The use of rabbits in atherosclerosis research. Diet and drug intervention in different rabbit models exposed to selected dietary fats and the calcium antagonist (-)-anipamil

evaluation and comparison of rabbit models

Mortensen, Alicja

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THE USE OF RABBITS IN ATHEROSCLEROSIS RESEARCH. DIET AND DRUG INTERVENTION IN DIFFERENT RABBIT MODELS EXPOSED TO SELECTED DIETARY FATS AND THE CALCIUM ANTAGONIST (-)-ANIPAMIL

Evaluation and comparison of rabbit models

Ph.D. Thesis

ALICJA MORTENSEN

Roskilde University
Institute of Life Science

National Food Agency
Institute of Toxicology

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Author: Alicja Mortensen.

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Department: Institute of Toxicology, Department of General Toxicology.

Address: Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.

Telephone: +45 39696600

Fax: +45 39660100

Abstract: Laboratory animal models play an important role in atherosclerosis research. One of the most popular laboratory animal species in this field of research is the rabbit. The rabbit fulfils most of the criteria for an animal model for human atherosclerosis. Three rabbit models were established and used for dietary or drug intervention: 1) the cholesterol-fed normolipidemic rabbit, 2) the 1% cholesterol-fed heterozygous Watanabe heritable hyperlipidemic (WHHL) rabbit and 3) the homozygous WHHL rabbit. The reproductive performance and physiological blood lipid levels in growing and adult heterozygous and homozygous WHHL and normolipidemic rabbit were characterized. The position of the rabbit models in atherosclerosis research was discussed. The characteristic features of cholesterol-fed and WHHL rabbis were compared.

Key words: Animal models, WHHL rabbit, cholesterol-fed rabbit, atherosclerosis.

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Front cover: A cross section of unopened aorta from 19 months old rabbit shows circumferential advanced lesion (x 25, elastic van Gieson).

GENERAL INFORMATION

The present thesis discusses the rabbit models in atherosclerosis research. The thesis is based on literature studies and on own experience and data obtained through the experimental work with the three rabbit models: homozygous Watanabe heritable hyperlipidemic (WHHL) rabbit, heterozygous WHHL rabbit fed 1% cholesterol, and cholesterol-fed rabbit based on a normolipidemic strain, the results of which are included in part III, chapters A - F.

The chapters III.A.1, III.B. III. C., III.D. and III.F. contain the results already published elsewhere. Chapter III.A.2. contains yet unpublished results. Chapter III.E. contains an unpublished paper but the abstract based upon this study has already been published.

In the published papers, the following work was performed by the author of this thesis and independently of the co-workers of the publications:

Paper in chapter III.A.1.: study design, development and maintenance of breeding colony of WHHL rabbits, characterisation of breeding performance of WHHL rabbits, statistical analysis and interpretation of data on physiological blood lipid levels in growing homozygous and heterozygous WHHL rabbits and the comparison of the blood lipid levels in growing WHHL rabbits to those in growing normolipidemic rabbits, and preparation of the manuscript;

Paper in chapter III.B.: design of the time course for breeding of the WHHL rabbits and the distinction between the heterozygous and homozygous offsprings, supervision of the clinical course of the study, *post mortem* examination, sampling of tissue for microscopic examination;

Papers in chapters III.C. and III.D.: study design, supervision of clinical course of the study, *post mortem* examination, sampling of tissue for microscopic examination, statistical analysis and interpretation of data on body weight, feed intake, blood lipids, cholesterol content in aorta (III.C.) and atherosclerotic lesions (III.C), preparation of the manuscripts;

Paper in chapter III.F.: design of the time course for breeding of the WHHL rabbits and the distinction between the heterozygous and homozygous offsprings, supervision of the clinical course of the study, *post mortem* examination, sampling of tissue for microscopic examination, statistical analysis and interpretation of data on body weight, feed intake and blood lipids.

The study reported in chapter III.A.2. was carried out and the manuscript was prepared strictly by the author of this thesis.

In the study reported in chapter III.E. the following work was performed strictly by the author of this thesis and independently of the co-workers: design of the time course for breeding of the WHHL rabbits and the

distinction between the heterozygous and homozygous offsprings, study design, supervision of the clinical course of the study, *post mortem* examination, sampling of tissue for microscopic examination, statistical analysis and interpretation of data on body weight, feed intake, blood lipids, cholesterol content in the aorta, atherosclerotic lesions, and preparation of the manuscript.

Furthermore, the author of this thesis gained practical experience with methods for measurement of blood lipids during the studies from chapters III.A.1. & 2. and III.C., and with preparation of the tissue for microscopic examination including fixation, embedding in paraffin, sectioning and staining, and the methods for macroscopic and microscopic qualitative and quantitative evaluation of atherosclerosis under the guidance of dr. med. Birgit Fischer Hansen on the material from the study in chapter III.B.

CONTENTS

PART I

INTRODUCTION

I.A. AIM OF THE PROJECT

The present project has been made in the Institute of Toxicology in the National Food Agency. The aim of the project was 1) to establish three rabbit models for human atherosclerosis, 2) to use the rabbit models for investigation of possible effects of dietary fats and drug intervention on atherosclerosis, and 3) to evaluate the rabbit models. For this purpose following has been performed:

- establishment of a breeding colony of Watanabe heritable hyperlipidemic (WHHL) rabbit and comparison of reproductive performance of WHHL and New Zealand White (NZW) females (chapter II.B., III.A.1.),
- description and comparison of the physiological blood lipid levels in growing (chapter III.A.1.) and adult (chapter III.A.2.) normolipidemic and spontaneously hyperlipidemic rabbits,
- evaluation of the morphology of spontaneous atherosclerosis in heterozygous and homozygous WHHL rabbits (chapter III.B.),
- comparison of the atherogenic effect of margarine (hydrogenated fat) and olive oil in the cholesterol-fed rabbit based on a normolipidemic strain and description of morphology of atherosclerotic lesions in this model (chapter III.C.),
- description of the morphology of experimental atherosclerosis in the heterozygous WHHL rabbit fed diet with 1% cholesterol and evaluation of the usefulness of this model to investigate the atherogenic effect of dietary fats using fats containing vegetable n-6 and marine n-3 polyunsaturated fatty acids (chapter III.D.),
- comparison of the effect of fish and olive oils on the development of spontaneous atherosclerosis in homozygous WHHL rabbits (chapter III.E.),
- investigation of the effect of the calcium antagonist (-)-anipamil on spontaneous atherosclerosis in homozygous WHHL rabbits (chapter III.F.),
- discussion of the position, usefulness and application of 3 rabbit models: cholesterol-fed rabbit based on a normolipidemic strain, homozygous WHHL rabbit and heterozygous WHHL rabbit in atherosclerosis research, and comparison of cholesterol-fed and homozygous WHHL rabbit models (chapter IV. A.1-3.).

I.B. ATHEROSCLEROSIS IN MAN

Atherosclerosis and its clinical sequela coronary heart disease (CHD) is a major cause of premature death in the modern world accounting for 40%

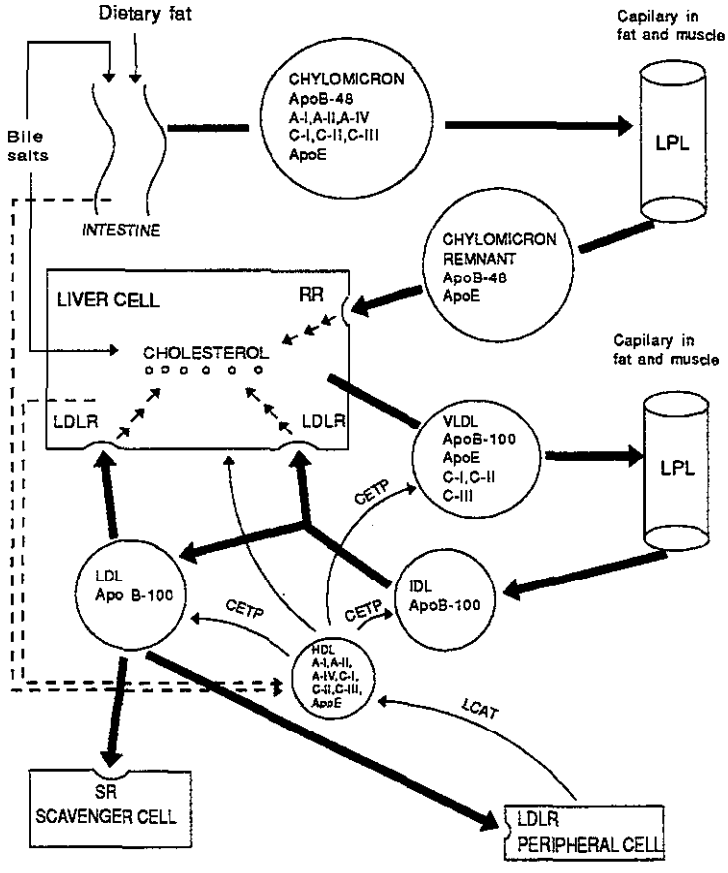
of death in middleaged men. In Denmark, people with cardiovascular disease comprise one of the largest patient groups, about 225000 people. Every year 25000 Danes die from CHD or expressed in another way one Dane dies from CHD every twenty minutes (*Hjertenyt august 1991*). CHD kills more than 180000 people in the UK and 500000 in the USA (*Durrington, 1993*). Besides CHD, atherosclerosis also contributes to cerebral infarction, gangrene and loss of function of the extremities. Therefore great efforts are being done to understand the pathogenesis of atherosclerosis in order to prevent and cure the disease.

