates with high molecular weight may have occurred as previously demonstrated [141]. The Maillard reactions may also impair protein nutritional value and reduce bioavailability of amino acids, lysine in particular. However, no reduction in lysine was observed, suggesting that the browning reaction was still in an early phase. Taurine, the exclusively free amino acid, however, was significantly reduced after heat treatments. Poaching affected the taurine loss more severe than baking due to a higher water loss. It has also previously been demonstrated that the taurine loss corresponds to water loss and processing [142].

6.2 Bioactivity and impact of household preparation

Prior to studying hypertension and atherosclerosis in animal models, the ACE inhibitory activity and the AOC measured as ORAC, were evaluated *in vitro*. As mentioned, the ACE inhibitory activity was measured in samples after a completed simulated digestion, whereas the ORAC was measured at different time points throughout a simulated digestion.

6.2.1 Angiotensin converting enzyme inhibitory activity

Fillets of wild and farmed cod and pieces of pork and beef were hydrolyzed in a simplified *in vitro* digestion model and thereafter evaluated for ACE inhibitory activity (paper I and II). The results were presented as the concentration of dried hydrolysates needed to inhibit 50 % of 1 mU ACE activity (IC_{50}) (**table 1**). When comparing the raw samples, farmed cod and pork showed the highest ACE inhibitory activity with an IC_{50} value of 60 µg/ml while beef showed the lowest activity with an IC_{50} value of 70 µg/ml. The differences were not significant. Previous measurements of ACE inhibitory activity in cod and salmon have been

conducted using the same model [119] showing similar results for cod and a slightly lower activity (88 µg/ml) for salmon. Although results on raw samples give an indication of potential antihypertensive effect and are valuable for comparison with previously reported results, it may be argued that it is more relevant to evaluate food proteins as they are typically consumed. In this project the cod fillets were poached or baked and the meat pieces were cooked in a heated pan. While poaching and baking slightly enhanced the ACE inhibitory activity of cod compared to the raw fillet, cooking reduced the ACE inhibitory activity of meat (significant only for pork). Since the results are presented per gram dried hydrolyzate, the differences observed cannot be attributed to differences in water content. After subjection to normal household preparations, it was evident that cooked fillets of cod showed significantly higher ACE inhibitory activity than cooked pieces of pork and beef. Poaching and baking are, as mentioned, more gentle methods of heat treatment than cooking in a heated pan. Although no differences in amino acid compositions were observed between raw and cooked samples, it is likely to assume that some protein aggregation occurred in the cooked meats, reducing the digestibility, leading to a lower ACE inhibitory activity. In addition, terrestrial meats are, as previously mentioned, higher in connective tissue which may impede digestibility.

This *in vitro* ACE inhibitory activity assay only considers the action of components on ACE and does not regard other possible BP reducing actions of other components, such as taurine. It is assumed that taurine may function as a BP reducing agent, although the mechanisms of actions are unclear. Taurine has shown BP reducing effects in several rat models [143], such as the SHR [144], the DOCA-salt rat [145], the salt-sensitive Dahl-S rat [146] and the renovascular hypertensive rat [147]. Since antihypertensive effects have been found in all models, it seems likely that taurine may reduce BP by multiple mechanisms. Cod had significantly higher taurine content than the meats (1.1 and 1.2 mg/g fillet compared to 0.3 and 0.4 mg/g meat), so it is likely that the *in vivo* antihypertensive effect of cod would be higher than that of meat. However, the differences found *in vitro* are not easily interpreted into *in vivo* conditions.

Table 1 Angiotensin converting enzyme (ACE) inhibitory activity (µg/ml) of digested raw or cooked pieces of different food protein sources.

Protein source	IC_{50} value
Wild cod, raw	63 ± 5
Wild cod, poached	55 ± 5
Wild cod, baked	54 ± 5
Farmed cod, raw	60 ± 5
Farmed cod, poached	58 ± 5
Farmed cod, baked	57 ± 5
Pork, raw	60 ± 3.2
Pork, cooked	80 ± 3.2
Beef, raw	70 ± 6.3
Beef, cooked	90 ± 3.2

Data are presented as the concentration of sample needed for 50 % inhibition (IC_{50}) of ACE in a 1 mU ACE-assay.

