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# Cholesterol-lowering and anti-atherogenic effects of oats in mice.

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# Abstract

The cholesterol-lowering effect of oats is well established, but the crucial properties eliciting this effect need to be further investigated to optimize the use of oats as functional foods. Furthermore, there are almost no reports investigating the effect of oats on atherosclerosis development. This thesis describes our work with finding suitable mouse models to study cholesterol-lowering and anti-atherogenic effects of oats, the mechanism behind, and how processing of oat foods might interfere with these beneficial effects.

We found that supplementation of oat bran to an atherogenic diet significantly reduced plasma cholesterol and LDL+VLDL concentrations in C57BL/6 mice. The responsiveness to oats did however differ between two substrain of mice. Oat intake resulted in reduced plasma cholesterol, increased faecal excretion of bile acids and cholesterol, and increased expression of the bile acid producing enzyme CYP7A1 in the C57BL/6NCrl substrain. None of these parameters were altered in the C57BL/6JBomTac mice. The different expression of CYP7A1 in the two substrains of C57BL/6 strongly supports the importance of increased bile acid excretion, together with increase of bile acid synthesis from cholesterol, for oats to reduce levels of cholesterol in plasma.

To address how processing of oats might interfere with its cholesterol-lowering properties, beta-glucans were enzymatically digested to different molecular weights and then fed to C57BL/6NCrl mice. Reducing the molecular weight of the beta-glucans affected its viscous properties *in vitro*. It also affected the production of short chain fatty acids in caecal contents of the mice, but did not influence the cholesterol-lowering properties. Thus molecular weight and viscous properties of beta-glucans do not seem to be crucial parameters for the cholesterol-lowering properties of oats in the C57BL/6 mice.

When studying effects of oats on atherogenesis and inflammation we used a mouse model developing more pronounced hypercholesterolaemia, the LDL-receptor deficient mice. Oats reduced plasma cholesterol and levels of LDL+VLDL in this model too, and also reduced plasma concentrations of the inflammation markers fibrinogen and vascular adhesion molecule-1 (VCAM-1). Most importantly oat bran in the diet reduced incidence of atherosclerotic lesions in both the aortic root and the descending aorta. These findings demonstrate that oats have anti-atherogenic properties, and support health claims that oats can reduce risk of cardiovascular disease.



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# List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. **Andersson KE**, Immerstrand T, Swärd K, Bergenståhl B, Lindholm MW, Öste R, Hellstrand P. Effects of oats on plasma cholesterol and lipoproteins in C57BL/6 mice are substrain specific.  
*Br J Nutr*, doi:10.1017/S000711450999211X, published online by Cambridge University Press 20 October 2009.
- II. **Andersson KE**, Andersson U, Xu J, Ahrné S, Mohlin G, Swärd K, Holm C, Hellstrand P. Substrain differences in cholesterol response to oats in C57BL/6 mice correlate with differences in liver enzyme expression but not glucose tolerance or diversity of intestinal microbiota. (Manuscript, 2009)
- III. Immerstrand T, **Andersson KE**, Wange C, Rascon A, Hellstrand P, Nyman M, Cui S, Bergenståhl B, Öste R. Effects of oat bran, processed to different molecular weights of beta-glucan, on plasma lipids and caecal formation of SCFA in mice. (Submitted, 2009)
- IV. **Andersson KE**, Svedberg KA, Lindholm MW, Öste R, Hellstrand P. Oats (*Avena sativa*) reduce atherogenesis in LDL-receptor deficient mice. (in revision, *Atherosclerosis*, 2009)





# Abbreviations

AACC	American association of cereal chemists
ABC	ATP-binding cassette protein
ACAT	Acyl-CoA-cholesterol acyltransferase
ASBT	Apical sodium-dependent bile acid transporter
B6JB	C57BL/6JBomTac
B6NC	C57BL/6NCrl
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7 $\alpha$ -hydroxylase
eNOS	Endothelial nitric oxide synthase
FDA	Food and drug administration (U.S.)
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
$\alpha$ -HC	7 $\alpha$ -hydroxy-4-cholesten-3-one
HDL	High density lipoprotein
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-CoA
HNF	Hepatocyte nuclear factor
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
LDL	Low density lipoprotein
LDLr <sup>-/-</sup>	LDL-receptor deficient mice
LRP	Low density lipoprotein receptor-related protein
LXR	Liver X receptor
MCP-1	Monocyte chemotactic protein 1
M <sub>w</sub>	Molecular weight
NO	Nitric oxide
NPC1L1	Nieman-Pick C1-like 1
NTCP	Na <sup>+</sup> -taurocholate cotransporting polypeptide
OATP	Organic anion transporter
OST $\alpha$ / $\beta$	Organic solute transporters
PPAR	Peroxisome proliferator-activated receptor

PPIA	Peptidylprolyl Coenzyme A
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCFA	Short chain fatty acids
SNP	Single nucleotide polymorphism
SR-BI	Scavenger receptors, class B, type I
SREBP	Sterol regulatory element binding protein
TNF	Tumour necrosis factor
TRFLP	Terminal restriction length polymorphism
VCAM-1	Vascular adhesion molecule-1
VLDL	Very low density lipoprotein

# Introduction

The main role of our diet is to provide enough macro- and micronutrients to satisfy our need for energy, growth and development. During the past decades the concept of “functional foods” has emerged, implying that some foods or components in foods are biologically active and can modulate various body functions, thereby being beneficial to health and reducing the risk of disease<sup>1</sup>. This function of foods is however not a modern concept since Hippocrates already 400 BC expressed it as: “Let your food be your medicine and your medicine be your food”. Present-time nutritionists and medical staff meet a challenge in providing consumers and patients with accurate information on biologically active foods, based on solid scientific research.

In western societies the most common cause of death are complications of atherosclerosis like myocardial infarction and stroke. Many genetic and environmental risk factors have been identified to drive the development of atherosclerosis in humans and experimental animals. Among the risk factors, elevated levels of serum cholesterol have been identified as the most important<sup>2</sup>. Hence the most common treatments for atherosclerosis are therapies directed at lowering cholesterol levels. Although current therapies effectively lower plasma cholesterol levels and reduce cardiovascular causes of death, many patients still experience adverse coronary events<sup>3</sup>. It is therefore a challenging work to find alternatives and/or supplements to the existing cholesterol-lowering drugs.

The cholesterol-lowering properties of oats were discovered already in the 1960's<sup>4</sup>. Almost half a century later, the mechanisms of action are not yet fully understood and there is also a lack of knowledge on how the health properties are affected by food processing, storage or packaging procedures. Furthermore, the ability of oats to reduce plasma cholesterol is often said to prevent cardiovascular disease, but very few reports have actually directly addressed effects of oats on atherosclerosis development or heart disease. The aim of the work presented in this thesis was to find a suitable mouse model in which cholesterol-lowering effects of oat preparations can be systematically studied, mechanisms of action investigated, and effects on atherosclerosis development evaluated.



# Background

## Oats & Cholesterol-lowering effects

Since it was first discovered in 1963<sup>4</sup>, the cholesterol-lowering effects of oats and oat beta-glucans have been extensively studied both in humans<sup>5-9</sup> and animals<sup>10,11,12-14</sup>. The cholesterol-lowering effect is usually ascribed to the mixed linked soluble (1→3),(1→4)-β-D-glucan fibres present in oats, referred to as beta-glucans below. The food and drug administration (FDA) of the United States allowed the use of health claims for the cholesterol-reducing effect of oat products in 1997, followed by the Swedish Nutrition Foundation in 2001 and the U.K. Joint Health Claims Initiative in 2004<sup>15,16,17</sup>. The majority<sup>7,8</sup>, but not all<sup>6,9</sup> trials investigating the hypocholesterolemic effect of oats and/or oat beta-glucans show significant reduction of the plasma cholesterol. When FDA stated the health claim they reviewed 33 clinical studies; 21 of these showed significant reduction of blood cholesterol, whereas 12 did not<sup>17</sup>. There could be several reasons for the variable results. Levels of initial plasma cholesterol in the test subjects have been suggested to influence the response<sup>18</sup>, and the nature of the oat product is of great importance. In some studies the product contained too low levels of beta-glucans to be likely to achieve an effect. There are also indications that processing, cooking and storage of oat products change the physicochemical properties of oat beta-glucans, and thus may also change their physiological effects<sup>19</sup>. The poor understanding of the mechanisms by which beta-glucans reduce cholesterol levels also contributes to difficulties in understanding the different outcomes of the studies.

## Cholesterol

Cholesterol is an essential building block in cell membranes and is a precursor for steroid hormones, bile acid production and dermal synthesis of vitamin D. Cholesterol can either be absorbed from the diet or synthesized in the body. The liver has traditionally been regarded as the primary organ for cholesterol biosynthesis, which however also occurs in extra-hepatic organs such as the central nervous system<sup>20</sup> and the intestine<sup>21</sup>. The total cholesterol concentration in human plasma ranges from 2.5 to 7.5 mmol/l (100-300 mg/dl), and European guidelines recommend levels less

than 5 mmol/l<sup>22</sup>. Cholesterol is not water-soluble and is therefore carried in the blood by lipoproteins, of which the four most common are chylomicrons, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL). There is no mechanism for degradation of cholesterol in the body, so the only route of elimination is via bile acid excretion in faeces<sup>20</sup>. Although vital for human life, cholesterol is often portrayed in a negative manner because the plasma cholesterol level represents the most important risk factor for development of cardiovascular disease<sup>23</sup>.

## Cholesterol synthesis

Acetyl-CoA is the precursor of cholesterol synthesis and is itself synthesized from various sources of fatty acids<sup>24</sup>. Acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase and then further to mevalonate by HMG-CoA reductase, resulting in cholesterol multiple steps later. It is the conversion to mevalonate by HMG-CoA reductase that is the rate-limiting step in the cholesterol biosynthesis<sup>23</sup>.

The cholesterol synthesis is tightly regulated by the amount of available cholesterol. Inhibition of cholesterol absorption increases the synthesis, whereas interference with HMG-CoA reductase increases the absorption of cholesterol. Short term control involves degradation of the enzyme. HMG-CoA reductase is anchored to the membranes of the endoplasmic reticulum (ER) by its N-terminal end, whereas the C-terminal end is soluble and contains all the catalytic activity. The attachment to the membrane makes the reductase stable only in sterol-depleted cells. When sterol concentration increases the enzyme is rapidly degraded<sup>25</sup>.

Long term control involves regulation of gene expression, and the delicate balance of cholesterol synthesis and elimination is controlled in part by transcription factors such as the liver X receptors (LXRs) and Sterol Regulatory Element Binding Proteins (SREBPs; see below). Crosstalk exists between these, and they down-regulate hepatic HMG-CoA reductase as a response to elevated intracellular levels of cholesterol<sup>20,23</sup>.

## Sterol Regulatory Element Binding Proteins (SREBPs)

SREBPs are synthesized in the endoplasmic reticulum (ER). They are transported to the Golgi complex where they are cleaved by proteases to become soluble and able to enter the nucleus and act as transcription factors. SREBPs activate all enzymes in cholesterol synthesis, most importantly the HMG-CoA reductase, and also the LDL-receptor. Consumption of high-fat diet results in accumulation of cholesterol in liver membranes, which blocks the transport of SREBPs to the Golgi. Instead SREBP are trapped in the ER membrane, with no access to target genes in the nucleus.

Therefore transcription of the target genes declines, contributing to less cholesterol production, and fewer LDL-receptors on the hepatocytes<sup>25,26</sup>.

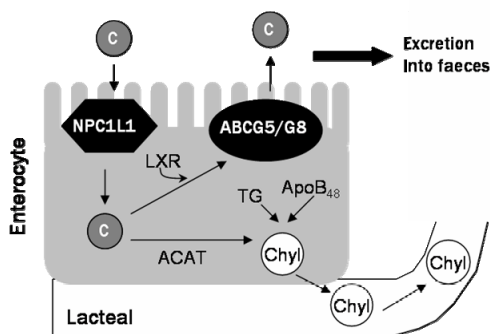
Besides cholesterol synthesis, members of the SREBP family induce transcription of various genes involved in metabolic pathways, such as insulin signalling, caveolin expression, phospholipid synthesis, fatty acid synthesis, citric acid cycle and scavenger receptor B1 (SR-B1) expression<sup>27</sup>. The SREBP family consists of SREBP-1a, SREBP-1c and SREBP-2 that are encoded by two separate genes *Srebf-1* and *Srebf-2*. There is overlap in functionality, but whereas SREBP-1a and 1c is more important for fatty acid metabolism, SREBP-2 rather activates genes important for cholesterol homeostasis<sup>28</sup>.