I.B.1. Pathogenesis of atherosclerosis

Atherosclerosis is a complex disease of the arterial intima and media due to interaction between plasma lipoproteins (Fig. 1, p. 11) and cells of the arterial wall. It is influenced by genetical and environmental factors of which the diet is assumed to be an important one. The effect of the diet on the development of atherosclerosis is, in part, mediated through the influence of dietary fats on plasma levels of low density lipoprotein (LDL) cholesterol and by possible thrombogenic or antithrombogenic properties of fatty acids (*Pyörälä et al. 1994*). Furthermore, other dietary components e.g. dietary fibres, complex carbohydrates and polyphenolic antioxidants in vegetable foods may play a role as protective factors (*Hennig et al. 1994, Hertog 1994*).

Several reviews concerning the pathogenesis of atherosclerosis are available (*Ross 1993, Getz 1990, Munro et Cotran 1988*). The presently most widely accepted hypothesis for the pathogenesis is that atherosclerosis is a response to an injury. Different factors may cause injury e.g. hypercholesterolemia, hypertension, diabetes, obesity, chemical toxin as cigarette smoke, bacterial toxins, different mechanical factors, immunological factors, viruses, homocysteine, oxidized LDL, oxidative stress, fatty acid composition of the diet. According to this hypothesis (*Ross 1993, Thompson 1994*), the injury factors alter the function of endothelial cells of intima of arterial wall and promote increased adherence of monocytes, macrophages and T lymphocytes to vessel endothelium. Subsequently, these cells migrate between the endothelial cells into the arterial wall and localize subendothelially and form cell aggregations. The macrophages accumulate fat and become large foam cells. These lipid rich macrophages together with T lymphocytes form fatty streaks - the earliest recognizable light microscopic lesion within the intima. Fatty streaks progress to intermediate fibrofatty lesions composed of layers of macrophages and smooth muscle cells and ultimativelly develop into fibrous plaques. Fibrous plaques increase in size as they accumulate more cells and the macrophages scavenge more lipids and become foam cells. These processes lead to transformation of the fibrous plaques to advanced lesions which are covered by dense caps of connective tissue with embedded smooth muscle cells that

Fig. 1. From: Mortensen A: Transgenic laboratory animal models in atherosclerosis research. Scand J Lab Anim Sci 1995, 22: 133-143



A schematic presentation of the lipoprotein metabolism. In the intestine the dietary cholesterol together with triglycerides is built into chylomicrons which are then transported by lymph into the bloodstream. In the capillary vessels the chylomicrons are hydrolyzed to chylomicron remnants by lipoprotein lipase (LPL) and hepatic lipase (HL). Chylomicron remnants are taken up by remnant receptors (RR) in the liver. Cholesterol from the chylomicron remnants is 1) secreted in the bile (as cholesterol or bile salts) into the intestines and 2) is used in the synthesis of very low density lipoproteins (VLDL), which are secreted into the blood. In the circulation VLDLs are hydrolized to intermediate density lipoproteins (IDL) by LPL and HL. IDLs 1) bind to the low density lipoprotein receptor (LDLR) and are transported into the liver cells, and 2) are catabolized to low density lipoprotein (LDL) in the blood stream. LDLs are 1) taken up by LDLR in liver cells and peripheral cells, and 2) some of them undergo modification in the circulation and then bind to the scavenger receptor (SR) and are transported into scavenger cells. High density lipoproteins (HDL) are directly produced both in the liver and intestine. HDL constituens are also derived from chylomicron and VLDL catabolism. There are two main types of HDLs: HDL_2 and HDL_3. HDLs serve as acceptors of lipids especially free cholesterol from varying tissues. Free cholesterol is then estrified due to reaction mediated by lecitin cholesteryl acyltransferase (LCAT). Cholesterol esters are then transferred from HDLs to other lipoproteins nonspecifically, as well as by cholesteryl ester transfer protein (CETP). HDLs contribute in the transport of cholesterol from periphery to the liver.

usually overlays a core of lipid and necrotic debris. Some of lipid laden macrophages in advanced lesions may migrate back to the lumen of the vessel pushing apart the endothelial cells. Sites where endothelial disjunction occurs become thrombogenic and platelet mural thrombi may form there. Platelet mural thrombi, activated macrophages and possibly also smooth muscle cells of the atherosclerotic lesion release many growth factors which induce the progression of advanced lesions. The ruptures or fissures in the advanced lesions lead to haemorrhage into the plaque, thrombosis and occlusion of the artery with the clinical consequence in the worst case being a sudden death from myocardial infarct.

I.B.2. Familial hypercholesterolemia in man

Atherosclerosis develops very slowly over the years. The clinical manifestation of the disease usually occurs in middleaged humans. However, people with genetic defects in the lipid metabolism may develop the disease earlier. One of the genetic syndromes leading to premature atherosclerosis is familial hypercholesterolemia (FH). FH is a dominant, inherited condition which affects approximately 0.2% of the population. It is a result of a defect in the LDL receptor due to mutation of its gene on chromosome 19. Since the LDL receptor plays a major role in the catabolism of LDL (see Fig. 1, p. 11), an LDL receptor deficiency or errors in its function result in accumulation of LDL in the plasma. There are five different classes of mutations of the LDL receptor which give rise to FH. Each mutation includes several distinct gene defects. In the class 1 mutation the immunologically detectable LDL receptors are absent. In class 2 the transport to the cell surface of any LDL receptors which are synthesized is either completely blocked or reduced. In class 3 functionally defective receptors are formed. In class 4 the LDL receptors miss the ability to cluster in coated pits, which prevents them from being internalized after binding LDL. In class 5 there is a failure in the delivery of LDL by receptors to endosomes or in recycling of LDL receptors to the cell surface (*Thompson 1994, Schneider 1990*). Until now, 150 different mutations have been described. Fifty four percent of them are class 2. FH occurs either in heterozygous form (inheritance of one mutant gene encoding for the LDL receptor) or homozygous form (inheritance of two mutant genes). The latter occurs very rarelly. Heterozygous patients have plasma cholesterol levels between 9 and 11 mmol/l[1]. Their triglyceride levels are usually in the normal range[2], rarely beyond 4 mmol/l. Homozy-

[1] For comparison, the average plasma cholesterol for a midle-aged man in United Kingdom is between 6 and 6.5 mmol/l (Durrington 1993) and the average plasma cholesterol in 50 years old Danes of both sexes is about 6.5 mmol/l (*data from the Glostrup study (1982) mentioned in LST publication nr. 176*).

[2]The normal range for humans is less than 2 mmol/l.

gous patients have total cholesterol levels about four times normal (*Thompson 1994, Durrington 1993, Davignon 1991*). Young men with homozygous FH may have heart attacks in their twenties. Less than 50% of affected and not treated men survive to the age of 60. Most male survivors will have either a heart attack or angina pectoris by this age. The same applies to 50% of females with FH, although only 15% will be dead at the age of 60 (*Durrington 1993*). Apart from atherosclerotic lesions in the arterial system, tendinous xanthomas on the dorsum of the hands and the Achilles tendons develop in most of adult humans with FH.

Another congenital condition leading to premature CHD despite the absence of the clinical features of FH is familial combined hyperlipidemia (FCH). The patients often have an increase in both plasma triglyceride and cholesterol levels and low levels of high density lipoprotein (HDL) cholesterol. This condition often results from a synergy between the various adverse effects of risk factors such as hyperlipidemia, hypertension, diabetes and a family history of CHD. FCH is more loosely defined as FH but this condition is more common, affecting between 1 in 50 and 1 in 200 people.

I.C. THE USE OF ANIMALS IN ATHEROSCLEROSIS RESEARCH

The information on pathogenic mechanisms on progression and regression of atherosclerosis is gathered through experiments in animal models and studies in man such as intervention trials, epidemiological studies, and to some extent studies in patients. Additonally, various *in vitro* model systems to study different aspects of lipid and lipoprotein metabolism play important role in understanding of pathogenesis of atherosclerosis (*Hussain et al. 1992*).

I.C.1. Advantages and limitations of human studies with respect to atherosclerosis

The advantage of human studies is that they provide the direct information about the effect of a chosen factor on the human organism. However, studies in man have several limitations. The intervention trials provide information on the short lasting effects of the treatment on clinical parameters (eg. blood lipids) only. Epidemiological studies are resource and time consuming and interpretation of the results is difficult because of the multifactorial nature of the effect. The experimental approaches on human patients are limited by the difficulty to control the effect of the treatment.

The control is handicapped by the slow rate of development/regression of the arterial lesions and the inability to make quantitative *ante mortem* determination of the extent and severity of the atherosclerotic lesions, because of the lack of noninvasive techniques that are sufficiently sensitive to measure the expected changes in the arterial wall of the arteries of greatest clinical interest, such as the coronary arteries.

I.C.2. Advantages and limitations of animal studies with respect to atherosclerosis

To overcome some of the limitations of studies in man, the animal models are widely used in atherosclerosis research. There are several advantages of using animal models. The experiments on animals can be performed in relatively short time. They also permit the obtaining of information on a long lasting effect of the diet or treatment, because of the short life span of laboratory animals. The clinical results are supported by post mortem examination of the arterial system. The influence of environmental factors on the results is minimalized by standardization of laboratory conditions and the use of the subjects with known genetic and health status (*Öbrink & Rehbinder 1993*). Furthermore, the studies in animal models are generally cheaper than most studies in man.