6.2.2 Oxygen radical absorbance capacity

The ORAC of pork and beef was measured throughout a simulated digestion of raw and cooked samples (paper II). For all the samples ORAC increased significantly during the pepsin digestion and leveled off during the pancreatic digestion. A similar development has previously been documented for saithe and shrimp [148]. After the pepsin digestion, the antioxidative peptides may have been released to such a degree that a high proportion of the antioxidative amino acid residues or side chains had been exposed, thus further digestion did not increase the AOC. The ORAC was significantly higher in pork than in beef both at the start and at the end of digestion. The amount of amino acids and the amino acid distribution was similar for pork and beef except the amino acid histidine which was present in higher amounts in pork meat. This amino acid has been shown to quench hydroxyl radicals [149], so the difference seen in the ORAC may be attributed to this amino acid residue or to the amino acid sequence of the released peptides, which was not investigated in this project. Peptides from marine origin released during digestion have previously been documented to exhibit antioxidative properties and have been thoroughly reviewed [150]. Peptides extracted from micro algae by-product has been digested with pepsin and the hydrolysates exhibited higher AOC compared to both Trolox and butylated hydroxytoluene [151] demonstrating that

antioxidants from food can be equally or even more effective than synthetic ones. Despite several *in vitro* assays demonstrating AOC and creating evidence for an association between oxidative stress and CVD, the preventive effect of antioxidative peptides *in vivo* remains to be proven.

6.3 Antihypertensive effect of cod hydrolysates in SHR

It has previously been revealed that peptides containing 2-3 amino acids exhibit the greatest ACE inhibitory effects [152] and indeed Fujita *et al*. [83] demonstrated that the tripeptide Leu-Lys-Pro exhibited higher ACE inhibitory activity compared to the prodrug peptide Leu-Lys-Pro-Asn-Met. The antihypertensive effect of cod hydrolyzate was evaluated in SHR and compared to salmon and haddock hydrolysates (paper III). Although results from the *in vitro* study were promising, neither of the fish hydrolysates significantly inhibited the natural BP rise in SHR (**figure 11**). This may be explained by the fact that the animals were administered with hydrolysates already subjected to a simulated digestion. A criterion for *in vivo* effect of bioactive peptides is that they remain intact during uptake in order to affect the cardiovascular system. The further *in vivo* digestion may have degraded the peptides to such a degree that single, free amino acids were released. Despite lack of significant inhibition, the BP in the rats administered with cod hydrolyzate leveled off at day 7 and did not increase further, suggesting some inhibitory activity of the cod hydrolyzate. As previously mentioned, it is difficult to establish a direct correlation between *in vitro* and *in vivo* conditions due to the bioavailability of the active ingredients after oral administration [153]. Fujita *et al.* [67] demonstrated this discrepancy in bonito and chicken peptides. Bonito peptides exerted higher *in vivo* antihypertensive activity than expected from the *in vitro* activity. On the contrary, peptides from chicken which exhibited high *in vitro* activity, failed to show antihypertensive activity after oral administration in SHR. Several animal studies have been performed evaluating BP reducing effect of different food protein sources. Peptides from tuna frame, tuna muscle, yellowfin sole, oyster and wakame have all been compared to Captopril showing maximum effect three hours after administration [76, 79-81, 84]. These peptides were, however, purified and the results are therefore not comparable with the results obtained in this project where the hydrolysates are mixtures of different peptides obtained from digestion of a protein meal.