## Intestinal cholesterol absorption

In the intestine mixed micelles are formed from cholesterol, bile acids, monoglycerides, phospholipids, lysophospholipids and fatty acids. The micelles reach jejunal enterocytes<sup>23</sup>, where the transport of cholesterol and other small sterols into the enterocytes is mediated by the protein Nieman-Pick C1-Like 1 (NPC1L1)<sup>20</sup>. Scavenger receptors, such as the class B, type I (SR-BI) have also been suggested to be involved. Once inside the enterocyte some of the cholesterol is transported back to the intestinal lumen via ABC transporters (ABCG5 and ABCG8), that are under regulation of the liver X receptor (LXR). The remaining cholesterol is esterified by acyl-CoA-cholesterol acyltransferase (ACAT) and packed into chylomicrons<sup>23</sup>. The cholesterol absorption is summarized in Fig. 1.

## Cholesterol metabolism

Both triglycerides and cholesterol are absorbed from intestinal mixed micelles by the enterocytes and packed into chylomicrons. The chylomicrons subsequently enter the blood circulation where lipoprotein lipase anchored to the endothelial cells in the vascular wall hydrolyses their triglycerides to fatty acids and monoglycerides, and

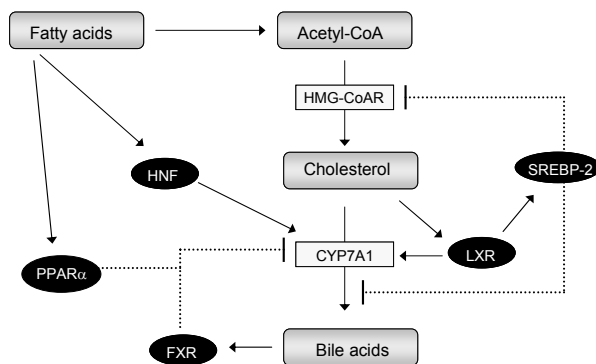


**Figure 1.** A schematic and simplified model of cholesterol absorption from the intestine. C: cholesterol, Chyl: chylomicrons, TG: triglycerides, NPC1L1: Nieman-Pick C1-Like 1, ACAT: acyl-CoA-cholesterol acyltransferase, ApoB<sub>48</sub>: apolipoprotein B<sub>48</sub>, LXR: liver X receptor. Modified from Charlton-Menys et al.<sup>20</sup>



these are either taken up locally or transported to the liver<sup>20</sup>. The remaining, small chylomicron remnants are taken up by the liver via the LDL receptor-like protein (LRP), which also involves binding to heparan sulphate<sup>29</sup>.

The cholesterol synthesized in the liver is secreted in VLDL particles. When VLDL is formed, triglycerides build a complex with apolipoprotein B<sub>100</sub> in the endoplasmic reticulum. This complex is further processed in the Golgi and then transported to secretory vesicles where additional triglycerides and unesterified cholesterol are added to the VLDL particle. Following release into the blood VLDL receives cholesteryl esters from HDL, mediated by the cholesteryl ester transfer protein (CETP)<sup>20</sup>. This does however not occur in mice, which lack CETP activity<sup>30</sup>. Just like chylomicrons, the VLDL particles are transported to peripheral tissue where the triglycerides are removed by lipoprotein lipase. The remaining, smaller, cholesteryl-rich particle is LDL<sup>20</sup>. LDL binds to LDL-receptors on the cell surface and internalizes the lipoprotein in coated pits. The LDL is degraded in lysosomes and the cholesterol is made available for further processing<sup>25</sup>. LDL supplies the tissues with cholesterol. If there is an excessive transfer of cholesterol to the tissues by LDL, this can be transported back to the liver in a process called reverse cholesterol transport mediated by HDL<sup>20</sup>.



**Figure 2.** Simplified model of the control of bile acid synthesis in relation to cholesterol metabolism. Dashed lines symbolise repression, whereas arrows symbolise activation. HNF and LXR activate CYP7A1, whereas FXR and PPAR $\alpha$  suppress its activity. SREBP-2 has dual effects –repressing both cholesterol synthesis and bile acid synthesis at a step downstream of CYP7A1. HMG-CoA reductase (HMGCoAR), cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), Farnesoid X receptor (FXR), Liver X receptor (LXR), Peroxisome proliferating factor (PPAR $\alpha$ ), Hepatocyte nuclear factor (HNF), Sterol regulatory element-binding protein (SREBP-2). Modified from Fuchs<sup>24</sup>

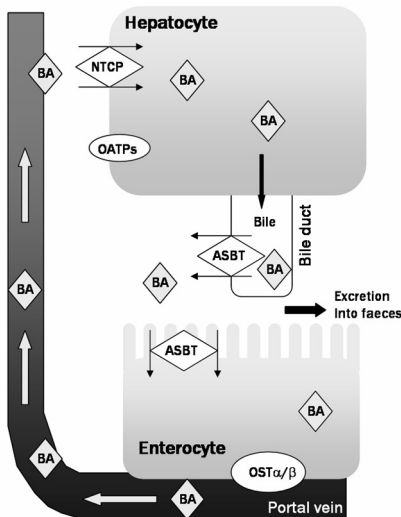
## Bile acid metabolism

Bile acids are produced in the liver and secreted into the small intestine where they emulsify lipids and cholesterol by forming mixed micelles, thereby facilitating their absorption.

Bile acids are synthesized from cholesterol in a series of reactions beginning with  $7\alpha$ -hydroxylation of cholesterol. This reaction is catalyzed by the enzyme cholesterol  $7\alpha$ -hydroxylase (CYP7A1), which is the rate-limiting enzyme in the bile acid synthesis pathway<sup>24</sup>. The hepatic bile acid synthesis is under the control of negative and positive feedback mechanisms from bile acids, hormones and nutrients. CYP7A1 can be regulated by several different pathways<sup>31</sup>, summarized in Fig. 2.

The majority of bile acids excreted in the intestine are re-absorbed and re-cycled in the enterohepatic circulation (Fig. 3). The terminal ileum is the main site for bile acid absorption. In ileum an apical sodium-dependent bile salt transporter (ASBT) mediates transport of bile acids into the enterocyte<sup>31</sup>. Deletion of ASBT in mice results in increase of both bile acid excretion and bile acid biosynthesis<sup>32</sup>. Knock out of the organic solute transporters (OST $\alpha$ /OST $\beta$ ) which transport bile acids from the enterocyte into the portal blood does, on the other hand, not result in increased bile acid excretion and leads to a reduction of CYP7A1 expression<sup>33</sup>.

Besides by the specific transporters, bile acids may also be taken up by passive transport in the small intestine<sup>31</sup>. The portal blood takes bile acids from the intestine to the liver, where uptake into hepatocytes is mediated by the basolateral sodium-dependent cotransporter Na<sup>+</sup>-taurocholate cotransporting polypeptide (NTCP), and also probably by multiple sodium-independent anion transporters belonging to the



**Figure 3.** Simplified model of the enterohepatic circulation, showing the important transporters of bile acids in the enterocytes and hepatocytes respectively. BA: bile acids ASBT: apical bile salt transporter, OST $\alpha$ /OST $\beta$ : organic solute transporters, NTCP: Na<sup>+</sup>-taurocholate cotransporting polypeptide, OATP: organic anion transporter. Modified from Ballatori et al.<sup>35</sup>

organic anion transporter (OATP) family. Transport of conjugated bile acids from the enterocyte to the canaliculus (small bile capillaries merging into ductules and finally the common hepatic duct) is mediated by the ATP-energized pump, BSEP (ABCB11)<sup>31</sup>.

Increased bile acid excretion promotes cholesterol synthesis and increases the expression of hepatic LDL-receptors<sup>31</sup>. Bile acid resins, a group of drugs reducing plasma cholesterol, block the re-absorption of bile acids in the ileum. This promotes production of new bile acids from cholesterol and contributes to reduced levels of cholesterol in the blood<sup>34</sup>.

## Atherosclerosis

Cardiovascular diseases (CVD) like coronary heart disease (CHD), myocardial infarction, peripheral vascular disease and stroke are all clinical complications of atherosclerosis and are the most common causes of death in western societies<sup>36,22</sup>. Elevated levels of plasma cholesterol are regarded as one of the most important risk factors for development of atherosclerosis and cardiovascular disease<sup>2</sup>, but many other genetic and environmental risk factors have been identified to drive the development of atherosclerosis. Abdominal obesity, hypertension, elevated LDL cholesterol, low HDL cholesterol, elevated triglycerides, insulin resistance ( $\pm$  glucose intolerance), pro-inflammatory state and pro-thrombotic state are all included in the cluster of risk factors for CVD commonly termed the metabolic syndrome. Cigarette smoking, family history of CHD, aging, physical inactivity and atherogenic diet are other important risk factors for atherosclerosis and CVD<sup>37</sup>.

### The atherosclerotic process

Atherosclerosis is a complex inflammatory process involving lipids, immune cells and pro-inflammatory molecules. The initial step of atherogenesis involves entrapment of LDL in the subendothelial space of the vascular wall, where LDL is modified by reactive oxygen species (ROS) to minimally modified (mmLDL) and oxidized LDL (oxLDL). These modified forms of LDL inhibit the production of nitric oxide (NO), a mediator of vasorelaxation in the vascular wall and moreover, stimulate endothelial cell activation. Activated endothelial cells produce pro-inflammatory cytokines and adhesion molecules including vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E- and P-selectins and monocyte chemoattractant protein (MCP-1), all contributing to the recruitment of monocytes to the arterial wall. The monocytes migrate into the intima of the artery, where they become

macrophages. After activation by peroxisome proliferator-activated receptors (PPARs) and stimulation by various cytokines such as TNF- $\alpha$  and IFN- $\gamma$ , the macrophages up-regulate expression of scavenger receptors on their surface that mediate uptake of oxLDL. This engulfment of oxLDL results in lipid-filled macrophages that are named foam cells. The death of foam cells leaves a growing mass of extracellular lipids and debris behind in the vascular wall. Concurrent with the accumulation of lipids and foam cells, smooth muscle cells from the media start to proliferate and migrate into the intima, creating a fibrous cap over the lipid core of the lesion. Lesions with a thin fibrous cap are more vulnerable than those with a thick cap, and the vulnerability of a plaque contributes more to thrombus formation than the severity of stenosis<sup>38</sup>.

## Inflammation

Inflammatory processes are important at all levels of atherosclerosis and therefore markers of inflammation are usually used to score the risk of disease. Plasma levels of serum amyloid A (SAA) have been shown to correlate with atherosclerotic lesion area in LDL-receptor deficient (LDLr<sup>-/-</sup>) mice fed a high-fat diet<sup>39</sup>. Liver-derived markers of chronic subacute inflammation, including SAA, C-reactive protein (CRP) and fibrinogen, have been demonstrated to independently predict future cardiovascular risk. Reduction of these inflammation markers after cholesterol-lowering therapy is associated with improved clinical outcome<sup>40,41</sup>.

## Nitric oxide

Nitric oxide (NO) is released by endothelial nitric oxide synthase (eNOS) in response to different stimuli, including blood flow and exposure to acetylcholine, and contributes to endothelium-dependent vasodilatation of the artery. NO has also been ascribed various anti-atherogenic properties, such as inhibition of smooth muscle cell proliferation, inhibition of platelet aggregation and leukocyte inactivation<sup>42</sup>. Although NO can have both pro- and anti-oxidative properties, it is regarded that the basal activity of eNOS contributes to an anti-oxidant mechanism to suppress lipid peroxidation and maintain vascular function<sup>43</sup>.

## Anti-atherogenic drugs

Most current therapies aimed to prevent atherosclerosis reduce plasma cholesterol levels. Below is a short presentation of the most widely used groups of compounds.