In spite of obvious practical advantages the animal studies have a great limitation - the extrapolation from animal results to the human situation is dubious. The conclusions one can draw from experimental atherosclerosis are limited because the experimental atherosclerosis is usually produced by exaggerating a single risk factor eg. by atherogenic diet, while multiple risk factors of genetic and environmental origin are involved in the development of human atherosclerosis. Furthermore, the factum that the experimental lesions develop during a relatively short period in conditions of high or extreme hypercholesterolemia while human lesions develop over decades at much lower plasma cholesterol concentrations should be taken into consideration. It is also important to note that hypercholesterolemia induced by cholesterol feeding in most species is associated with unusual types of lipoproteins: β-very low density lipoproteins (β-VLDL) or large LDL which normally do not occur in significant quantities in man.

I.C.3. Relevance of animal studies in atherosclerosis research

The relevance of animal studies in atherosclerosis research is beyond doubt. The fact that the animal and human cells have in general the same structure (e.g. cell membrane) and biochemistry (e.g. glycolysis, β-oxidation, and the Krebs cycle) serves as the basis for extrapolation from animal results to the human situation (*Calabrese 1983*). The lipoprotein metabolism and the cholesterol metabolism at the cellular level are similar in man and laboratory animals despite some species differences e.g. the apopro-

tein(a) and the cholesteryl ester transfer protein (CETP) are absent in the mouse but present in man. The animal studies and investigations in man demonstrate the same response to known blood lipid lowering compounds and to dietary fats. Therefore, despite some differences in experimental atherosclerosis in laboratory animal models and the disease process in humans (I.C.2.), the studies in animal models provide reliable information on how different factors accelerate atherosclerosis. Furthermore, more definitive answers to the question on development and regression of atherosclerosis in human have to await better techniques for noninvasive evaluation of atherosclerosis. Therefore the use of animal models continue to be critical for studies to uncover the cellular and biochemical events responsible for the development and regression of atherosclerotic lesions. Experiments in animals permit investigation of the biology of the arterial wall in each stage of plaque development and regression and under defined conditions. Although the effect of a diet or a therapeutic agent on the atherosclerotic process in the arterial wall must ultimatively be demonstrated in humans, the primary screening has to be done in animals. The results from animal studies indicate direction for intervention trials and to some extent for epidemiological studies. Furthermore, they provide support for particular conclusions reached in the population studies.

I.C.4. Most common animal models in atherosclerosis research

The use of animal models in atherosclerosis research goes back to the beginning of this century. In 1908, Ignatovsky induced atherosclerosis in rabbits by feeding them milk, meat and eggs (*Glueck 1979, Jokinen et al. 1985*). In 1913, Anitschnikov obtained similar lesions feeding rabbits pure cholesterol (*Glueck 1979*). Since then different animal species have been used to study atherosclerosis (*Vesselinovitch 1975, McCauley & Bull 1980, Malinov 1983, Jokinen et al. 1985, Armstrong & Heistad 1990, Overturf & Loose-Mitchell 1992*). The most common species have been: pigeons among birds, swine and rabbit among non-primate mammalians and Old World monkeys among non-human primates. The advantages and disadvantages of these models have been widely discussed (*McCauley & Bull 1980, Jokinen et al. 1985, Armstrong & Heistad 1990, Overturf & Loose-Mitchell 1992*) and are summarized in the Table below (p. 16). Recently, the development of transgenic techniques and identification of human genes coding for lipoprotein transport proteins opened the possibility to create various transgenic mice to study lipoprotein metabolism. Some of these transgenic mice seem promising animal models for atherosclerosis research (*Aalto-Setälä 1992, Breslow 1993 & 1994, Maeda 1993, Rubin & Smith 1994, Mortensen 1995*). However, their commercial availability remains very restricted and their usefulness for atherosclerosis research is still under establishment. Thus until now, the rabbit has been the most popular animal model in atherosclerosis research (*Mortensen et al. 1994*).

Table: Advantages and disadvantages of four most common animal models of human atherosclerosis.

Animal model	Advantages	Disadvantages
Pigeon	Lesions occur spontaneously, can be exacerbated or induced by dietary cholesterol. Morphological resemblance of lesions to those in human. Strain differences in susceptibility to lesion formation well established.	Plasma lipoproteins different from those in man. Topography of lesions different than in human. Herbivorous. Avian. Reproduce slowly. Small size of body and arteries.
Swine	Lesions occur spontaneously, can be induced by atherogenic diet. Topography and morphology of lesions and lipoprotein profile similar to those in human. Size of heart and vessels sufficient for studies of cardiovascular function. Omnivorous.	Lesions develop slowly. Large size[a]. Difficult to handle. Expensive to maintain.
Rabbit	Experimental lesions easily induced by dietary cholesterol. Spontaneous and induced lesions and blood lipids well characterized. Strains developing spontaneous atherosclerosis with morphological resemblance to that in humans available. Easy to acquire, maintain, relatively inexpensive.	Herbivorous. Physiologically low plasma cholesterol compared to man. Topography of lesions different than in human. Exaggerated cholesterol feeding leads to extreme hypercholesterolemia and lipid storage in many organs.
Non-human primates	Lesions occur spontaneously, are exacerbated by dietary cholesterol. Morphology of lesions similar to those in man. Lipoprotein profile similar to human. Close phylogenetic relationship to humans. Omnivorous.	Expensive to acquire and maintain. Long gestation and few progeny. Most species are moderately resistant to atherosclerosis. Species with small body size not suitable for collection of large blood samples.

[a]:This applies also to mini-pig which body weiht is relatively big compared to body weight of the pigeon, rabbit and most of the laboratory non-human primates. The body weight of 6 months old minigris is about 25 kg but a grown-up mini-pig may reach 50-70 kg (*Skydsgaard 1988*).

I.D. THE RABBIT MODELS IN ATHEROSCLEROSIS RESEARCH

I.D.1. Suitability of the rabbit for atherosclerosis research

The suitability of an animal model in atherosclerotic research is evaluated based on three criteria: 1) the nature of atherosclerosis in the animal model, 2) biology of the chosen species, 3) features of the chosen species in the relation to the experimental procedure.

The purpose of the criteria concerning the nature of atherosclerosis in the animal model is to ensure the resemblance of the experimentally induced or spontaneous disease in the model and that occurring naturally in man. This includes pathogenesis, morphological structure of lesions, their progression with time and topographical development as well as the occurrence of the specific complications of the disease like myocardial or cerebral infarction, aneurysm, gangrene and loss of function of extremities.

The present rabbit models fulfil the most important of these demands. Atherosclerosis in the rabbit models develops due to factors comparable to those existing in man e.g. due to exogenous factors such as diets (cholesterol-fed rabbit model) or endogenous factors like genetic lipid disorders (e.g. WHHL rabbit, Kurosawa and Kusanagi rabbit[3] or St. Thomas Hospital rabbit[4]). The atherogenic factors lead to development of atherosclerotic lesions in arterial wall. As in man the lesions in the rabbit progress in time from fatty streaks (i.e. subendothelial band-like accumulation of foam cells) to fibrous plaques (i.e. intimal thickenings with occasional foam cells) and advanced lesions (i.e. accumulation of foam cells in fibrous stroma with formation of deep seated plaque). Occasionally, the disease in the rabbit can be complicated by myocardial infarction, which, however, is due to atheromatous embolism in contrast to the usual atherothrombotic pathogenesis in humans.

The shortcomings of the rabbit concerning the nature of atherosclerosis are the following differences from man: 1) topography of the lesions, 2) morphology of advanced lesions, 3) origin of hypercholesterolemia in cholesterol-fed rabbit and 4) extreme hypercholesterolemic response to high cholesterol and high fat diet. The rabbit mainly develops atherosclerotic lesions in the aortic arch and in the thoracic aorta and not in the abdominal aorta as in man. The complications of atherosclerotic plaques such as pronounced calcification, ulceration, haemorrhage and thrombosis leading to luminal stenosis never occur. The hypercholesterolemia in the cholesterol-fed rabbit occurs because the physiological mechanism of lipoprotein

[3]Kurosawa et al. 1995 in the reference list.

[4]La Ville et al. 1987 and Seddan et al. 1987 in the reference list.

clearance are overwhelmed by large amount of dietary cholesterol while hypercholesterolemia in man is caused by genetic or acquired abnormalities in synthesis or degradation of plasma lipoproteins. If rabbits are fed high cholesterol (1-3% w/w or more) and fat diet (4-10% w/w), they develop extreme hypercholesterolemia, lesions consisting mostly of lipid-laden macrophages and lipid infiltrations in different parenchymatous organs (e.g. *III.D.: Mortensen et al. 1995*). The extreme hypercholesterolemia and lipid infiltrations in different organs are a clinical and morphological manifestations of disturbed lipid metabolism respectively. The disturbed lipid metabolism is considered as a disadvantage in an animal model to study the effect of dietary factors or drugs on atherosclerosis. This is because the massive hypercholesterolemia in the model may mask any effect of the test compound on blood lipid levels and (or) aortic cholesterol accumulation. However, the disturbed lipid metabolism due to high cholesterol and fat diet should be regarded as a failure in experimental design rather than a shortcoming of the rabbit itself. Concerning the morphology of atherosclerotic lesions in the cholesterol-fed rabbit the following should pointed out: the morphology of the lesions due to a reasonable atherogenic diet (cholesterol < 0.5%) is comparable to that of lesions induced in other species. In addition, none of the popular laboratory animal models of atherosclerosis, inclusive the rabbit models, develops atherosclerotic lesions identical to those in man.