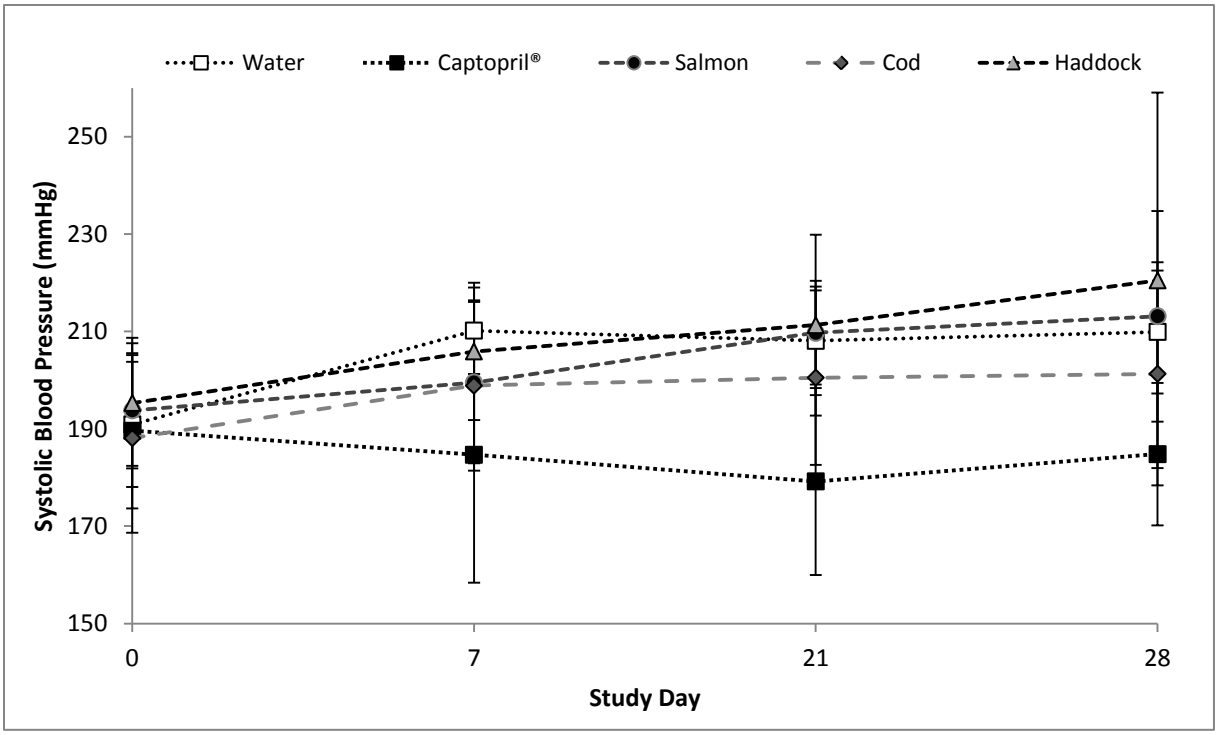

Figure 11 Development of systolic blood pressures (mmHg) of rats treated with hydrolysates of Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), water (as negative control) or Captopril® (as positive control). The dosages were 200mg/kg body weight the first 21 days and 500 mg/kg body weight the last 7 days. Captopril was dosed at 20 mg/kg body weight.

6.4 Effect on atherosclerosis in apolipoprotein E-deficient mice

The effect of seafood protein on atherosclerosis was evaluated using female *apo* $E^{-/-}$ mice fed Western type diets (paper IV). Cod and scallop were combined 1:1 on nitrogen basis to create a lean marine protein source high in taurine and glycine, whereas chicken was chosen as a lean protein source of terrestrial origin. The total plaque burden in the aortas of mice fed cod-scallop protein was significantly lower compared to the plaque burden of mice fed chicken protein (**figure 12**). The plaque burdens in the aorta thoracic, abdominal as well as total area were reduced by 46%, 56% and 24% in mice fed cod-scallop compared to mice fed chicken. As the serum concentration of triglycerides did not differ between the two groups, other mechanisms seemed more likely to be involved in this plaque burden reduction. The cholesterol content in the chicken diet was slightly higher compared to the cod-scallop diet (2.0 g/kg and 1.9 g/kg, respectively) but this difference is not sufficient to cause a reduction in aorta atherosclerosis. Taurine and glycine are known to reduce cholesterol levels and the higher dietary level of these in the cod-scallop fed mice may have contributed to a reduced level of circulating cholesterol by increasing cholesterol clearance from blood circulation and excreting cholesterol as bile (taurocholic and glycocholic) acid from intestines [154]. The

reduction of serum cholesterol in the cod-scallop-fed mice compared to the chicken fed mice was, however, not significant. The atherosclerotic development is known to be accelerated by oxidative stress [155] with subsequent up-regulation of endogenous cellular antioxidants such as the protein paraoxonase2 (PON2) [156]. We have previously demonstrated higher AOC of cod compared to meats *in vitro* (unpublished results). In the cod-scallop fed mice the expression of the *Pon2* gene was lower compared to chicken-fed mice, suggesting less oxidative stress and a possible explanation for the reduced atherogenesis. Taurine, known to possess AOC [157], may have contributed to the lower oxidative stress in the cod-scallop fed group. The expression of the *Vcam1* gene encoding for the cellular adhesion molecule VCAM-1 was also lower in the cod-scallop fed mice. This protein mediates firm adhesion of leukocytes which then migrate into the sub-endothelium. The *Vcam1* gene is normally up regulated by hyperlipidemia and by inflammatory cytokines [158], proposing a potentially higher inflammatory condition in the chicken fed mice, but hepatic expressions of inflammatory marker genes did not differ between the two groups.

The mice fed chicken protein gained more weight and had higher concentration of leptin compared to cod-scallop-fed mice. Leptin is recognized as a key hormone in regulation of food intake and energy expenditure [159] and recently it has also been demonstrated to play a direct role in almost every step in the atherosclerotic plaque development [160].

Another indication that may explain the lower atherosclerotic burden in cod-scallop-fed mice, is the lower glucose concentrations in this group. An elevated blood glucose level over a prolonged time may lead to insulin resistance and eventually diabetes type 2, which is a known risk factor for CVD.