### *Statins*

Statins bind to and inhibit HMG-CoA reductase, the rate limiting enzyme for endogenous cholesterol production. The lowered cholesterol levels in the liver increase expression of hepatic LDL-receptors via activation by SREBP. This stimulates increased uptake of LDL particles, which are digested intracellularly, making cholesterol available for metabolic purposes. The amount of cholesterol in the liver is maintained at normal levels, whereas the blood total and LDL cholesterol concentrations are kept low<sup>26</sup>. As a compensatory mechanism absorption of cholesterol via the intestine is up-regulated when cholesterol synthesis is decreased by statins<sup>23</sup>. A treatment reducing the intestinal cholesterol absorption would therefore be a good complement to statin treatment.

### *Ezetimibe*

Ezetimibe reduces plasma cholesterol by selective inhibition of intestinal absorption of cholesterol by binding to Niemann-Pick C1 like 1 (NPC1L1). Ezetimibe does not block absorption of bile acids or triglycerides<sup>44</sup>.

### *Resins*

Resins (i.e. cholestyramine) are bile acid sequestrants consisting of a flexible skeleton covered by positively charged groups that enable binding of bile acids both hydrophobically and electrostatically, thereby increasing the excretion of bile acids and increasing bile acid biosynthesis by a factor of four to six<sup>31</sup>. The actual amounts of bile acids excreted *in vivo* are however much less than the theoretical capacity of the resins, necessitating administration of high doses with resulting side effects and cost-ineffective treatment<sup>34</sup>.

### *Fibrates*

Fibrates are amphiphatic (mainly hydrophobic) carboxylic acids that modify plasma lipid levels due to their agonist effect on peroxisome proliferator-activated receptors (PPARs), especially PPAR $\alpha$ . They have mainly triglyceride-lowering effects, but also lower LDL-cholesterol moderately, and raise HDL-cholesterol levels. The activation of PPAR $\alpha$  increase e.g. lipoprotein lipase activity, thereby decreasing triglyceride content in remnant lipoproteins and inducing production of LDL particles with higher affinity for LDL-receptors, thereby reducing levels of LDL<sup>45</sup>.

By far the most used drugs for reduction of blood cholesterol are the statins (HMG-CoA reductase inhibitors)<sup>36</sup>. Although the statins effectively lower plasma cholesterol levels and reduce cardiovascular causes of death, many patients still experience adverse cardiovascular events<sup>3</sup>. Statin treatment may also cause adverse effects such as rhabdomyolysis, which, albeit rare, is a widespread and highly discomfoting death of

skeletal muscle cells<sup>46</sup>. It is therefore a challenging work to find alternatives and/or supplements to the existing cholesterol-lowering drugs.

## Dietary fibres

A dietary fibre can be defined as “...the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine...” as stated by the American Association of Cereal Chemists (AACC) in 2001. Dietary fibres with beneficial physiological effects, such as laxation or reduction of blood cholesterol, have been given the name functional fibre. The physiological effects of fibres are associated with their physicochemical properties, including their solubility, viscosity and fermentability<sup>1</sup>.

Wholegrain products are, besides fruit and vegetables, the most important sources of dietary fibres. Most dietary fibres in grains are polysaccharides, but non-digestible oligosaccharides are also present. The cereals have different amounts and composition of the different fibres, and the technology applied when preparing the cereal products also affects their amount and composition<sup>47</sup>.

### Formation of short chain fatty acids (SCFA) by fermentation of fibres

Fermentable dietary fibre is the most important source for formation of SCFA in the large intestine. Beta-glucans, pectin and resistant starch are fermented to 70-100 %, whereas for example cellulose is usually not fermented at all<sup>48</sup>. The major SCFAs produced after fermentation of dietary fibres are acetate, propionate and butyrate. The properties of the microflora and the composition of the substrate (dietary fibre) are important factors both for the total and individual SCFA formed<sup>48</sup>.

The SCFAs are physiologically active in different ways. Acetate is readily taken up from the intestine and transported to the liver where it can serve as a substrate for cholesterol biosynthesis<sup>49</sup>. Butyrate serves as a fuel for epithelial cells and can also regulate cell proliferation and differentiation<sup>49,50</sup>. Recently butyrate was suggested to influence lipid metabolism by regulation of intestinal fat absorption<sup>51</sup>. Propionate may contribute to hypocholesterolemic action by either inhibiting HMG-CoA reductase<sup>49,52,53</sup> or by preventing utilization of acetate for cholesterol synthesis<sup>54,55</sup>.

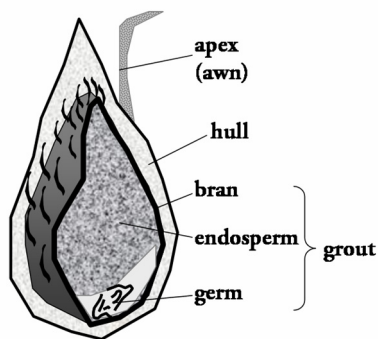
A substantial amount of bacteria is present in the ileum, but the vast majority of bacteria exist in the proximal large bowel, where fermentation also takes place. It is in the caecum and ascending colon that the SCFA production reaches its highest concentration in humans<sup>1</sup>. The gastrointestinal microbiota play important roles in

health and disease, but the diversity of the microbiota is poorly defined and yet far from completely characterized<sup>1</sup>. The amount and type of dietary fibre ingested influences the composition of the intestinal microbiota<sup>56</sup>.

## Oats (*Avena sativa*)

Oats (*Avena sativa*) are cereals rich in dietary fibres, antioxidants, proteins and unsaturated fat, which makes them interesting as functional food ingredients. There are many types of dietary fibres present in oats; cellulose, arabinoxylans and beta-glucans. Cellulose is a (1→4)-β-D-glucan, where the beta-glucoside bond makes the cellulose indigestible and insoluble. The mixed linked (1→3),(1→4)-β-D-glucan are composed of β(1→4)-linked glucose units with a single β(1→3)-linked glucose every two or three units. It is the (1→3) linkages that make beta-glucans soluble<sup>6</sup>. The beta-glucans are high molecular weight polysaccharides which form highly viscous solutions<sup>57</sup>.

The oat grain is composed of the oat grout (kernel, caryopsis) and the hull (husk), and it is the dehulled grout that is of interest for human nutrition. The grout contains three compartments which are separated both morphologically and chemically – the bran, the starchy endosperm and the germ (Fig. 4). The bran represents the surface layer that envelopes the kernel<sup>58</sup>. Whereas commercial whole groat rolled oats normally contain approximately 4 % beta-glucans, oat bran typically contains 6-10 % beta-glucans<sup>59</sup>. In the oat bran used for the studies in this thesis the beta-glucan content was 6.3-7.5 %. The main composition of Swedish oat grout is 53-73 % starch, 12-23 % protein, 5-14 % lipids and 5-13 % total fibre, of which soluble beta-glucans represent 3-6 %<sup>60</sup>. Compared to other grains, oats contain relatively high levels of proteins, lipids (unsaturated fatty acids), vitamins, antioxidants, phenolic compounds and minerals<sup>48</sup>.



The definition of oat bran is not completely obvious and can have both morphological and production aspects. The bran is the outermost layer of the grout (but does not contain hull). It is obtained after milling the whole groat and then separating the courser bran from the fine particles<sup>59</sup>.

**Figure 4.** Composition of the oat grain. Modified from Butt et al<sup>61</sup>

According to a definition by AACC in 1989, the oat bran fraction should not be more than 50 % of the starting material, and have a total beta-glucan content of at least 5.5 % (dry weight basis) and a total dietary fibre content of at least 16 % (dry weight basis)<sup>57,59</sup>. The chemical composition of oat bran may vary depending on sources of the bran<sup>57</sup>.

## Proposed mechanisms of cholesterol-lowering effect of oats

Several possible mechanisms have been suggested for the cholesterol-lowering actions of oats. These are described below.

### *I. Increased unstirred layer*

In the intestine the beta-glucans absorb fluids, thereby contributing to increased viscosity of the intestinal contents<sup>62</sup>. The increased viscosity of the small intestinal contents leads to an increase of the unstirred layer present close to the mucosa<sup>63</sup>. Since the unstirred layer serves as a physical barrier to absorption of nutrients<sup>64</sup>, the thickening of the layer reduces absorption of dietary cholesterol and reabsorption of bile acids.

### *II. Binding of bile acids*

Binding of bile acids (directly or indirectly) by beta-glucans in the small intestine has been suggested to contribute to the cholesterol-lowering effect<sup>65</sup>. The precise mechanism of this interaction is not known, but Bowles et al (1996) found no chemical binding between isolated beta-glucans and bile acids<sup>66</sup>. Other *in vitro* experiments have revealed that the binding between extrudates of dietary oat fibres and glyco-conjugated bile acids is dependent on the composition of the oat product, on the fine-structure of the bile acid and on the pH of the medium<sup>48</sup>. There are also suggestions that beta-glucans entrap whole micelles or their components, thereby increasing the excretion of bile acids and cholesterol. This hypothesis arose after findings in ileostomy patients that not only excretion of bile acids and cholesterol were increased after oat intake, but also the net fat excretion<sup>67,68</sup>.

Normally, the bile acids are almost completely reabsorbed and transported back to the liver via the enterohepatic circulation. The mechanisms proposed under I and II above lead to increased bile acid excretion following consumption of oat fibres as shown in numerous studies<sup>53,67,65</sup>. The major pathway for elimination of cholesterol is synthesis and excretion of bile acids. The increased bile acid excretion stimulates bile acid production with increased uptake of cholesterol from the circulation<sup>65</sup>, followed by decreased levels of cholesterol in the plasma<sup>69</sup>.



### *III. Reduced glucose & insulin response*

Another possible mechanism of action is that the increased intestinal viscosity contributes to a reduced postprandial glucose and insulin response<sup>70-72</sup>. This may result in a reduced hepatic cholesterol synthesis<sup>70</sup>.

### *IV. Inhibition of endogenous cholesterol synthesis by propionate*

Finally, it has been implied that propionate, one of the SCFAs produced when beta-glucans are fermented by microorganisms in the large intestine, enters the blood stream and suppresses hepatic cholesterol synthesis either by impairing the utilization of acetate as a substrate for acetyl-CoA formation<sup>54,55</sup> or by inhibition of HMG-CoA reductase<sup>52,53</sup>. *In vitro* experiments showed that cells (Caco-2/TC-7 enterocytes) stimulated with propionic or butyric acid decreased the levels of HMG-CoA reductase by 16 and 33 % respectively, evaluated with mRNA analysis<sup>73</sup>.

## Possible anti-atherosclerotic effects of oats

The most obvious mechanism for oats to prevent atherosclerosis is of course the cholesterol-lowering effect. However, since oxidation of lipoproteins and inflammation are hallmarks of atherosclerosis, dietary micronutrients (organic chemicals and trace elements) with anti-oxidative or anti-inflammatory properties may well contribute to the atheroprotective action of oats. Results from studies in animal models of atherosclerosis and epidemiological research indicate that anti-oxidants can reduce the atherosclerotic process and the risk of cardiovascular disease<sup>74</sup>. Oats contain several components with documented or suggested anti-oxidative or anti-inflammatory effects that can possibly contribute an anti-atherosclerotic effect in addition to reduced levels of plasma lipids (summarized in Fig. 5).

### *Avenanthramides & other polyphenols*

Phenolic acids and polyphenols are present in whole-grain foods and have various bioactive functions, including anti-inflammation, anti-oxidation and anti-proliferation. The major phenolic compounds in oats are the avenanthramides, but there are also small amounts of free phenolic acids (i.e. caffeic,  $\rho$ -coumeric, ferulic, sinapic, vanillic acid) and flavonoids (i.e. apigenin, kaempferol, luteolin)<sup>75,76</sup>.