The purpose of the criteria concerning the biology of the chosen species is to ensure a well characterized biological organism for the study in order to facilitate the interpretation of the results. Therefore the demands are directed towards defined genetic background, similarities between the species and man in anatomy, physiology, clinical chemistry and function of the cardiovascular system, to the availability of the information on physiological levels on blood lipids, spontaneous and induced atherosclerosis in the species, and possible strain differences in response to atherogenic stimuli.

The rabbit models fulfil most of these demands. The genetics, physiology and pathology of the rabbit is well known. The physiological levels of blood lipids in normolipidemic and spontaneously hypercholesterolemic rabbits have been described (*Fillos & Mann 1956, Roberts et al. 1974, Watanabe 1980, Lind et al 1990, Norido et al. 1993, Esper et al. 1993a, La Ville et al. 1987, Havel et al. 1982*). The normolipidemic rabbit and man are both "LDL mammals" which means that the sum of the VLDL, intermediate density lipoproteins (IDL), and LDL accounts for more than 50% of the total plasma lipoproteins in both species. Lipoprotein metabolism in the rabbit bears some important resemblances to that of humans: 1) apo B-100 is the major or sole form of apo B secreted in the liver, 2) an active mechanism for transfer of cholesteryl esters between lipoproteins also exist in the rabbit and 3) the LDL receptor in the rabbit is readily regulated by diet and drugs that also affect the activity of the hepatic LDL receptors in humans (*Havel et al. 1989*). The spontaneous atherosclerosis in normolipidemic rabbits and in spontaneously hypercholesterolemic rabbits (WHHL and St. Thomas Hospital rabbits) has been described (*Haust &*

More 1965, Schenk et al. 1966, Buja et al. 1983, Rosenfeld et al 1987 ab, Sedon et al. 1987, III.B.: Hansen et al. 1994). The experimental atherosclerosis due to cholesterol feeding has been characterized by light microscopy (*Constantinides 1965, Pollack 1965, Lee et al. 1978*). The strain and age variations in response to cholesterol diet have been described (*Pollack 1965, Spagnoli et al 1991*).

The most frequently mentioned shortcomings of the rabbit concerning the biology are that 1) the rabbit is herbivorous while the man is omnivorous, and 2) the rabbit has physiologically low plasma cholesterol compared to man. Among the physiological differences in lipid metabolism between the rabbit and human a reduced activity of hepatic lipase and lower conversion of VLDL to LDL, and a higher activity of cholesteryl ester transfer protein in the rabbit compared to man should be noted. The fact that the major carrier of the plasma cholesterol in the cholesterol-fed rabbit is β-VLDL while it is LDL in man, is no longer regarded as a shortcoming of the model because a comparable condition also occurs in man known as broad-β disease (type II hyperlipidemia) (*Overturf & Loose-Mitchell 1992*).

The purpose of the criteria concerning the features of the chosen species in relation to experimental procedure is to facilitate the realisation of the experiment. The animals should have a proper size to allow the desired blood sampling frequency and total volumen of a single sample, the experimental manipulation e.g. angiography, histological examination of the aorta and also the coronary arteries. The induced lesions or lesions due to inherited hypercholesterolemia should develop in relatively short time to enable a reasonable duration of the experiment. Animals should be easy to acquire, handle and house, especially when large number of animals is needed, and of reasonable cost. The rabbit fulfils most of these demands.

I.D.2. Reasons for choosing the rabbit for this project

As presented above the rabbit fulfils most of the criteria for an animal model in atherosclerosis research. Some of the shortcomings such as different topography of the lesions and lack of complications of the advanced lesions specific for the man also exist in other animal species, and they are of minor importance compared to the fact that the rabbit and man share common points in the mechanism of lesion formation. The shortcoming that the rabbit is herbivorous and that the normolipidemic strains have lower plasma cholesterol than man became less important when spontaneously hypercholesterolemic rabbit strains were developed. Thus, keeping in mind the demands torwards the nature of atherosclerosis in a laboratory animal model and torwards the biology of the chosen species, the advantages arising from the features of the chosen species in relation to experimental procedure, and the capacity of the animal facilities in our laboratory, the rabbit was chosen to investigate links between the dietary fats and atherosclerosis in this project. Also of great importance for this decision

was the opportunity to obtain WHHL rabbits, not commercially available at that time, for the study of the development of spontaneous atherosclerosis with the intent of using this strain for dietary and medicamental intervention in the future.

I.D.3. Characteristic features of the rabbit models chosen in the project

The cholesterol-fed rabbit

The cholesterol-fed rabbit based on physiologically normolipidemic strains is a classical rabbit model. Due to exogenous cholesterol added to the diet the rabbits develop hypercholesterolemia with the β-VLDL as the major carrier of plasma cholesterol and atherosclerotic lesions. The atherosclerotic lesions have fibrocellular morphology when caused by low cholesterol doses (0.1-0.3 g/rabbit/day). The higher cholesterol doses lead to extreme hypercholesterolemia, formation of lesions consisting of lipid laden macrophages, and extracellular lipid infiltration in parenchymatous organs. This rabbit model is widely used to study the role of dietary factors in the development of atherosclerosis, the lipoprotein metabolism and for screening of hypocholesterolemic compounds. This rabbit model has been chosen to study effect of hydrogenated fat on development of experimental atherosclerosis in this project (III.C.).

Homozygous Watanabe heritable hyperlipidemic rabbit

This rabbit strain was obtained by inbreeding from a single hyperlipidemic mutant discovered in 1973 by Watanabe (*Kondo & Watanabe 1975*). These rabbits are genetically deficient in LDL receptors and have strong hypercholesterolemia, moderate triglyceridemia, and spontaneously develop atherosclerosis. The LDL receptor deficiency is inherited as a single gene mutation. Cholesterol synthesis, concentration, composition and metabolism of lipoproteins and morphology of arterial atherosclerosis have been intensively studied in this rabbits (*Dietschy et al. 1983, Havel et al. 1982, 1989, Buja et al. 1983, Goldstein et al. 1983, III.B.: Hansen et al. 1994*). Several similarities between the disease process in homozygous WHHL rabbits and humans with FH have been established. In these animals and FH patients 1) the massive hypercholesterolemia results from a defect in the gene for LDL receptor, 2) the lipoproteins that carry endogenous cholesterol are elevated (LDL, IDL, VLDL) while the lipoproteins that carry dietary (=exogenous) cholesterol are at normal levels (chylomicrons), 3) the HDL cholesterol may be at normal or reduced level compared to physiological levels, 4) the arterial atherosclerosis develops and progress with age. Furthermore, 5) the morphology of atherosclerotic lesions in homozygous WHHL rabbits closely resemble that of human atherosclerotic lesions, 6) and tendinous xanthomas were reported also in the homozygous WHHL rabbits. The difference between the FH in humans and the disease process in homozygous WHHL rabbits is the presence of triglyceridemia in the rabbits and its absence in man. This difference originates

from the species difference in lipoprotein metabolism: the rabbit LDL has a relatively high triglyceride content in comparison to human LDL. The homozygous WHHL rabbit has been used to study the development and progression of atherosclerotic lesions (*Rosenfeld et al. 1987 ab*), and for dietary and medical interventions (*Clubb et al. 1989, Lichtenstein & Chobanian 1990, Carew et al. 1987, Nagano et al. 1989, Mao et al. 1991, Mortensen et al. 1995*). Until recently the WHHL rabbits were not commercially available. The colonies existed only in a few laboratories. This rabbit model has been used in this project to monitor blood lipids in growing and adult rabbits (III.A.1.-2.), to study the morphology of spontaneous atherosclerosis (III.B.) and for dietary and drug interventions (III.E. & III.F.).

The cholesterol-fed heterozygous WHHL rabbit

Heterozygous WHHL rabbits have plasma cholesterol levels significantly lower than their homozygous siblings. The levels are comparable to the levels in normolipidemic rabbits (*Esper et al. 1993 a, III.A.1. & 2.*) or intermediate between those in normal rabbits and those in WHHL homozygotes (*Goldstein et al. 1983*). The heterozygous WHHL rabbits develop only minimal spontaneous atherosclerosis observable at the age of approximately two years (*Atkinson et al. 1989, Esper et al. 1993 b, III.B.: Hansen et al. 1994*). Analogously to the normolipidemic rabbits cholesterol feeding leads to hypercholesterolemia and to development of experimental atherosclerosis. The high cholesterol doses induce disturbances in the lipid metabolism manifested by extreme hypercholesterolemia and extravascular lipid deposition in various organs (*III.D.: Mortensen et al. 1995*). Furthermore, the high cholesterol and the high fat diet may lead to severe liver changes (*Simonsen et al. 1995*). The morphology of atherosclerotic lesions is fibro-cellular. These lesions are not too different from the spontaneous lesions in 6 months old homozygous WHHL rabbits (*Atkinson et al. 1989, III.D.: Mortensen et al. 1995*) and from lesions seen in cholesterol-fed rabbits receiving low doses of cholesterol (*III.D.: Mortensen et al. 1995*). The cholesterol-fed heterozygous WHHL rabbit was proposed as a new rabbit model in atherosclerosis research in 1989 (*Atkinson et al. 1989*). However, the use of this model seems very limited as no reports from other laboratories have been published until now. In our laboratory the heterozygous WHHL rabbit fed low cholesterol doses has been used to study the atherogenic effect of different fats (*Andersen et al. 1993, Mortensen et al. 1994*). In this project the heterozygous WHHL rabbit fed 1% cholesterol was tested as a model for investigations of atherogenic effect of dietary fats (III.D.)