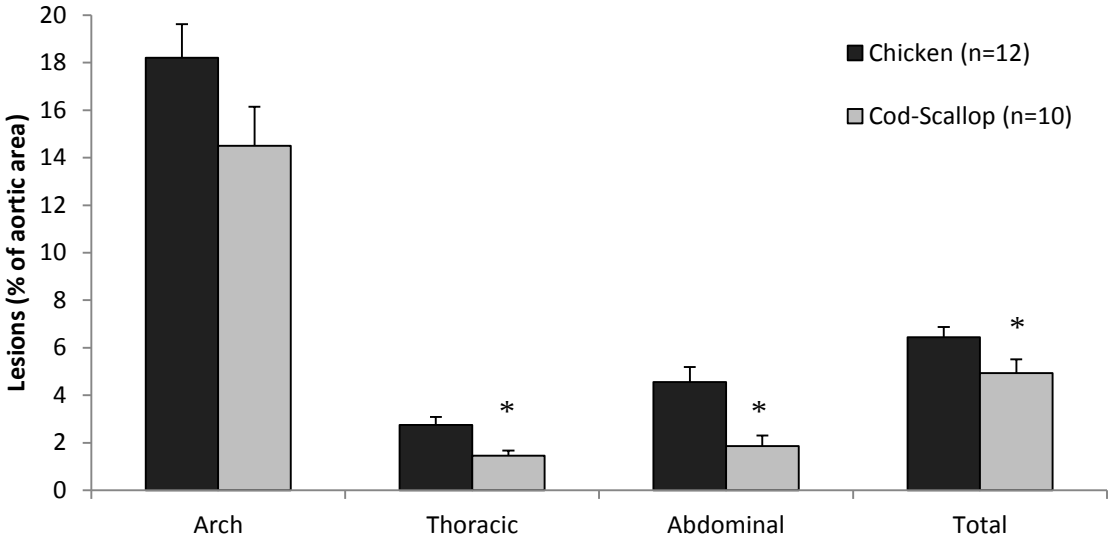

Figure 12 Atherosclerotic plaque burden, expressed as area percentage covered by lipid Oil Red O staining, in female *apoE*$^{-/-}$ mice fed diets containing different protein sources for 13 weeks. Data are mean ± standard error of the mean and were analyzed using the independent samples t-Test. *Atherosclerotic lesion is significantly different between the dietary groups ($p<0.05$).

7 Conclusions and further work

The nutritional content of cod and terrestrial meats was comparable. The main differences were lower fat content in cod and three times higher taurine content compared to meats. Traditional household preparations mainly influenced the water content and thus the relative amount of lipids and protein. The composition of amino acids in proteins did not change after cooking, but the exclusively free amino acid taurine was lost correspondingly to the water loss. Cooked cod provided higher ACE inhibitory activity compared to cooked meats, but the *in vivo* effect remains to be evaluated.

The antihypertensive effects of different fish hydrolysates were evaluated in SHR and the results indicated that cod hydrolyzate have potential to inhibit BP rise, though not proven significant in this study.

The cardioprotective effects of seafood proteins were compared to those of chicken protein in *apoE*$^{-/-}$ mice. To enhance the taurine and glycine content in the marine protein diet fed to mice, scallops were included. It was demonstrated that seafood proteins exhibited a beneficial effect on overall atherosclerotic burden, probably due to a combination of factors, such as slightly lower serum cholesterol concentrations, lower leptin and glucose, and less oxidative stress in the mice fed cod-scallop.

During the work of this project, limitations of the studies have been disclosed and new questions have arisen, revealing the need for follow-up studies. The nutritional composition of chicken, and it´s *in vitro* bioactivities should be evaluated for comparison with lean marine protein and red meats. Both animal studies should be expanded to cover a longer time span, probably giving more consistent results and maybe result in more pronounced between-group differences. The rats ought to be given cod as the protein source included in normal feed to give valuable insight in the antihypertensive effect of our diet *per se*. As BP measurements in humans are non-invasive and relatively simple to perform, a human clinical trial aimed to investigate antihypertensive effect of cod as the protein source would be possible to conduct. Also during the experiment on evaluation of anti-atherogenic effects of seafood, some study design limitations were revealed, proposing follow-up studies. Faeces samples should be collected, to calculate true digestion, energy expenditure and clarify the excretion mechanisms involved in for instance cholesterol metabolism. Such knowledge could improve understanding the mechanisms underlying the atherosclerotic process.

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Additional papers

During the period of my PhD, I have contributed to other articles and book chapters that may serve as background material on nutritional and bioactive components in fish and seafood. These are, however, not regarded as part of this thesis.

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Paper I

Paper II

Paper III

Paper IV