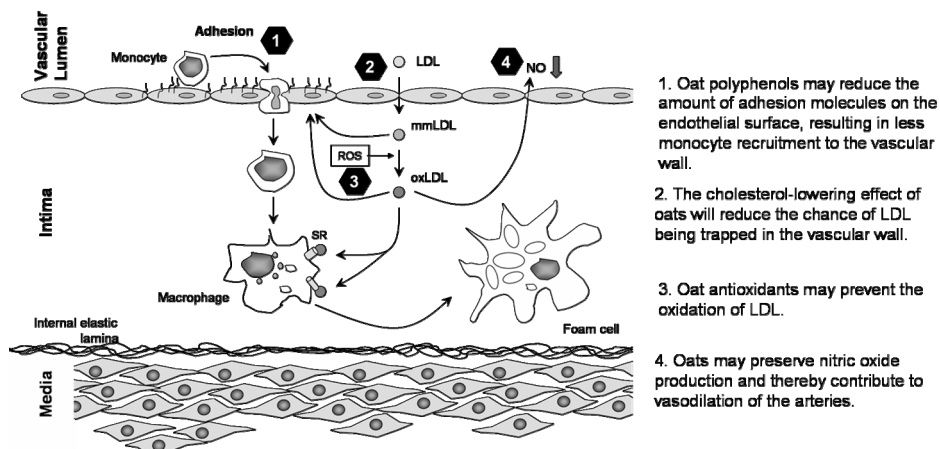
Avenanthramides are polyphenols unique for oats that are most abundant in the bran and outer layers of the kernel. They have been shown to exert anti-oxidative properties by preventing oxidation of LDL and thereby also the formation of foam cells<sup>76</sup>. In endothelial cells *in vitro* avenanthramides suppressed expression of the adhesion molecules VCAM-1 and E-selectin and of the pro-inflammatory cytokine

IL-6<sup>77</sup>, and also up-regulated mRNA expression of eNOS in both endothelial and smooth muscle cells<sup>78</sup>.

It has recently been shown that avenanthramides are bioavailable<sup>79</sup>, and if the above-mentioned effects appear also *in vivo* this might lead to less monocyte recruitment to the vascular wall, less engulfment of oxLDL by macrophages and maintained NO production, all contributing to less atherosclerotic lesion development. Hence avenanthramides are important candidates as anti-atherogenic components in oats.

### Vitamin E (Tocopherols & Tocotrienols)

Four isoforms ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -) of tocopherol and the corresponding tocotrienols are included in the generic term “vitamin E”. Vegetable oils are the natural sources for vitamin E<sup>80</sup>, but they are also present in grains like oats and barley<sup>75</sup>. The E-vitamins are taken up by enterocytes in the intestine and are transported in chylomicrons to the liver, where they are incorporated in VLDL particles that are finally developed to LDL. Studies *in vitro* have shown that E-vitamins are superior anti-oxidants to prevent oxidation of LDL due to their lipid solubility and the fact that they are incorporated into the lipoprotein particle<sup>80</sup>. Further atheroprotective properties of vitamin E was shown in LDLr<sup>-/-</sup> mice, where the progression of atherosclerosis was inhibited by increased nitric oxide (NO) production and suppressed inflammatory and oxidative events, but total plasma cholesterol was unaffected<sup>81</sup>. Expression of the adhesion molecules ICAM-1 and VCAM-1 was reduced after E-vitamin supplementation to rabbits fed atherogenic diet, as was the amount of macrophages attached to the blood vessel wall<sup>82</sup>.



**Figure 5.** The initiating events of atherosclerosis, and hypothetical potential of oats to prevent the atherogenesis. NO: nitric oxide, LDL: Low density lipoprotein, mmLDL: minimally modified LDL, oxLDL: oxidized LDL, ROS: Reactive oxygen species, SR: scavenger receptor. Modified from Glass and Witztum<sup>2</sup>.

### *Plant sterol & stanol*

There are three major sterols present in oats:  $\beta$ -sitosterol,  $\Delta^7$ - and  $\Delta^5$ -avenasterol. The latter was shown to have anti-oxidative effects<sup>75</sup>, but reports on bioactive effects of oat sterols or stanols are scarce. Sterols and stanols from other plant sources have however documented cholesterol-lowering effects and the suggested mechanisms are as follows: i) the stanols/sterols replace cholesterol in micelles in the intestine and therefore reduce the cholesterol present in an absorbable form, ii) the stanols/sterols block the cholesterol uptake via NPC1L1 in the intestine (but this was not supported in C57BL/6J mice in Plösch et al), iii) the stanols/sterols interfere with the esterification process in the enterocytes, which inhibits the formation of chylomicrons<sup>83</sup>. Plant stanol esters derived from vegetable oil and wood have been shown to reduce atherosclerosis development in mice<sup>84</sup>.

### Glucose response to oats

The increased viscosity of the intestinal contents provoked by oat dietary fibres results in an extended digestion period that might have positive effects on the glucose response to a meal, and oat beta-glucans have indeed been shown to lower the postprandial glucose response<sup>62,85</sup>. An extensive review of the glycemic index (GI) of various food products revealed that oats show variable results but usually have a low to medium GI, with for example porridge having a GI value of 55<sup>86</sup>. Jenkins et al (2002) claim that enrichment of oat foods with additional beta-glucans is required to obtain a low GI of oat products<sup>85</sup>.

### Processing of beta-glucans, molecular weight & bioactivity

The physico-chemical properties of beta-glucans, including their molecular weight and solubility, affect their viscous properties and are suggested to be crucial for their beneficial effects on plasma cholesterol and glucose response. Both solubility and molecular weight may be altered during processing of oats: i) mild extraction procedures may not deactivate endogenous beta-glucanases and can hence increase degradation of the beta-glucans, reducing the molecular weight<sup>87</sup>, ii) freezing and storage are believed to reduce the extractability of beta-glucans in the intestine<sup>19</sup>, iii) food processing like heat treatment, addition of endogenous enzymes and shear forces may reduce the molecular weight of beta-glucans<sup>57,88,89</sup>. The beta-glucans can be broken down differently depending on how they are digested, which is illustrated by the fact that beta-glucans digested with enzymes reduced the postprandial glycemia, whereas beta-glucans digested by an aqueous method did not<sup>90</sup>.

Reduction of postprandial glucose and insulin levels in blood has been shown to depend on the viscosity of the beta-glucans<sup>70,72,91</sup>, whereas freezing of an oat drink did not affect the glucose response compared to a non-frozen control<sup>62</sup>.

High viscosity of beta-glucan water extracts has been shown to be important for the cholesterol-lowering effect in hamsters<sup>92</sup> and rats<sup>14</sup>. Also, in man, beta-glucanase-treated oat bran did not reduce plasma cholesterol, whereas intact oat bran did<sup>93</sup>. More knowledge on how different processing of the fibres changes their molecular weight and viscous properties coupled to their bioactivity needs to be generated, especially regarding interference with the cholesterol-lowering effect.

## Why mice?

Animal models for studying effects of dietary factors are needed because human studies are not always safe, practical or affordable. The majority of animal studies evaluating the effects of oats have been performed on rats<sup>4,11-13</sup>. Rats are, however, relatively resistant to induction of hypercholesterolemia and atherosclerosis<sup>13</sup>. A mouse model would be attractive because of the large number of genetic variants available with increased propensity for hypercholesterolemia and atherosclerosis. Mice also consume small amounts of food, which is advantageous since only small amounts of the isolated oat fractions will be available in the initial developmental phase of new food products. Finally mice are relatively inexpensive and convenient animals to handle. A suitable mouse model for evaluating the cholesterol-lowering effect of oats and isolated components from oats would be an important tool when developing new functional food ingredients.



# Aim

From an industrial point of view it would be useful to have a controlled biological system in which new candidates for functional foods can be systematically tested. Our overall objective was therefore to find a suitable mouse model in which the cholesterol-lowering effects of oats can be studied. Once established, the mouse models were used to address the following questions:

Can the proposed mechanisms of action for the cholesterol-lowering effect be confirmed or rejected in the mouse models?

Can oat bran reduce development of atherosclerotic lesions in mice fed a high-fat diet?

Can oats reduce systemic and vascular inflammation?

Do changes of the physico-chemical properties of beta-glucans, such as viscosity and molecular weight, affect the cholesterol-lowering capacity?

Do other components in oats, besides beta-glucans, contribute to the cholesterol-lowering effect?



# Methods

For detailed materials and methods descriptions please refer to the individual papers I-IV. This methods section provides an overview and additional information on some methods used in this thesis.

## Mouse models

In their natural state, mice are animals with very low proportions of circulating VLDL and LDL in plasma, having the majority of their lipoproteins in the HDL fraction. All experimental methods for inducing hypercholesterolemia and atherosclerosis in mice involve a change in this balance, either by dietary regimens or by genetic means<sup>94</sup>. In the studies included in this thesis we use two different mouse models of hypercholesterolemia:

- 1) The wild type strain of C57BL/6 mice was used when the cholesterol-lowering effects of oats were the main end-point of the study. C57BL/6 mice develop hypercholesterolemia when fed a high-cholesterol, high-fat diet<sup>95,96</sup>.
- 2) When evaluating if oats in the diet can inhibit the development of atherosclerosis the LDL-receptor deficient mice (LDLr<sup>-/-</sup>) were used. LDLr<sup>-/-</sup> mice are commonly used for evaluation of hyperlipidemia and atherosclerosis<sup>97-99</sup>.

Mice are used as model for human disease within many fields of research. When expressed in relation to body weight the total cholesterol pool is similar in humans and rodents<sup>100</sup>. There are however many significant differences between man and mouse regarding lipid metabolism, digestion of food and atherosclerotic disease that should be considered:

- Mice normally never develop hypercholesterolemia and atherosclerosis. This needs to be induced by atherogenic diet in wildtype mice or by using transgenic mice predisposed for hyperlipidemia<sup>94</sup>, as mentioned above.
- Mice are coprophages, which means that they re-ingest their faeces. This could possibly influence the action of dietary fibres in the bowel. From experiments in rats, also coprophages, it was concluded that the faecal re-ingestion did not influence the cholesterol-lowering effect of oats bran<sup>101</sup>.
- Mice lack the cholesterol ester transfer protein (CETP). In humans this is an important protein for delivery of HDL cholesterol to the liver by first transferring the cholesterol from HDL to apoB-containing lipoproteins (LDL, VLDL)<sup>102</sup>.



Despite these differences mice have proved to be widely useful in studies of cholesterol-reducing and anti-atherogenic effects of drugs and different dietary compounds, and reliable assays to evaluate atherogenesis have been developed. However, as mentioned above, very few studies of effects of oats in mice have been reported.

## Diets

By designing and producing the atherogenic diets ourselves, we had precise control of dietary and nutritional factors that could be of importance for evaluation of the oat fibres. Lichtman et al (1999) claim that from a nutritional perspective it is better to produce experimental diets from scratch rather than adding new ingredients to existing commercial diets. Chow diets contain a diverse array of soluble and insoluble fibres and a multitude of possible biologically active phytochemicals such as carotenoids and flavonoids. The latter might have antioxidant actions that could influence the results in studies of hypercholesterolemia and atherosclerosis<sup>103</sup>. Similar atherogenic diets as the one used in our studies (Table 1, Paper I) have been published before<sup>104</sup> and are also recommended by Jackson Laboratories on [www.jax.org](http://www.jax.org). The major modification we made from the formerly published atherogenic diets was to exchange some of the sucrose for corn starch and maltodextrin as carbohydrate sources, and to add a larger proportion of butter in relation to corn oil. The total cholesterol concentration in our diets was 0.8 % compared to 1 % in the previous diets, and we modified the cholate concentration from 0.5 % to 0.1 %. When producing the western diet given to the LDLr<sup>-/-</sup> mice the same diet formula was used, but with no extra cholesterol or cholic acid added.

We chose oat bran as source of oats to ensure that no processing would have affected the oats negatively. The concentrations of oat bran used in our study were 40 and 27 %, which correspond to beta-glucan concentrations of approximately 3 and 2 % in the diet. We chose a high concentration for the initial studies to be certain to see an effect. The oat bran was milled to a particle size of 0.8 mm, since the extractability of beta-glucans has been shown to increase when the particle size of the oat product is reduced<sup>105</sup>.

We wanted to evaluate the precise effect of oat fibres and therefore the control diet contained the same amount of fibres as the oat bran diet, but from a different source, cellulose, which has also been used as a negative control to oats in previous studies in rats<sup>106</sup> and hamsters<sup>10</sup>.