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PART II

METHODS

II.A. INDUCTION OF EXPERIMENTAL ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBIT

II.A.1. Methods for cholesterol addition to the rabbit diet

Experimental atherosclerosis in the cholesterol-fed rabbit is induced by addition of exogenous cholesterol to the diet. There are several methods for addition of cholesterol to the diet. A first method is dissolving the cholesterol in a solvent e.g. chloroform (*Kritchevsky 1970*) or ethyl ether (*Leth-Espensen et al. 1988*) and pouring it on the pelleted rabbit diet and mixing them thoroughly. When the solvent evaporates the cholesterol added diet is ready for use. This method allows preparation of a so called cholesterol "fat-free" rabbit diet. This means that the diet does not contain any additional fat but only the fat which is a native component of standard rabbit chow[1] (*Kritchevsky 1970*). A second method is dissolving cholesterol in the test fat(s) and adding it (them), with continuous mixing, to the pelleted rabbit diet. The mixture of fat and cholesterol is usually heated to facilitate the dissolving of cholesterol. A third method is suspending cholesterol in the test fat(s) and adding the suspension, with continuous mixing, to the rabbit pelleted diet. A fourth method is addition of cholesterol to the powdered standard rabbit diet and pelleting afterwards. This method allows preparation of so called cholesterol "fat-free" diet to which test fat(s) can be added, if necessary. Addition of the fat is done by pouring the fat on the pellets and mixing thoroughly. This method was used in this project (III.C. & D.).

An important point was raised by Kritchevsky (*1970*) concerning the differences in atherogenic potential of the same cholesterol dose added to the diet as a suspension in the fat or dissolved in the same type of fat. The heating of some fats in order to facilitate dissolution of cholesterol in them increases the content of free fatty acids in these fats and their atherogenicity. For instance this is the case for corn oil but not for olive oil. Thus the diet added cholesterol dissolved in a test fat may be more atherogenic than the diet containing cholesterol suspension in this test fat. This should be kept in mind when designing experiments.

II.A.2. Cholesterol feeding regimens

There are different methods for dosing the rabbits with cholesterol in the diet in order to induce experimental atherosclerosis. The following classification of the methods is proposed: 1) fixed single cholesterol level

[1]The fat content in standard rabbit diet is 2-5% (*Hagen 1974*).

regimen, 2) controlled one cholesterol level regimen, and 3) controlled individual cholesterol dose regimen.

Fixed single cholesterol level regimen is a classical design for induction of experimental atherosclerosis in the rabbits. All groups receive the same fixed dose of cholesterol in the diet during the whole experimental period regardless of dietary or medicamental treatment. The hypercholesterolemia is not controlled deliberately but in extreme cases of unsatisfactory changes in plasma cholesterol (to high or to low increase) the dose may be changed. This design allows to study the effect of treatment on plasma lipids and experimental atherosclerosis. This regimen preceded by a 4 weeks adaptation period to experimental diets was used to induce experimental atherosclerosis in heterozygous WHHL rabbits in order to study its morphological picture in aorta and to compare effect of n-6 and n-3 polyunsaturated fatty acids in this project (III.D.).

Controlled one cholesterol level regimen is a modification of the first method. All groups receive the same cholesterol dose as the control group. The cholesterol dose for the control group is adjusted at an interval of a few days in order to maintain the mean plasma cholesterol in this group at a certain predetermined level. This method prevents the unsatisfactory changes in plasma cholesterol which may occur using the fixed single cholesterol level regimen. This design also allows to study the effect of treatment on plasma lipids and experimental atherosclerosis. This regimen has been used in our laboratory to induce experimental atherosclerosis in heterozygous WHHL rabbits in order to compare the atherogenic effect of different fats (*Andersen et al. 1993, Mortensen et al. 1994*) but it was not used in this project.

Controlled individual cholesterol dose regimen is more resource consuming. All rabbits regardless of the treatment group receive individual cholesterol doses which are adjusted at an interval of a few days in order to maintain all the rabbits at the same plasma cholesterol level chosen for the study. This regimen diminishes the differences in hypercholesterolemic response to exogenous cholesterol between hypo- and hyper-responders. It allows to study the effect of test compounds on distribution of plasma cholesterol between lipoproteins and on experimental atherosclerosis due to other mechanism than a change in total plasma cholesterol. This regimen has been used to induce experimental atherosclerosis in normolipidemic rabbits used in drug trials (*Holm et al. 1995*) to compare the atherogenic effect of different fats (*Leth-Espensen et al. 1988*) and olive oil and margarine in this project (III.C.).

The duration of cholesterol feeding varies in different studies depending on the aimed degree of atherosclerosis and the type of lesions. In general, the low cholesterol doses e.g. 0.1-0.3% are applied for several weeks in order to obtain the fibrocellular atherosclerotic lesions in aorta.

II.B. BREEDING OF WHHL RABBITS

All WHHL rabbits in this project were obtained from own breeding colony established in 1990 from a parent generation obtained with the permission of dr. Y. Watanabe from prof. Jansen, University of Leiden, The Netherlands.

The LDL receptor deficiency in WHHL rabbits is inherited as a single gene mutation. This is a simple Mendelian trait with partial expression in the heterozygous state and complete expression in the homozygous state. All offspring of homozygous parents inherit the LDL receptor deficiency. The offspring of heterozygous WHHL parents are of different genetic status: free for LDL receptor deficiency, heterozygotes for LDL receptor deficiency and homozygotes for LDL receptor deficiency in the ratio 1:2:1 respectively. The offspring of homozygous parent and the heterozygous parent are either homozygotes or heterozygotes for LDL receptor deficiency in the ratio 1:1. All the offspring of homozygous parent and normolipidemic parent are heterozygotes for LDL receptor deficiency.

The breeding of WHHL rabbits using homozygous parents is difficult as homozygous WHHL females show decreased reproductive performance compared to heterozygous WHHL females or normolipidemic females genetically free for LDL receptor deficiency (*III.A.1., Shiomi et al. 1987*). Additionally, frequent occurrence of stillbirths in homozygous WHHL females and birth deformities of the offspring has been reported (*Esper et al. 1993*). A few cases of pregnancy toxemia (*Bergdall & Dysko 1994*) and decreased conception rates in homozygous WHHL females were seen in our breeding colony. The heterozygous LDL receptor deficiency condition in WHHL females does not significantly affect the reproductive performance compared to normolipidemic females (*III.A.1.*). Therefore mating of homozygous WHHL males with heterozygous WHHL females is the preferred system to obtain the homozygous offspring in our colony while heterozygous WHHL offspring were obtained either of homozygous males with New Zealand White (NZW) females or heterozygous WHHL females. Comparison of the reproductive performance of homozygous and heterozygous WHHL and New Zealand White breeding females from our colony is presented in Table 1 in chapter III.A.1.

The distinction between the heterozygous and homozygous offspring obtained from homozygous WHHL males and heterozygous WHHL females is based on the total plasma cholesterol level which is measured at 4 weeks of age (preliminary selection) and 6 weeks of age (final selection at the end of weaning) in our colony. All offspring with a total cholesterol higher than 10 mmol/l at the end of weaning is considered homozygous. The distinction between homozygous and heterozygous WHHLs based on the plasma cholesterol level at the end of weaning is applied in several laboratories (*Shiomi et al. 1987, Rich et al. 1989, Esper et al. 1993*).

II.C. MEASUREMENT OF BLOOD LIPIDS

Determination of blood lipids includes measurement of the concentration of total cholesterol, triglycerides and assay of the concentration of cholesterol and triglycerides in lipoproteins. The results are expressed as mmol/l. In some countries the concentration of blood lipids is still expressed in mg/dl[2]. In this project, the blood lipids were measured in plasma. Therefore, all blood samples were collected into tubes containing EDTA or natrium heparinat. Then the blood samples were centrifuged for 10 min at 4000 rpm in order to separate plasma from blood cells.

II.C.1. Measurement of total plasma cholesterol

In this project the measurement of total plasma cholesterol was performed automatically in a COBAS MIRA autoanalyzer using CHOD-PAP (Boehring Mannheim) method based on an enzymatic colorimetric test (*Seidel et al. 1983, Kattermannet al. 1984*) the principle of which is presented below:

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \Delta\text{- cholestone} + H_2O_2$$

$$2\,H_2O_2 + \text{4-}\textit{aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{4(}\textit{p-benzoquinone-monoimino}\text{)-phenazone} + 4\,H_2O.$$

Five µl plasma was added 35 µl H_20 and 350 µl reagent MPR2 CHOL 1442341 (total volume 390 µl) and the extinction was measured at 500 nm. The reagent MPR2 CHOL 1442341 was prepared before the measurement: the content of the bottle with the reagent was added 100 ml of MiliQ water and placed for 10 min at room temperature. The Roche lipid control serum art. nr. 0737194 was used for calibration. The coefficient of variation within the day is 0.8% (n=25) and the coefficient of variation beween days is 4.7% (n=7).