## Lipoprotein distribution

HDL, VLDL and LDL were separated on thin agarose gels by their different electrophoretic mobility as described by Noble et al.<sup>107</sup>. Gels were stained with Sudan black after drying and the intensity of the bands was evaluated by densitometric scanning, revealing the relative distribution between HDL and LDL+VLDL. Values of VLDL and LDL were summed since the bands were not always clearly distinguishable. Since Sudan black stains all lipids (cholesterol, triglycerides and phospholipids) the percentage given for each lipoprotein does not exactly correspond to HDL- and LDL-cholesterol, but rather reflects the lipid distribution among lipoproteins. Only 2 microliters of plasma is needed for the analysis, which is a benefit when handling mouse samples. The electrophoretic mobility of the lipoproteins depends on their apolipoprotein content. Therefore this method is sometimes used for detecting changes in lipoprotein assembly<sup>108</sup>.

## Evaluation of atherosclerotic lesions

Determination of lesion area in the aortic root and the descending aorta are two commonly used methods for assessing atherosclerosis in experimental mouse models<sup>109</sup>. The lesions develop earlier in the aortic root than in the descending aorta<sup>110</sup>, so in order to get a complete picture of the atherosclerosis development both sites were evaluated in the LDLr<sup>-/-</sup> mice in paper IV:

The descending aorta (from 1 mm below the left subclavian artery to the iliac bifurcation) was cut open longitudinally, put on a glass slide, fixed and stained *en face* with Oil-Red-O as described by Brånén et al.<sup>111</sup>. Lesion area was quantitated blindly by microscopy and computer-aided morphometry using Image ProPlus 4.5 software. Lesion size was expressed as lesion area in percent of total aortic surface area.

The aortic root was placed in HistoChoice®, embedded in OCT and cryo-sectioned. Five sections from each mouse at equal distance from each other were used to provide an average lesion area, counted as n=1. The sections were stained with Oil-Red-O and counterstained with haematoxylin. Blinded quantification of the lesion size was performed by computer-aided morphometry and expressed as mean lesion area per section.

## Liver mRNA expression analysis

The mRNA expression of various enzymes, receptors and transcription factors in the liver was measured by real-time, reverse transcription polymerase chain reaction (RT-PCR). RT-PCR enables quantification of the expression of a gene by analysing the amount of its mRNA. First, DNA is made from the mRNA by reverse transcription, then the PCR amplifications are run, where the amount of DNA produced during

each cycle is monitored by measuring the fluorescent light emitted by a dye that binds to the DNA. We used the dye SYBR-Green-I, which emits fluorescent light when bound to double-stranded DNA. The cycle at which the fluorescence from the dye is so high that it is first detectable is called the crossing point (CP) value (sometimes also referred to as the Ct value). CP values are used for quantitative calculations; the more mRNA from start in the sample the lower the CP value<sup>112</sup>.

The real-time RT-PCR was performed with a MxPro 2005P system (Stratagene, Agilent technologies Inc., Santa Clara, USA) with Quantifast™ SYBR® Green RT-PCR kit and QuantiTect primer assays (all from Qiagen) in a one-step reaction according to the manufacturer's instructions. These ready-made reaction mixes are optimized by the manufacturer to yield a good quality PCR-reaction without additional optimizing procedures.

The target genes were normalized to the mean of two reference genes, Gapdh and Ppia, and the oat bran group was compared with the control group according to the Pfaffl equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}}(\text{control-sample})}}$$

E is the PCR efficiency. The efficiency is calculated from a dose-response curve made for each primer pair, where the slope of the curve is used for the equation  $E = 10^{(-1/\text{slope})}$ .  $\Delta\text{CP}_{\text{target}}$  is CP deviation of control – sample of the target gene and  $\Delta\text{CP}_{\text{ref}}$  is the CP deviation of control – sample of the reference genes<sup>113</sup>. If there is no difference between control and sample the ratio will be 1.

For extraction of RNA livers were snap-frozen directly after sacrifice of the mice and stored in -80°C until assayed. Since there are known zonal differences in the expression of HMG-CoA reductase and CYP7A1 within the liver<sup>27</sup>, livers were pulverized in liquid nitrogen to ensure homogenous sampling. RNA was extracted from the pulverized tissue with RNeasy Mini Kit with simultaneous DNase treatment (Qiagen Inc. Valencia, USA). The choice of genes that were analysed with RT-PCR in paper II is listed in Table 1.

**Table 1.** The different liver proteins analyzed for their mRNA content with Real-time RT-PCR and their respective function in lipid metabolism.

Gene	Protein	Role in lipid metabolism
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Reference gene
Ppia	Peptidylprolyl coenzym A (PPIA)	Reference gene
Ldlr	LDL-receptor (LDLr)	The main receptor responsible for hepatic clearance of plasma lipoproteins <sup>114</sup> .
Lrp1	LDLr-related protein (LRP)	Another member of the LDL-receptor family that acts together with LDLr in the hepatic clearance of plasma lipoproteins <sup>114</sup>
Hmgcr	3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA)	Rate limiting enzyme for endogenous cholesterol synthesis in hepatocytes and in extrahepatic organs <sup>20</sup> .
Cyp7a1	Cholesterol-7 $\alpha$ -hydroxylase (CYP7A1)	A microsomal cytochrome P-450 enzyme involved in the first step of bile acid synthesis in the liver. It is of great regulatory importance and complex control and feed-back mechanisms regulate the expression of CYP7A1 <sup>115</sup> .
Ppara	Peroxisome proliferator-activated receptor- $\alpha$ (PPAR $\alpha$ )	A nuclear transcription factor with regulatory effects on metabolic and inflammatory signalling. PPAR $\alpha$ is the liver isoform, and this is the target for the hypocholesterolemic drugs fibrates <sup>116,117</sup> .
Srebf2	Sterol regulatory element-binding protein (SREBP-2)	A transcription factor that induces transcription of various genes involved in metabolic pathways and especially cholesterol uptake and synthesis, such as HMG-CoA reductase and LDL-receptors <sup>26,28</sup> .

## Terminal Restriction Length Polymorphism (TRFLP)

TRFLP is a gut community finger-printing technique that gives an overview of differences in gut microbiota communities between individuals. The technique uses DNA-amplification of the bacterial 16S rRNA-gene that contains both highly conserved and variable regions. The DNA-amplicons are then digested with restriction enzymes. Because different bacterial species have different variable sequences in their 16S rRNA the terminal restriction fragments (T-RF) will have different sizes, in our case ranging from 20 bp to 600 bp. Variation will thus be found in the number and size of peaks, providing quantitative information on the compositional differences of gut microbiota communities<sup>118</sup>.

## Ethics

All animal experiments performed in this thesis followed national guidelines for care of animals and were approved by the Malmö/Lund regional ethical committee for laboratory animals.

# Results & Discussion

If not otherwise stated, C57BL/6 mice below refer to C57BL/6NCrl mice that responded to oat bran intake with reduced plasma cholesterol. The oat non-responsive substrain C57BL/6JBomTac is discussed separately under the “substrain difference” headline.

## Oats & effects on plasma lipids

### Cholesterol

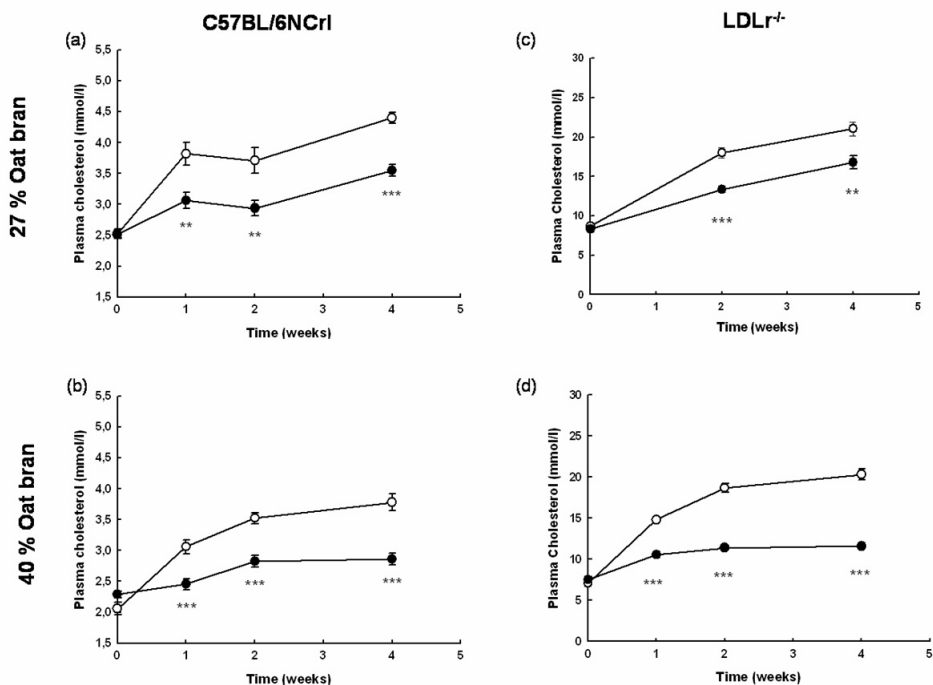
We have repeatedly shown in our experiments that oat bran significantly reduces plasma cholesterol in both wild type C57BL/6 (Paper I, II, and III) and genetically modified LDLr<sup>-/-</sup> mice (Paper IV) fed atherogenic or western diet respectively. We have thereby established two experimental mouse models in which the cholesterol-lowering effects of oat products can be studied.

In the initial experiments the rather high concentration of 40 % oat bran (approx 3% beta-glucan) was included in the diet. A lower concentration of 27 % (approx 2% beta-glucan) was however also shown to yield substantial and statistically significant reduction of plasma cholesterol in both C57BL/6 and LDLr<sup>-/-</sup> mice (Fig. 6). The concentration seems to affect the level of cholesterol reduction, at least in the LDLr<sup>-/-</sup> mice. The 27 and 40 % oat bran diets cannot however be directly compared since the total amounts of fibre in the two diets differ (4.4 *vs.* 6.5 %). In previous animal experiments concentrations of 30 % oat bran or 1.5-4.0 % beta glucans have been shown sufficient to lower plasma cholesterol in rats and hamsters<sup>10,12,13</sup>.

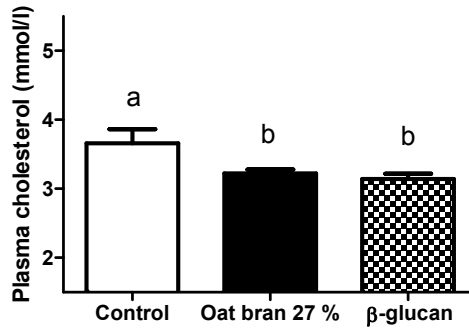
The FDA health claim concerning whole oats specifies that the daily intake of oat beta-glucan fibre in a human should be 3 g and that one serving should contain at least 0.75 g beta-glucans<sup>15</sup>. Assuming that an average human weighs 75 kg, eating 3 g of beta glucans gives an intake of 0.4 mg beta-glucan per gram body weight. The corresponding value for a mouse (weighing 25 g) fed 27 % oat bran is 1.6 mg beta-glucan per gram body weight (feed intake 2 g/mouse & day, of which 2 % is beta-glucans). An intake of beta-glucan relative to body weight of 4 times that recommended by FDA is thus sufficient for substantial (20 %) and significant cholesterol-lowering effects in the mice (Fig. 6). This is of course a crude comparison,

but it would be interesting to perform dose-response studies in the mice to find the lower limit for cholesterol reduction by beta-glucans.

Our goal with the mouse models is to enable studies of various food components with possible cholesterol-reducing effects. We recently conducted an experiment in which pure (97 %), high viscous beta-glucans, purchased from Megazymes International Ltd. (Ireland), was included in the atherogenic diet. The pure beta-glucans reduced plasma cholesterol to the same level as oat bran (Fig. 7). This supports the role of beta-glucans as the main active cholesterol-lowering components in oats.



**Figure 6.** Plasma cholesterol are reduced in C57BL/6 (a and b) and LDLr<sup>-/-</sup> (c and d) mice after intake of 40 or 27 % oat bran. Figures modified from paper I and IV.



**Figure 7.** Isolated beta-glucans lower plasma cholesterol to the same level as oat bran in the C57BL/6 mice fed atherogenic diet. n=10, statistics was performed with ANOVA followed by Tukey's multiple comparison test. Bars with different letters differ significantly,  $p < 0.05$ .

## Lipoprotein distribution

Elevated plasma concentration of LDL is a potent risk factor for vascular disease, while high levels of HDL are considered to protect from atherosclerosis development<sup>119</sup>. Naturally, mice have high levels of HDL and low proportions of circulating VLDL and LDL. When C57BL/6 mice were fed atherogenic diet there was a shift in this balance, resulting in elevated LDL+VLDL *vs.* HDL. Oat bran significantly reduced this shift, and there were lower levels of LDL+VLDL in oat bran fed mice compared to control (Table 4, Paper I). There was however no significance of the differences found for the several oat products investigated in Paper III (Table 4). A possible explanation for this is loss of statistical power due to the multiple comparisons performed in the analysis of the many experimental groups.

The LDLr<sup>-/-</sup> mice have elevated LDL+VLDL already at baseline, and the western diet only provoked a small increase, which was inhibited by adding oat bran to the diet (Fig 1C, Paper IV). Our results suggest that oat bran contributes to a less atherosclerotic lipoprotein distribution in both C57BL/6 and LDLr<sup>-/-</sup> mice.

## Plasma triglycerides (TAG)

The results on triglycerides differed between C57BL/6 and LDLr<sup>-/-</sup> mice, both in response to the diets and to oat bran: LDLr<sup>-/-</sup> mice on a western diet increased their triglyceride content compared to baseline by threefold, and it was reduced significantly by oat bran (Figure 1B, Paper IV). Plasma triglyceride levels of C57BL/6 mice fed atherogenic diet on the other hand, decreased compared to baseline levels



and was not further decreased by oat bran (Table 5, Paper I). The reduced levels of triglycerides after atherogenic diet in wild type mice has been observed before<sup>104</sup> and has been proposed to be a result of cholate being a ligand for the nuclear hormone receptor FXR. This receptor regulates expression of a number of genes involved in lipoprotein metabolism<sup>94</sup>. The elevated triglyceride levels seen in LDLr<sup>-/-</sup> mice on western diet may be due to their lack of LDL-receptors, which are responsible for clearance of triglyceride-rich remnant lipoproteins produced by hydrolysis of VLDL and chylomicrons<sup>114,120</sup>.

Although debated, hypertriglyceridemia is regarded as an independent risk factor for cardiovascular disease<sup>121</sup>. Compared to fasting values, the concentration of triglycerides in blood are higher throughout much of the day. This contrasts with LDL and total cholesterol concentrations, which are unaffected by meals<sup>122</sup>. Recent epidemiological data reveal that non-fasting triglyceride values are better predictors of cardiovascular disease than fasting values, and postprandial responses to triglycerides are believed to trigger a number of pro-atherosclerotic processes, such as inflammation, oxidative stress and vasoconstriction<sup>123</sup>. When evaluating an effect of a diet on triglycerides I therefore find it more interesting to compare postprandial rather than fasting values, and in future studies postprandial levels of triglycerides rather than fasting values will be analysed.

Regarding effects of oats on triglycerides the data in the literature are inconclusive. In a comprehensive meta-analysis no evidence that triglycerides were reduced by oats in humans (probably based on fasting values) was found<sup>124</sup>. A reducing effect on triglycerides was however found in rats fed oats<sup>11</sup>. Our divergent results on triglyceride levels may thus be due to organism/strain differences in lipoprotein metabolism.

## Oats & atherosclerosis

### Oat bran reduces atherogenesis in LDLr<sup>-/-</sup> mice.

Despite numerous studies on the cholesterol-lowering effect of oats, information is scarce on how oats affect the development of atherosclerosis. Therefore we evaluated atherosclerotic lesions in LDLr<sup>-/-</sup> mice fed a western diet with or without 40 % oat bran. After 16 weeks, mice fed oat bran had significantly smaller lesion area both in the descending aorta and in cross sections of the aortic root (Fig 3, Paper IV), showing that oats are indeed anti-atherogenic. The direct demonstration that intake of oats can reduce atherosclerotic lesion development is to our knowledge a novel finding. There is one report in the literature that oat beta-glucans reduced the content

of esterified cholesterol in aortas of hamsters<sup>10</sup>, but other than that all studies have focused on plasma cholesterol and mechanisms behind reduced cholesterol levels. Recent work also provides some information about effects of oats on vascular inflammation<sup>125</sup>.

The most important factor for the reduced atherogenesis in our study is probably the reduction in plasma cholesterol, but as mentioned in the background section there are many micronutrients present in oats, such as E-vitamins, avenanthramides and other poly-phenols, that can exert important anti-oxidative and anti-inflammatory effects to prevent the development of atherosclerosis<sup>77,79-81</sup>. With our study design we could not discriminate whether the reduced atherogenesis solely is a consequence of the reduced plasma cholesterol or if other components in oat bran contributed to the effect. This remains to be elucidated, but the reduced atherogenesis observed supports the health claims that oats reduce risk of cardiovascular events.

## Effects on Inflammation

There was no effect of oat bran supplementation on the plasma inflammatory markers fibrinogen, SAA and TNF- $\alpha$  in C57BL/6NCrl mice fed an atherogenic diet (Table 5, Paper I). In the LDLr<sup>-/-</sup> mice there was however a significant reduction of both fibrinogen and soluble VCAM-1 and also a trend to lower concentrations of SAA in mice fed oat bran (Figure 2, Paper IV). Moreover, in the LDLr<sup>-/-</sup> mice oat bran intake led to a fourfold reduction of VCAM-1 expression in the aortic wall, analysed by immunohistochemistry (Figure 2E, F, Paper IV).

Our *in vivo* results of reduced VCAM-1 after feeding mice oat bran are in line with *in vitro* experiments where a reduced expression of VCAM-1 on endothelial cells was found after treatment with avenanthramides<sup>77</sup>. From the study design in Paper IV it is however not possible to unequivocally ascribe the reduced inflammation to avenanthramides or other anti-inflammatory components in the oat bran. The reduced inflammation could also originate from the reduced cholesterol *per se*. We can however conclude that oat bran indeed reduces inflammation in these mice and that the LDLr<sup>-/-</sup> strain is a suitable model for studying anti-inflammatory effects of oat components, in contrast to the C57BL/6 strain fed atherogenic diet.

The different outcomes in the two mouse models could be explained by the fact that LDLr<sup>-/-</sup> mice generally show more inflammation than the wild type C57BL/6 mice, which will make it easier to detect a reducing effect of the oat diet. Furthermore, the systemic inflammation was evaluated after 4 weeks on the experimental diet in the C57BL/6 mice, but after 16 weeks in LDLr<sup>-/-</sup> mice. The discrepancy could also be due to different oat bran concentrations: the LDLr<sup>-/-</sup> were fed 40 % oat bran whereas the C57BL/6 mice were fed 27 % oat bran. If it is the micronutrients in oats that contribute to the anti-inflammatory effect it may be significant that they are present in higher concentration in the diet containing 40 %

oat bran. The higher concentration was also more efficient in reducing plasma cholesterol. Hence the reduced inflammation could also originate from secondary effects of the reduced plasma cholesterol.

## Nitric oxide production

Production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) contributes to the protection against cardiovascular disease<sup>42</sup>. Experiments in cell cultures have revealed that avenanthramides can up-regulate the expression of eNOS on endothelial cells<sup>78</sup> and vitamin E has been shown to increase production of NO in LDLr<sup>-/-</sup> mice<sup>81</sup>. In our study LDLr<sup>-/-</sup> mice fed 27 % oat bran had greater protein expression of eNOS in the aortic arch than controls (Figure 4B, C, Paper IV). This suggests that oats can contribute to an improved endothelial function, possibly by an anti-oxidative action<sup>43</sup>. This could be one factor contributing to less atherosclerosis development.

## Mechanisms of the cholesterol-lowering effect

### Faecal excretion of bile acids & cholesterol

The excretion of cholesterol and bile acids in faeces after oat bran intake was significantly increased in both C57BL/6 (Table 3, Paper I and Figure 2A, Paper II) and LDLr<sup>-/-</sup> mice (Figure 1D, E, Paper IV). The levels of excretion were, not surprisingly, higher in the C57BL/6 mice since the atherogenic diet fed to them contained additional amounts of both cholesterol and bile acid in the form of cholic acid. Our results correspond well to earlier reports showing increased bile acid excretion with oat regimens in both humans<sup>67,68,126-128</sup> and rats<sup>53</sup>. However, several human studies reported no increased excretion of cholesterol after intake of oat products<sup>67,93,126</sup>. Taken together, the available results support the conclusion that increased excretion of bile acids and the concomitant increase in bile acid synthesis are important mechanisms for the cholesterol-reducing effects of oats. The effects observed in many ways resemble that of resins (i.e. cholestyramine), which bind bile acids and thereby increase the excretion of bile acids and increase bile acid biosynthesis<sup>34</sup>.

The total faecal output did not differ between control and oat bran groups in our studies (Paper I, III, IV). These data are in line with previous reports that various dietary fibre sources, such as cellulose, wheat bran and oat bran, increase stool weight

compared to a non fibre/low fibre diet, but no difference in stool weight was found between the fibres<sup>127</sup>.

## mRNA expression of liver proteins

The mRNA expression of the rate-limiting enzyme of bile acid production, CYP7A1, was up-regulated by fourfold in mice fed oat bran (Figure 3A, Paper II). Up-regulation of CYP7A1 has also been found in rats after intake of barley beta-glucans<sup>52</sup> and in human studies, where oat bran consumption nearly doubled the plasma concentration of 7 $\alpha$ -hydroxy-4-cholesten-3-one ( $\alpha$ -HC) compared to intake of wheat bran.  $\alpha$ -HC is a metabolite in the synthesis of bile acids and is used as a marker of bile acid synthesis since its plasma concentration correlates well with the activity of CYP7A1<sup>65</sup>. All the above-mentioned results support up-regulation of bile acid synthesis as an important mechanism for the cholesterol-reducing effect of oats.

There was a small but statistically significant increase in the expression of the lipoprotein receptors LDLr and LRP in the oat bran group. This indicates increased uptake of (mainly apoB and E containing) lipoproteins from the circulation to the liver. However, the fact that reduced plasma cholesterol levels are seen in LDLr<sup>-/-</sup> mice fed oat bran (Paper IV), implies that increased number of LDL-receptors is not a crucial mechanism for the cholesterol-lowering effect of oats. There was no effect of oat bran on mRNA expression of HMG-CoA reductase, PPAR $\alpha$  or SREPB-2 in the C57BL/6NCrl mice.

## Substrain difference in response to oats in C57BL/6 mice.

The mouse substrain difference in response to oats, summarized in Table 2, was a serendipitous discovery made when we temporarily had to change animal facility within the university and therefore also changed supplier of the C57BL/6 mice. After return to our regular facility we confirmed that the effect correlated with mouse strain and not housing environment. However, the mice were obtained at 7-8 weeks of age from two different suppliers, so we cannot exclude that the breeding environment plays a role.

There are more than 20 substrains of C57BL/6 available on the market, all originating from the first stock established by CC Little in 1921 (thereof the “C” in C57BL/6). The strain was taken to the Jackson laboratories in 1948. The C57BL/6N (NIH) substrain was separated from the C57BL/6J (Jackson) in 1951. Significant genetic differences have been found between the C57BL/6 substrains<sup>129</sup>. From the two main substrains N and J there are numerous sub-substrains available – the C57BL/6NCrl (B6NC, Charles River) and C57BL/6JBomTac (B6JB, Taconic) strains used in our studies being two examples. It must be emphasized that all

substrain differences discussed in this thesis are between these two particular substrains, not N and J generally.

Our first finding regarding differences between the substrains was that total plasma cholesterol was reduced by the oat diet in B6NC, whereas no sustained effect was found in B6JB (Fig 1, Papers I and II). In accordance with this finding, levels of LDL + VLDL were reduced in B6NC, but not in B6JB (Table 4, Paper I). While the effects on plasma lipids were accompanied by increased faecal excretion of both cholesterol and bile acids in B6NC this was not the case in B6JB (Table 3, Paper I and Fig 2, Paper II).

The mouse substrain differences in response to oats are interesting from two perspectives. First, they are of practical value when evaluating the cholesterol-lowering effects of oat products experimentally. Surprisingly few studies have evaluated effects of oats in mice<sup>130,131</sup>, although mice are used extensively for experiments with hypocholesterolemic agents. Secondly, the substrain difference can serve as a tool for elucidating mechanisms involved in the cholesterol-lowering effect of oats.