II.C.2. Measurement of total triglycerides

In this project the measurement of triglycerides was performed automatically with a COBAS MIRA autoanalyzer. The analysis is based on an

[2]Conversion of values from mg/dl to mmol/l: for cholesterol divide mg/dl by 38.7, for triglyceride divide md/dl by 88.5. Conversion of values from mmol/l to mg/dl: for cholesterol multiply mmol/l by 38.7, for triglyceride multiply mmol/l by 88.5.

enzymatic colorimetric test GPO/PAP. This test is performed using the Unimate TRIG, an *in vitro* diagnostic reagent system (Hoffman La-Roche). The principle of the method is as follows:

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightleftharpoons{\text{GK}} \text{glycerol - 3 - P} + \text{ADP}$$

$$\text{Glycerol - 3 - P} + O_2 \xrightleftharpoons{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

In presence of peroxidase, the hydrogen peroxide formed affects the oxidative coupling of 4-chlorophenol and 4-aminoantipyrine to form a red coloured quinoneimine derivative. The colour intensity is directly related to the triglyceride concentration and is measured photometrically.
Four µl plasma was added 36 µl H_2O and 300 µl reagent (total volume 340 µl) and red at 500 nm. The reagent Unimate 5 was prepared before the measurement: the content of the bottle with the reagent was added 30 ml MiliQ water and placed in room temperature for 10 min. The Roche lipid control serum art. nr. 0737194 was used for calibration. The coefficient of variation within the day is 1.9% (n=25) and the coeficient of varation between the days is 2.9% (n=10).

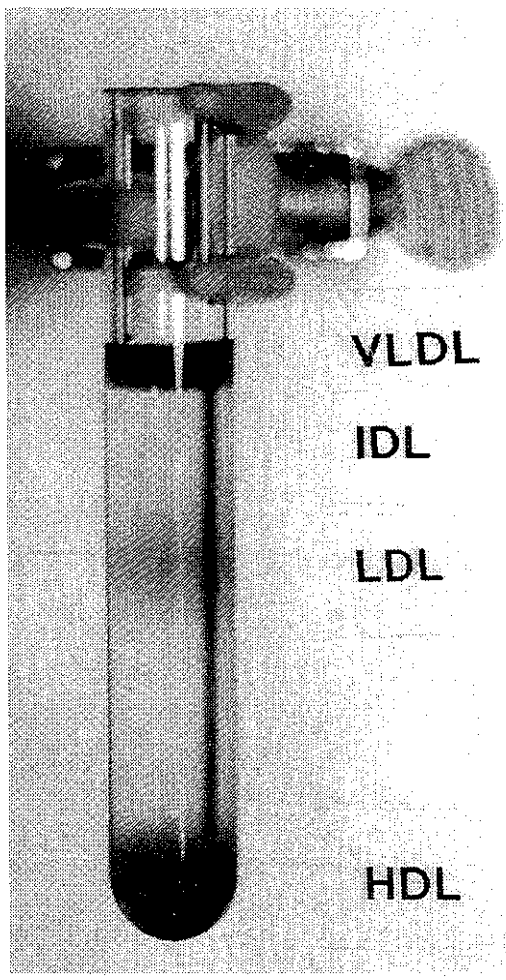

Fig. 2. Lipoprotein fractions in plasma from NZW rabbit

II.C.3. Measurement of total cholesterol and triglycerides in lipoproteins

In this project, the lipoproteins were separated by density gradient ultracentrifugation by the method of *Terpstra et al. (1989)*. Kalium bromide (KBr Merck 4905) 228 mg and 50 mg sucrose (D(+)-saccharose Roth 4621.1) were placed in each ultracentrifuge tube. Subsequently 2 ml of plasma was pipetted into each tube and finally 0.2 ml of Sudan black (Sudan schwartz B Serva 35610) solution was added to prestain the plasma. Thereafter the content of each tube was overlayered with 4.8 ml MiliQ water. The tubes were centrifugated for 18 h at 84000 x g_{av} ≈ 112000 x g_{max} at room temperature. After the centrifugation five layers were seen in each tube (Fig. 2). Starting from the bottom, the first fraction was HDL, the third LDL, the fourth IDL and the fifth HDL. The second fraction was not used in the assay. The density ranges of isolated fractions were: VLDL $\rho<1.0063$ g/ml, IDL $1.0063<\rho<1.019$ g/ml, LDL $1.019<\rho<1.063$ g/ml, HDL $1.063<\rho<1.21$ g/ml. The fractions were removed from the tube separately. The content of cholesterol and triglycerides in each fraction was determined as described above (II.C.1 and II.C.2).

II.D. EVALUATION OF ATHEROSCLEROSIS

Atherosclerosis in the rabbit can be evaluated by macroscopic, microscopic and biochemical methods.

II.D.1. Macroscopic quantitative evaluation

Macroscopic quantitative evaluation measures disease extent. It is performed on longitudinally opened aorta using different methods.

Naked eye evaluation. In this method the extent of atherosclerotic lesions in the aortic intima is estimated visually and usually it is performed separately in the ascending, the thoracic and the abdominal parts (Fig. 3). The estimation is performed on fresh tissue in connection with the autopsy. Different grading systems are used. *Wu et al. (1988)* estimated the extent of atheromatous plaques in the intima in these three parts of aorta using a four grade system: grade 0 indicated no lesions present, grade 1 indicated focal distribution of lesions, grade 2 indicated that less than 50% of aorta is covered by lesions, and grade 3 indicated that more than 50% of area is covered by lesions. *Nordestgaard & Lewis (1991)* used a more detailed

grading system. The area of the aortic intimal surface covered by lesions was graded visually as 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. Then the average percent for the whole aorta was calculated. In this project, the extent of atherosclerotic lesions in the intima of the ascending, the thoracic and the abdominal aorta was recorded at 5% intervals and it was always performed by the same person (III.B.).
The advantage of naked eye evaluation is that 1) it is performed immediately after euthanasia, 2) it is not time consuming, 3) it demands no advanced instruments, and 4) it leaves the "intact" aortic tissue for other and more advanced procedures e.g. histology or biochemical analysis. The disadvantage of this method is that it is subjective. The use of a more detailed grading system can give rise to a divergence in the evaluation of the extent of atherosclerosis. Therefore the estimation should be performed by the same person in the same study.

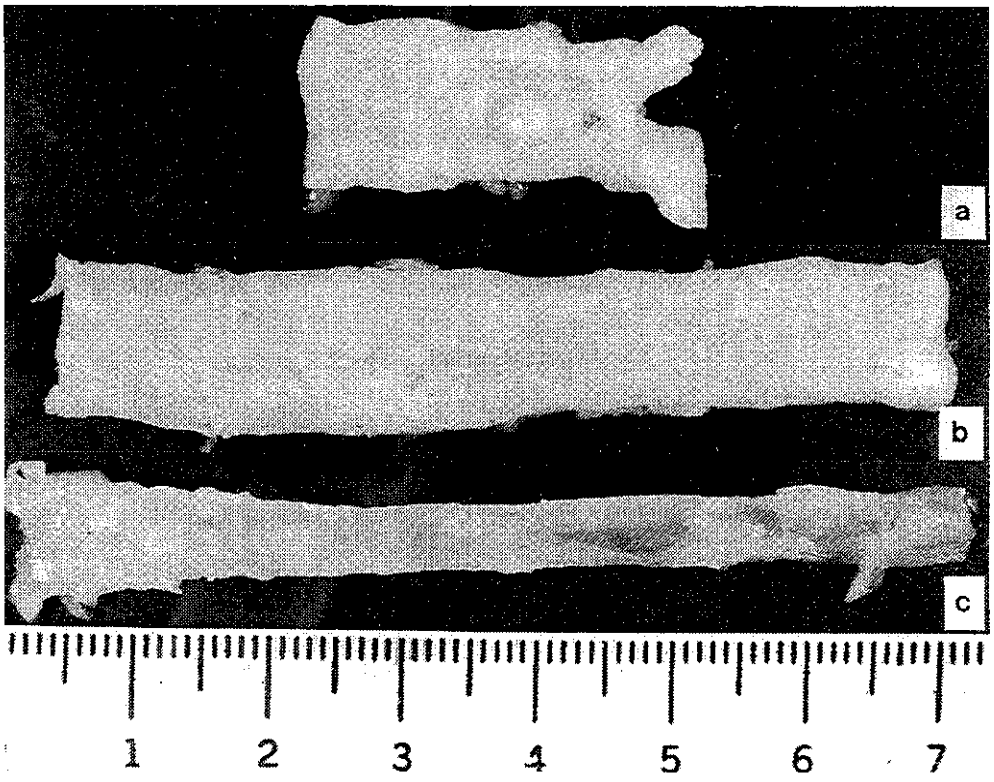

Fig. 3. Macroscopic picture of aortic atherosclerosis in longitudinally opened aorta from 19 months old homozygous WHHL male rabbit. a: ascending aorta, b: thoracic aorta, c: abdominal aorta.

Evaluation of lipid positive areas. Quantitation of aortic lipid positive area can be done on the whole aorta or separately in its ascending, thoracic and abdominal parts. Directly after removal from the body the aorta is opened longitudinally, pinned endothelial side up, photographed, and fixed

in formalin. After fixation the aorta is stained with Sudan IV - the lipid positive areas become red - and the vessel is photographed again. The photographs are used to estimate the extent of sudanophilia by planimetry using a computer assisted planimetry system. The results are expressed as percent of total surface area covered by plaques. This method is used not only in the rabbit (*Atkinson et al. 1989, Clubb et al. 1989, Rich et al. 1989*) but also in swine (*Cornhill et al. 1985*) and in mice (*Palinski et al. 1994*). This method is more objective than naked eye evaluation. It is also a relatively quick method as the calculation of the results is done using computer assistance. This method was not used in this project.