In the attempt to find mechanisms behind the above-mentioned differences we ran RT-PCR mRNA-analysis of liver samples. The most striking finding from this experiment was that in contrast to the up-regulation of CYP7A1 in B6NC, this enzyme was down-regulated in B6JB after oat bran treatment (Fig 2, Paper II). This finding supports the notion that increased bile acid excretion and production is crucial for the cholesterol-lowering effect of oats<sup>52,65,68</sup>. The question remaining is of course why CYP7A1 expression is up-regulated in one substrain but down-regulated in the other. What pathway(s) lead to this difference? A single nucleotide polymorphism (SNP) in gene(s) coding for CYP7A1 or its regulatory proteins could be one possible explanation. In humans, polymorphisms in the CYP7A1 gene make subjects more or less responsive to dietary and pharmacological cholesterol-lowering treatments<sup>132-134</sup>. It has also been shown that cholesterol-lowering response to dietary fibres, including oat bran, varies depending on different phenotypes of apolipoprotein-E<sup>128,135,136</sup>.

**Table 2.** Effects of oat bran on different parameters in two substrains of C57BL/6 mice fed atherogenic diet with or without 27 % oat bran.

Substrain	Plasma cholesterol	LDL +VLDL	Cholesterol excretion	Bile acid excretion	CYP7A1 mRNA	Microbiota diversity
B6NC	↓	↓	↑	↑	↑	-
B6JB	-	-	-	-	↓	↓

B6NC = C57BL/6NCrl, B6JB = C57BL/6JBomTac. ↓↑ represent sustained, significantly different (p<0.05) effects of 27 % oat bran compared to control diet.

The mRNA expression of SREBP-2 was moderately but significantly up-regulated in B6JB but not in B6NC mice (Fig 2, Paper II). SREBP-2 is important for regulation of cholesterol uptake and biosynthesis<sup>28</sup>, and does for example repress HMG-CoA reductase expression after input from LXR<sup>23</sup>. There was however no difference in the expression of HMG-CoA reductase in either substrain. Since the clearance of cholesterol via CYP7A1 seems to be impaired in the B6JB substrain, one might expect that there is an accumulation of cholesterol in the hepatocytes. Theoretically this would trap the SREBP-2 in the ER-membrane and inhibit its action<sup>25</sup>. Possibly this lack of a SREBP-2-mediated response induces transcription of the *Srebp-2* gene by a feed-back mechanism.

We also speculated that the differences in plasma cholesterol could originate from a divergent microbiota in the intestine of B6NC and B6JB. Results from the experiment performed to address this are presented under the “Microbiota diversity & plasma cholesterol” headline below.

## Mechanisms behind elevated bile acid excretion and production.

The fact that two different substrains of mice react differently to oat bran regarding plasma cholesterol, bile acid excretion and expression of CYP7A1 makes it unlikely that the mechanism behind the increased bile acid excretion would originate only from rheological properties of oat fibres resulting in an increased unstirred layer at the absorptive surface of the intestine. Besides possible polymorphisms in the CYP7A1 gene itself, one might speculate that alterations in genes or genetic expression of bile acid transporters or regulators of bile acid production can be found between the two substrains, and there are many targets of interest for further analysis.

The ASBT transporter that mediates the uptake of bile acids from the intestine<sup>35</sup>, is perhaps the most interesting target in the enterohepatic circulation (Fig. 3), although the importance of the other transporters cannot be excluded. HNF and LXR both induce CYP7A1 transcription (figure 2), whereas FXRs are important inhibitors of CYP7A1 transcription<sup>24</sup>. Furthermore, alterations in the intestinal cholesterol uptake or regulatory proteins involved in cholesterol synthesis cannot be ruled out as possible targets. In this respect proteins mediating uptake of cholesterol from the intestine to the enterocytes, NPC1L1 and possibly also SR-BI are natural targets to analyse in jejunal samples. LXR could be a target both in the liver, where it down-regulates cholesterol synthesis, and in the intestine, where it increases the expression of ABC transporters mediating transport of cholesterol and other sterols from the enterocyte back into the intestinal lumen<sup>23</sup>.

In an *in vitro* experiment it was shown that beta-glucans bind more strongly to dihydroxy- than to trihydroxy bile acids<sup>48</sup>. Chenodeoxycholic acid (CDCA), with 2 hydroxyl groups, is hence more likely to bind to beta-glucans than cholic acid (CA) with 3 hydroxyl groups. Production of a proportionally larger amount of CDCA in a

subject would therefore augment the probability of increased bile acid excretion following an oat beta-glucan intake. Alterations in the “neutral” and “acidic” bile acid synthesis pathways with different activities of a variety of enzymes involved in bile acid synthesis such as CYP8B1, CYP27A1, CYP7B1 and CYP39A1 result in different amounts of CA and CDCA produced<sup>24</sup>. Details about this are beyond the scope of this thesis, but altered expression of the different CYP-enzymes could possibly also be an explanation for the different response to oats found between the two C57BL/6 substrains and also between human subjects.

## Production of SCFA & plasma cholesterol

The SCFAs produced after intestinal fermentation vary depending on the substrate source, gut transit time and the number and types of bacteria present in the large intestine.

In Paper III we compared the amount of different SCFAs in the caecal contents from C57BL/6 mice fed either control diet with insoluble cellulose fibres or oat bran preparations with different molecular weight of the soluble beta-glucan fibres. The total SFCA pool was generally increased by all oat bran preparations, as was the amount of propionic acid (Table 5, Paper III). The ratio of propionate/acetate was also increased by the oat preparations compared with control. Since propionate is suggested to inhibit cholesterol biosynthesis (see Background), one might therefore expect that this contributed to the cholesterol-lowering effect of the oat preparations. However, we found that the propionate/acetate ratio was reduced by low molecular weight preparations of oat bran but had no detectable impact on the ability to reduce plasma cholesterol (Table 6, Paper III). In a study in rats Levrat et al could not establish a specific cholesterol-lowering effect of propionate and concluded that instead increased excretion of bile acids is an important feature of the cholesterol-lowering effect of fibre<sup>137</sup>. The collected results suggest that the level of propionate, or the ratio of propionate/acetate, is of minor importance for the cholesterol-reducing effect of fibre and likely to be obscured by effects on bile acid excretion and production. Further results supporting this are all studies made in ileostomy patients where oat products have been shown to reduce plasma cholesterol<sup>67,68,126</sup>. This argues against interference of SCFA with hepatic cholesterol synthesis as an important mechanism behind the cholesterol-lowering effect of oats, since the major fermentative action takes place in the large intestine. Bacteria are present in ileum, but production of SCFA by bacterial fermentation is very small in ileal samples<sup>93</sup>.

## Microbiota diversity & plasma cholesterol

Intestinal fermentation of different dietary fibres promotes growth of specific bacterial species<sup>1,55</sup>. It is therefore likely that feeding mice different fibres contributes to bacterial growth affecting the microbiota diversity. Our experimental diets contain beta-glucans and cellulose respectively, and in paper II the effect of the diets on microbiota diversity was evaluated in the two substrains of C57BL/6.

In the B6NC mice diversity of microbiota and microbial community was similar with and without oats in the diet, but in the B6JB mice oat bran in the diet resulted in lower diversity and smaller differences in microbial communities compared to controls (Figures 7 and 8, Paper II). Since oat bran reduced plasma cholesterol in B6NC but not in B6JB, we conclude that microbiota diversity as such is not crucial for effects on plasma cholesterol.

Comparison of the pattern of terminal restriction DNA fragments in the oat bran group of the two substrains, revealed that some peaks (i. e. groups of bacteria) were only present in one of the two substrains (Table 1, Paper II). Perhaps specific groups of bacteria present, rather than the diversity of the intestinal microbiota, can influence plasma cholesterol. Supporting this hypothesis is the finding that the probiotic bacteria *Lactobacillus plantarum* were shown to reduce plasma cholesterol in C57BL/6 mice with concomitant increase in bile acid excretion and CYP7A1 expression. It has been speculated that the increased faecal excretion of bile acids could originate from coprecipitation of the lactic acid bacteria with the bile acids<sup>138</sup>. Different amounts and sources of dietary fibres have already been shown to influence the composition of the intestinal microbiota, as for example barley decreased numbers of coliforms and *Bacteroides* and increased numbers of *Lactobacillus*<sup>56</sup>, the latter a genus commonly used as probiotics.

## Beta-glucan molecular weight & effect on plasma cholesterol

The molecular weight and solubility of beta-glucans are suggested to affect their cholesterol-lowering effect. In paper III beta-glucans in oat bran preparations were digested by beta-glucanases to reduce the molecular weight (Mw) of the fibres. The *in vitro* viscous properties of beta-glucans strongly correlated with their average molecular weight (Mw; Figure 1c, Paper III). The plasma cholesterol-reducing effect was however not affected by the reduced Mw of the beta-glucans (Figure 2A, B, Paper III), suggesting that Mw and viscous properties of beta-glucans may in fact not be



crucial parameters for reducing plasma cholesterol. Similar results have been found for barley beta-glucans in both human and animal studies<sup>139,140</sup>.

Another argument against molecular weight as a critical factor for cholesterol-lowering effects of beta-glucans is that these fibres are degraded in the small intestine. Different studies show a Mw of 35-100 kDa of beta-glucans isolated from small intestinal contents<sup>141,142</sup>. This indicates that beta-glucans are probably degraded to a larger degree by passage through the intestine than by processing before ingestion<sup>142</sup>.

Although the molecular weight of beta glucans does not seem crucial for the cholesterol-lowering effect, there are reports in the literature that processing of oats or oat fibres attenuates the cholesterol-lowering effect<sup>19,57,87-89</sup>. Results in our laboratory also support this. In a pilot study with LDLr<sup>-/-</sup> mice we fed the diets as pellets and there was significant reduction of the plasma cholesterol of oat bran compared to control. Halfway into the study we started to administer the diet as powder and found that the plasma cholesterol in the oat bran group was further reduced compared to when it was administered as pellets (data not shown). The “process” of making pellets included adding water, making pellets by hand, and then drying the pellets under vacuum at room temperature (freeze-drying without freezing the product). The physicochemical changes caused by the pellet production are unknown, but this pilot experiment illustrates that processing of oat bran can attenuate its cholesterol-reducing effect. Our results do however indicate that modifications in bioactive properties induced by processing cannot be ascribed to reduced molecular weight of the beta-glucans, at least regarding cholesterol-lowering effects.

## Glucose Response

Oats and oat beta-glucans generally contribute to a reduced postprandial glucose response to foods<sup>62,70,143</sup>, but there are inconsistent reports in the literature<sup>88,128</sup>.

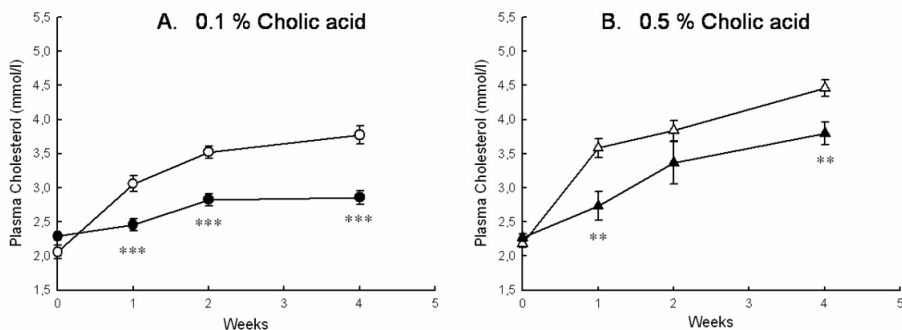
In paper II we evaluated the glucose tolerance and insulin sensitivity after long-term intake of oat bran, in contrast to the postprandial experiments referred to above. The oral glucose test did not reveal any effect of the oat bran on glucose or insulin responses (Fig 5A, B, Paper II). The insulin tolerance test did however show that mice fed oat bran had a reduced clearance of glucose (Fig 6A, Paper II), suggesting insulin resistance of the oat fed mice. The mice in the oat bran group had higher body fat mass and increased more in weight than their control counterparts and the increased body fat mass can explain the impaired insulin sensitivity. All experiments were performed in both C57BL/6NCrl and C57BL/6JBomTac, and we could conclude that there were no differences in body weight gain, body fat mass or glucose response

to oats between the two substrains. It has been suggested that oat foods need to be enriched with additional beta-glucans to achieve a low glycemic index<sup>85</sup>. If this is true it is likely that the fibres need to be present in the intestine to exert the glycemic effect as in postprandial experiments, whereas they may not cause long term effects on glucose and insulin responses as investigated in Paper II.

## Modifications of the diet

### Atherogenic diet & Gallstones

In our initial studies 0.5 % cholic acid was added to the diet to make it atherogenic, according to recipes of atherogenic diets from Nishina et al.<sup>95</sup>. The atherogenic diet is sometimes also called lithogenic, due to its ability to induce gallstones in susceptible mice strains (as C57BL/6)<sup>144,145</sup>. We found significant cholesterol-lowering effects of 40 % oat bran with this diet but reproducibility was poor due to large scatter in the data. Therefore we compared addition of 0.5 % with 0.1 % cholic acid in one experiment and specifically evaluated incidence of gallstones. Nine mice out of 28 investigated formed gallstones or pregallstone structures with 0.5 % cholic acid, whereas no gallstones were observed using 0.1 % (Table 2). The increase in plasma cholesterol was slightly less pronounced on 0.1 % cholic acid compared to 0.5 %, but still significant compared to baseline levels. Oat bran reduced the plasma cholesterol levels in both diets, but the mice fed 0.1 % cholic acid had a more even distribution of plasma cholesterol concentrations over time and between individuals than those fed 0.5 % cholic acid (Figure 8).



**Figure 8.** Total plasma cholesterol in C57BL/6 mice after 4 weeks of atherogenic diet with either A. 0.1% or B. 0.5% cholic acid. Open symbols represent control and black symbols represent oat bran 40 %. n=14, \*\*p<0.01, \*\*\*p<0.001, statistics were calculated with Student's t-test.

**Table 5.** Gallstone formation in C57BL/6 mice fed atherogenic diets with either 0.1 or 0.5 % cholic acid. The oat bran concentration was 40 %.

	<b>Incidence Pre-gallstone Structures</b>	<b>Incidence Gallstones</b>
	(n/N)	(n/N)
0.1 % CA, Control	0/14	0/14
0.1 % CA, Oat bran	0/14	0/14
0.5 % CA, Control	0/14	0/14
0.5 % CA, Oat bran	3/14	6/14

n/N = number of mice that formed gallstones/total number of mice.  
CA, cholic acid

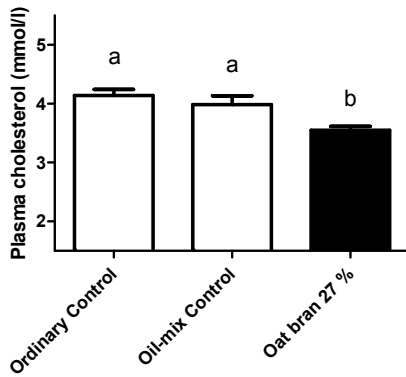
An interesting observation from this experiment was that all nine mice with gallstone structures were in the oat bran group with 0.5 % cholic acid. This is probably due to the fact that less bile acids are (re-)absorbed when soluble oat fibres are present. Reduction of the bile acid pool will result in supersaturation of the bile fluid and the excessive amount of cholesterol will precipitate out into gallstones. Similar observations were made when comparing a synthetic diet containing cellulose as fibre source with a chow diet, probably containing soluble fibres, with the same amounts of cholesterol and cholic acid<sup>95</sup>.

We conclude that 0.1 % cholic acid is preferable over the 0.5 % commonly used when effects of soluble fibres on plasma cholesterol are studied using atherogenic diets.

## Modifications of fat content in the diet

As discussed in Paper I, a possible drawback of using oat bran in the diets is that it is difficult to exactly compensate for its lipid and protein contents. As a result of this the fatty acid composition in control and oat bran diets differ slightly (Paper I, Table 2). We did however perform a control experiment where the fat in oat bran was compensated by a mixture of peanut oil (35 %), sunflowerseed oil (49 %) and rapeseed oil (16 %) instead of butter in the control diet, which made the fatty acid composition in the two diets equal. The plasma cholesterol in the group fed “ordinary” control diet did not differ from the “oil-mix” control group and the oat bran diet had significantly lower plasma cholesterol concentrations than both control

diets (Fig. 9). These findings make it unlikely that the results obtained in our studies are influenced by the minor differences in lipid composition between control and test diets. However, to achieve maximum similarity between control and test diets we will in future experiments use the oil mix resembling the fatty acid composition in oats to compensate for its lipid contents.



**Figure 9.** Comparison of ordinary control and control with oil mix supplement of the oat bran fat. n=9-10, Bars with different letters differ significantly ( $p < 0.05$  analysed with ANOVA, followed by Tukey's Multiple Comparison test).



# Conclusions & Future Perspectives

Many questions (raised decades ago!) remain regarding the cholesterol-lowering mechanisms of oats, and about components in oats besides the beta-glucans that may contribute to the effect. The contribution to the field described in this thesis has been to develop an experimental mouse model system in which these questions can be further addressed in a systematic way:

We have shown that C57BL/6 mice on an atherogenic diet serve as a good model for evaluation of the cholesterol-lowering effects of oat preparations, but that substrain differences exist in the responsiveness to oats.

C57BL/6 mice fed an atherogenic diet are useful when the cholesterol-lowering effect is the main endpoint. For studies of effects on inflammation and atherogenesis a model developing more pronounced hypercholesterolaemia, as the LDLr<sup>-/-</sup> mice, is preferable.

Our finding that oat bran reduces atherosclerosis in LDLr<sup>-/-</sup> mice directly demonstrates that oats have anti-atherogenic properties, and supports health claims that oats can reduce risk of cardiovascular disease.

The different expression of CYP7A1, and different excretion of bile acids, in the two substrains of C57BL/6 strongly support the importance of increased bile acid excretion together with increase of bile acid synthesis from cholesterol, for the cholesterol-lowering effect of oats. This mechanism probably dominates over effects of SCFA on cholesterol biosynthesis as a basis for the cholesterol-lowering action of oats. The mechanisms behind the increased bile acid production need however to be further investigated.

The molecular weight and viscous properties of beta-glucans do not seem to be a crucial parameter for the cholesterol-lowering properties of oats in the C57BL/6 mice.

Since oat bran was found to reduce plasma cholesterol without affecting HMG-CoA reductase expression, the possibility exists that synergistic effects might arise when an oat diet is combined with statin treatment, of potential interest for secondary

prevention in patients with cardiovascular disease. Collaborative studies to explore this using our mouse model are underway.

# Populärvetenskaplig sammanfattning

Om man säger havre tänker nog de flesta på gröt, flingor eller müsli. Men om du fick höra att man kan förhindra hjärtinfarkt med hjälp av havre skulle du säkert undra hur det hänger ihop. I min avhandling har jag delvis försökt förklara hur havre kan påverka och förhindra de processer i kroppen som till slut leder till att en hjärtinfarkt uppstår.

Så först, hur uppstår en hjärtinfarkt? Det hela startar med att en sorts fett i blodet, kolesterol, fastnar i blodkärlens väggar. Kolesterolet oxideras av fria radikaler och det stimulerar till inflammation i kärlväggen som gör att vita blodkroppar och ännu mera kolesterol tar sig in där och fastnar. Till slut bildas en stor ansamling av fett och döda celler som kallas plack. Man brukar kalla processen för åderförkalkning (fast ett bättre namn egentligen ju är åderförfettning). Om placket blir så stort att det helt hindrar blodflödet, eller om ett plack lossnar från sitt fäste och följer med blodbanan till ett annat mindre kärl där det helt täpper till blodflödet blir följden hjärtinfarkt eller stroke, beroende på om stoppet uppstår i hjärtat eller hjärnan.

Det finns många riskfaktorer som bidrar till åderförfettning t ex, rökning, övervikt, högt blodtryck, ärftlighet och diabetes. Förhöjda värden av kolesterol i blodet anses dock vara den kanske viktigaste riskfaktor för att utveckla åderförfettning, och det är här havre kommer in i bilden:

Havre har nämligen kolesterolsänkande egenskaper. De komponenter i havre som troligtvis står för den största effekten är beta-glukaner, en slags lösliga fibrer. Trots att många kliniska studier visat kolesterolsänkande effekter av havre har det saknats direkta bevis för att havre skulle minska uppkomst av plack i kärlen. Förutom beta-glukanerna innehåller havre också andra små kemiska komponenter som kan verka både anti-inflammatoriskt och anti-oxidativt, dessa kan ytterligare bidra till att förhindra åderförfettningensprocessen.

I ett delarbete i avhandlingen undersökte vi om tillsats av havre i kosten påverkar åderförfettning. Vi gav möss en fettrik diet med eller utan havrekli. Kontroll- och havreklidiet hade samma energiinnehåll och sammansättning av fett, protein, kolhydrater och fibrer. Den grupp som fick havrekli hade 40 % lägre halter kolesterol i blodet. Den hade också mindre spår av inflammation i både blodet och kärlväggen, och hade dessutom färre och mindre plack i kärlväggen. Våra resultat stödjer tesen att



havre kan förebygga hjärt-kärlsjukdom. Om det enbart är de kolesterolsänkande effekterna, eller om andra anti-oxidativa och anti-inflammatoriska komponenter i havre också verkar skyddande mot uppkomst av plack i kärlen återstår att ta reda på.

Trots att beta-glukaner är några av de bäst dokumenterade livsmedelskomponenterna med kolesterolsänkande egenskaper, kvarstår många frågetecken om mekanismerna bakom dessa. Effekterna har t ex visat sig variera mellan olika individer. Dessutom verkar effekterna också vara beroende av storlek och struktur på havrefibrerna, och dessa kan förändras vid tillverkningsprocessen och förvaringen av livsmedel. För att på ett standardiserat sätt kunna utveckla havrebaserade livsmedel som ger de önskade kolesterolsänkande effekterna är det nödvändigt att kvaliteten hos fibrerna utvärderas före och efter eventuell processning. En annan del av mitt avhandlingsarbete gick därför ut på att utveckla en musmodell i vilken havrefibrernas fysiologiska effekter kan utvärderas innan de tas i storskalig produktion för humant bruk, och där mekanismerna bakom de kolesterolsänkande effekterna kan studeras.

Igen gav vi mössen en fettrik diet med eller utan havrekli. I detta arbete fann vi att två sorters möss reagerade olika på havrebehandlingen: Den ena sorten fick tydligt lägre nivåer av kolesterol i blodet, ökade utsöndringen av kolesterol och gallsyror i avföringen, samt hade högre nivåer av ett ämne i levern som producerar gallsyror. Ingen av dessa effekter hittades i den andra sorten. Detta fynd stödjer starkt hypotesen att minskat upptag av gallsyror i tarmen är en viktig mekanism bakom de kolesterolsänkande effekter man ser av havre. Kroppen måste då producera nya gallsyror, vilket den gör med kolesterol som byggsten, varför nivåerna av kolesterol i blodet sjunker. Resultatet att olika möss svarar olika på havrebehandling är viktigt ur två avseenden: i) Ur ett praktiskt perspektiv då man ska välja rätt sorts möss för studier av kolesterolsänkande effekter av olika havreprodukter och andra lösliga fibrer. ii) Man vet att vissa människor får sänkta kolesterolvärden när de äter havre, men andra inte. Olikskheterna i mössen kan hjälpa oss att förstå varför. Det kan också ge värdefulla ledtrådar om mekanismerna bakom de kolesterolsänkande effekterna.

Slutligen ville vi studera hur processning av beta-glukaner påverkar de kolesterolsänkande egenskaperna hos havre. Med hjälp av enzymer bröt vi ner Beta-glukanerna till olika storlek, och jämförde dem med intakta beta-glukaner. Med den här typen av enzymatisk processning kunde vi dock inte se att kapaciteten att sänka plasmakolesterol förändrades med storleken på beta-glukanerna.

Sammanfattningsvis kan vi konstatera att storleken på beta-glukanerna inte verkar ha någon betydelse för den kolesterolsänkande effekten, att utsöndring och produktion av gallsyror är avgörande för att havre ska sänka kolesterolet i blodet, och att havre i kosten i en musmodell förhindrar åderförfettningprocessen i blodkärlen och därmed minskar risken för hjärtkärlsjukdom.

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