Point-counting. The ascending, thoracic and abdominal parts of aorta are photographed. The pictures of aortic parts are covered with the transparent point-grid. The number of points hitting atherosclerotic lesions (P-lesions) and the number of points hitting the whole aortic surface (P-aorta) on the photograph are recorded. The percent intima with lesions is then calculated: P-aorta/P-lesions x 100. This method was used in this project (III.B.).

II.D.2. Microscopic qualitative and quantitative evaluation

Qualitative evaluation. In this project the qualitative evaluation in parts II.B., III.D., III.E. and III.D. was performed as recording of three types of atherosclerotic lesions: 1) fatty streaks i.e subintimal accumulation of foam cells, 2) fibrous plaques i.e. localised intimal thickenings with occasional foam cells, and 3) advanced lesions i.e. intimal thickenings with many foam cells and or cholesterol crystals usually localized in deep-seated pools. In III.C. a qualitative estimation of atherosclerotic lesions in aorta and other chosen arteries was done using the following graduation: 0: not present, +: slight changes, ++: moderate to severe changes.

Quantitative evaluation. This evaluation is a measure of the severity and extent of atherosclerosis. The microscopic quantitation of atherosclerosis was performed by point-counting in this project (III.B., III.C., III.E., III.F.). The microscopic picture of transversely cut arteries stained with elastic-van Gieson or elastic Hematoxylin-eosine was projected to point-counting grid. The grid shows regularly spaced points on a white background. The extent of an atherosclerotic lesion is quantitated by counting the number of points hitting the lesion. The degree of magnification of the microscopic picture and the distance between the points in the grid are constant. Quantitation was performed in two to four serial sections of the artery, and the result was recorded as a mean value of counting results.

Aorta. In cross sections of unopened ascending, thoracic and abdominal aorta the points covering the intimal lesions (P-intima) and points covering media of the aortic wall (P-media) were recorded. The severity of atherosclerotic lesions was then calculated as ratio R = P-intima/P-media

(Ratio I/M). Furthermore, the severity of an atherosclerotic lesion was recorded in size of lesion in mm^2. This calculation is based on knowledge of both exact degree of magnification of the microscopic picture and of exact distance between points in the grid. In our estimation 1 mm of arterial tissue was magnified to 2.1 cm on the grid where points were regularly placed 1 cm apart. According to the formula: 1 point = 1 x 1 / 2.1 x 2.1 = 0.227 (*Gundersen et al. 1988*) the quantitation recorded in points could be converted into mm^2.

Left coronary artery. The degree of luminal stenosis in regular cross-sections of the left coronary artery was estimated as described below. In an artery without atherosclerotic lesions the internal elastic lamella is the demarcation of arterial lumen. This area is quantitated (P-IEL: number of points covering the total area luminal to the elastic lamella). In the diseased artery there is a reduction in size of lumen - this reduced lumen is quantitated (P-lumen: the number of points covering the free luminal space). The percentage stenosis was then calculated: % stenosis = (1 - P-lumen / P-IEL) x 100.

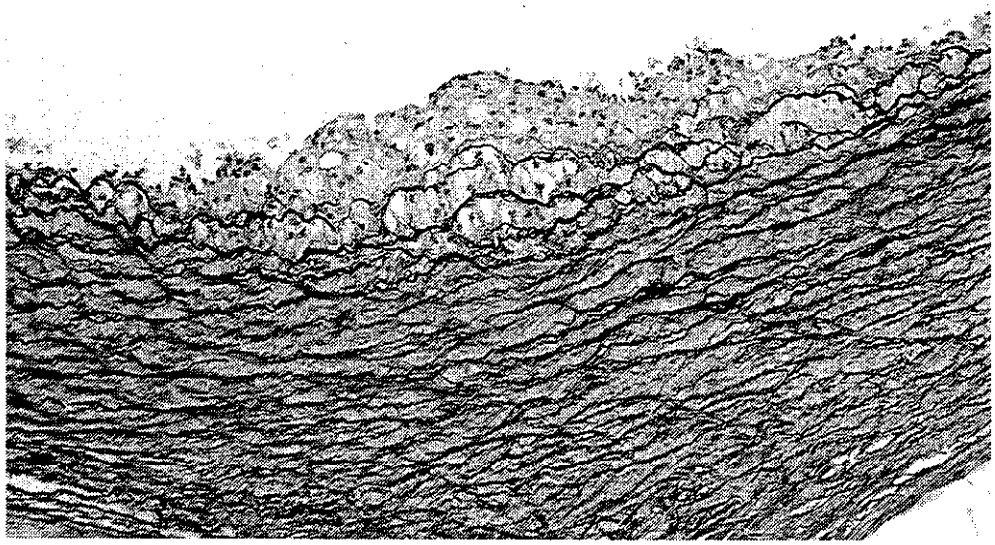

Fig. 4. Typical fatty streak in aorta of homozygous WHHL rabbit: subendothelial band-like accumulation of foam cells (Elastin van Gieson, x 100)

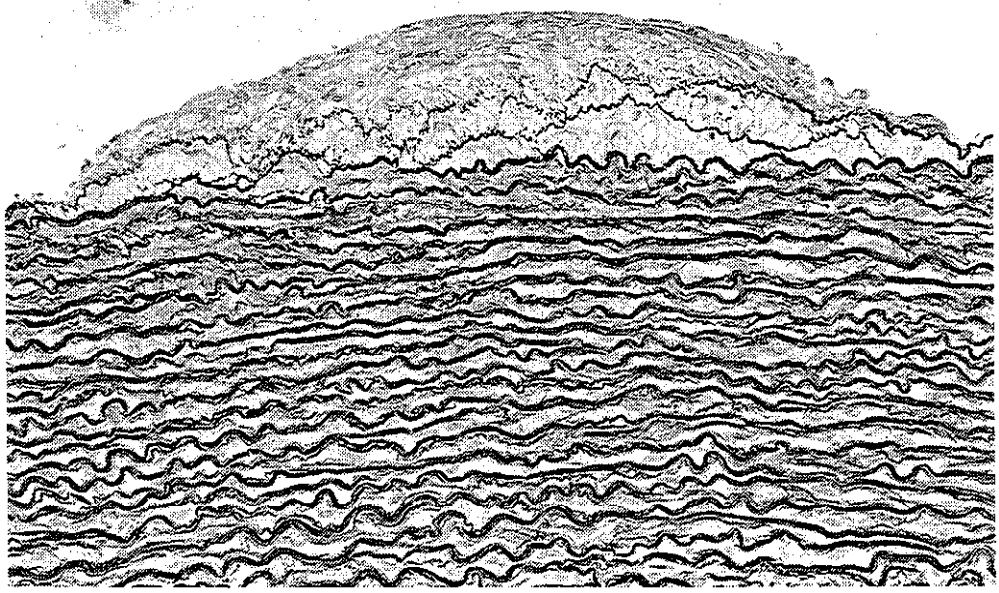

Fig. 5. Typical fibrous plaque in aorta of homozygous WHHL rabbit: localized intimal thickenings with occasional foam cell (elastin van Gieson, x 40).

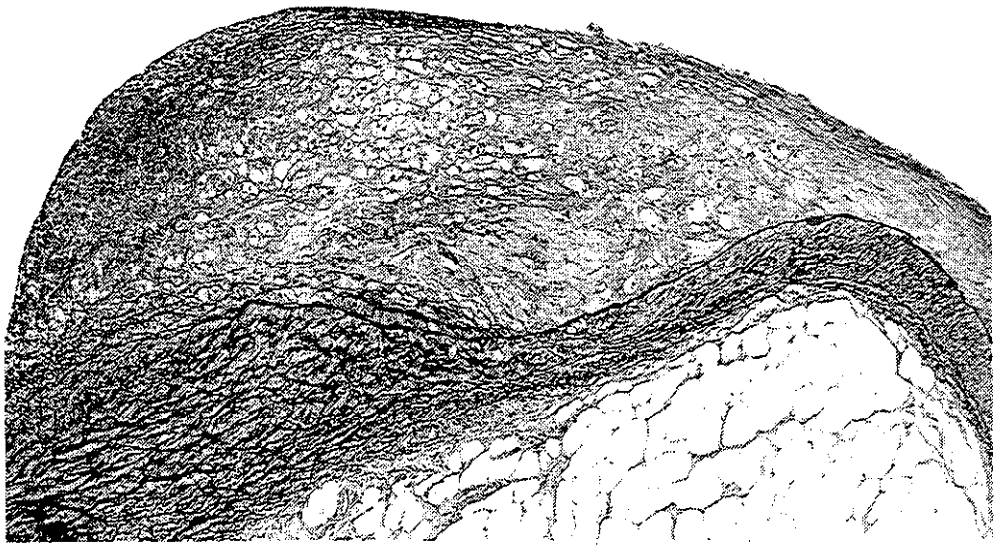

Fig. 6. Typical advanced lesion located where elastic (aortic) artery changes into muscular (coronary) artery in WHHL rabbit: accumulation of foam cells in fibrous stroma with formation of deep seated plaque (elastin van Gieson, x 100).

Right coronary artery. The degree of atherosclerotic lesions in longitudinal sections from the first part of the right coronary artery was also estimated by point counting technique. The point-grid was circular. We know (III.B.: *Hansen et al. 1994*) that lesions are most pronounced exactly where the arterial wall changes from elastic type (aorta) to muscular type (coronary artery, see Fig. 3b in III.B). The quantitation was performed in a restricted area, the centre of which was defined as this shift in arterial wall structure. Points covering the intimal lesion (P-intima) and points covering the media (P-media) were recorded and used to calculate the ratio I/M. The severity of an atherosclerotic lesion was also recorded in size of lesion mm^2 (see above).

II.D. 3. Biochemical determination of cholesterol content in aorta

This quantification of aortic atherosclerosis is regarded as a combined measurement of atherosclerosis extent and severity. In this project, the aortic specimens containing aortic intima-inner media from ascending, thoracic and abdominal aorta were minced with scissors and the lipids were extracted with 20 volumes of chloroform/methanol 2:1 or 1:1 (v/v) during 24 h. Lipids in the supernatant and proteins in the precipitate were separated by the method of *Folch et al. (1957)*. Total cholesterol was determined by the Liebermann-Burchard method after saponification (*Ness et al 1964*). The amount of protein was determined by the method of *Lowry et al. (1951)*. The results were presented either as nmol cholesterol per mg wet weight and nmol cholesterol per mg protein (III.C.) or µmol cholesterol per cm^2 aorta (III.B.) or mg cholesterol per g tissue (III.E.) depending on the study.

II.E. BASIC FEEDING REQUIREMENTS OF THE RABBIT AND FEEDING SYSTEMS

Rabbits are usually fed a pelleted diet made up of grains, hay and certain supplements (*Hagen 1974, Hunt & Harrington 1974*). According to *Hagen* (1974) rations of maintenance or breeding diet should provide the following:

	Maintenance diet[1]	Breeding diet[2]
Crude protein %	12-15	16-20
Fat %	2-3.5	3-5.5
Fiber %	20-27	15-20
Nitrogen-free extract %	43-47	44-50
Ash or minerals %	5-6.6	4.5-6.5

[1]: Rations for dry does, herd busks and growing rabbits.
[2]: Rations for pregnant or lactating females.

The rabbit diet most commonly used in our laboratory is Altromin 2110 (Altromin Tier-Labor-Service, D-4937 Lage, Germany). The composition of Altromin 2110 is as follows:

Crude protein	15.0%
Crude fat	3.5%
Crude fiber	19.5%
Ash	8.0%
Moisture	12.0%
Nitrogen-free-extract	42.0%

This diet in a pelleted form is referred to as Altromin 2113 and as a powder it is referred to as Altromin 2111. For content of vitamins, amino acids, minerals and trace elements see Appendix (p.44).

There are two systems for feeding the rabbits: 1) restricted feeding and 2) feeding *ad libitum*. In the first system the rabbits receive a measured amount of feed each day. This system permits recording of the feed intake. Therefore this system is usually applied in dietary studies. In the second system the daily portions are not limited.

In this project, the rabbits had free access to tap water and were fed once daily. The type of feed and size of the daily ration depended on the study design and were stated in "methods" section under each study.

II.F. HOUSING AND CLINICAL OBSERVATION OF RABBITS

In this project, all the rabbits from the 6 weeks of age were housed individually in steel cages under controlled environmental conditions. The temperature was maintained at 18 ± 2°C. The relative humidity was 55-

65%. The light was on from 8:00 to 20:00h. The body weight of rabbits in experiments was recorded once weekly. All the rabbits were observed at least twice a day for any abnormalities in the clinical condition.

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Zuchtdiät Kaninchen rohfaserreich

ALTROMIN 2110 rohfaserreich, Zuchtdiät für Kaninchen, ist eine Alleindiät - auf alle Zuchtphasen und auf das Wachstum der Jungtiere bis zum Lebensalter von 10 Wochen abgestimmt. Es wird empfohlen, die Preßlinge zur freien Aufnahme zu reichen. Stetige Frischwasserversorgung ist zu sichern.
In Plastiksäcke verschweißt, kann ALTROMIN 2110 direkt in SPF-Bereiche eingeschleust werden. Eine weitere Behandlung der Diät ist überflüssig. Zum Einschleusen ist lediglich eine Oberflächendesinfektion des verschweißten Plastiksackes erforderlich (z. B. Tauchtank).

ALTROMIN 2110 Rabbit Breeding Diet is a recently developed diet rich in crude fibre. With this diet it is not necessary to offer supplements e. g. hay etc. The diet should be offered ad libitum together with an ample supply of fresh water Sealed in polyethylene lined sacks, ALTROMIN 2110 can be passed directly into the SPF facility following surface disinfection.

Raumtemperatur/room temperature 15 - 21° C[1)]

relative Luftfeuchtigkeit/relative humidity 50-60 %

Ø Futteraufnahme g/Tag / Food absorption g/day

	Wachstum growth phase	Laktation lactation	erwachs.T. adult
Kaninchen/ Rabbits	bis 200 g	bis 500 g	ca. 150 g

ALTROMIN 2110 ist lieferbar:
ALTROMIN 2110 is available in:

	Mehl/Powder	2111
4,5 mm	Preßlinge/Pellets	2113

ALTROMIN Standard-Diäten - das Produkt jahrelanger Erfahrung
ALTROMIN Standard Diets - the result of long years' experience

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Breeding Diet Rabbits rich in crude fibre

Rohnährstoffe*	Nutrients*	
Rohprotein	Crude protein	15,0
Rohfett	Crude fat	3,5
Rohfaser	Crude fiber	19,5
Asche	Ash	8,0
Wasser	Moisture	12,0
N-freie Extraktstoffe	Nitrogen-free extract	42,0
Umsetzbare Energie[2)]	Metabolizable Energy[2)]	
Kcal/kg	Kcal/kg	2.300,0
MJ/kg	MJ/kg	9,7

Mineralstoffe*	Minerals*	
Calcium	Calcium	0,8
Phosphor	Phophorus	0,6
Magnesium	Magnesium	0,3
Natrium	Sodium	0,3
Kalium	Potassium	1,6

Aminosäuren* Amino Acids*	
Lysine	0,70
Methionine	0,30
Cystine	0,20
Phenylalanine	0,60
Tyrosine	0,50
Arginine	0,80
Histidine	0,30
Tryptophane	0,20
Threonine	0,60
Isoleucine	0,60
Leucine	1,00
Valine	0,70

Spurenelemente** Trace elements**		
Mangan	Manganese	95,0
Eisen	Iron	200,0
Kupfer	Copper	14,0
Zink	Zinc	55,0
Jod	Iodine	0,8
Fluor	Fluorine	15,0

Vitamine***	Vitamins***	Standard-Diet		Standard-Diet fortified	
Vitamin A	Vitamin A	12.000,0	IU	25.000,0	IU
Vitamin D_3	Vitamin D_3	480,0	IU	1.000,0	IU
Vitamin E	Vitamin E	60,0	mg	125,0	mg
Vitamin K_3	Vitamin K_3	2,0	mg	5,0	mg
Vitamin B_1	Vitamin B_1	15,0	mg	30,0	mg
Vitamin B_2	Vitamin B_2	10,0	mg	20,0	mg
Vitamin B_6	Vitamin B_6	8,0	mg	15,0	mg
Vitamin B_{12}	Vitamin B_{12}	20,0	mcg	40,0	mcg
Nikotinsäure	Nicotinic acid	30,0	mg	60,0	mg
Pantothensäure	Pantothenic acid	18,0	mg	35,0	mg
Folsäure	Folic acid	2,0	mg	3,0	mg
Biotin	Biotin	48,0	mcg	100,0	mcg
Cholin	Choline	480,0	mg	1.000,0	mg
Vitamin C	Vitamin C	28,0	mg	60,0	mg

* % in der Diät (Mittelwert) / average % content in the diet
** mg in 1 kg Diät (Mittelwert) / average mg content in 1 kg diet
*** Zusatz in 1 kg Diät / additive in 1 kg diet
1) EG-Empfehlung / EC recommended
2) berechnet / calculated

ALTROMIN Standard-Diäten garantieren größte Sicherheit bei Versuchen
ALTROMIN Standard Diets warrant experimental safety

Appendix: Altromin 2110, data from manufacturer.

PART III

RESULTS - SPECIFIC STUDIES

III. A. REPRODUCTION AND PHYSIOLOGICAL BLOOD LIPID LEVELS IN GROWING AND ADULT NORMOLIPIDEMIC AND SPONTANEOUSLY HYPERLIPIDEMIC RABBITS

III.A.1. Reproductive performance and changes in blood lipids in breeding females and in growing Watanabe heritable hyperlipidemic and New Zealand White rabbits

Alicja Mortensen & Henrik Frandsen
National Food Agency of Denmark, Institute of Toxicology, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

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SUMMARY

The aim of the study was to compare the reproductive performance of homozygous and heterozygous WHHL and NZW females, to record the changes in blood lipids due to gestation and lactation in the heterozygous WHHL and NZW females, and to investigate the changes in blood lipids from 4 to 11 weeks of age in homozygous and heterozygous WHHL and NZW rabbits. The conception rate of homozygous WHHL females was 14% lower than that of NZW and heterozygous WHHL females. The litter size and the weaning rate of homozygous WHHL females were significantly lower than in NZW and heterozygous WHHL females. In heterozygous WHHL and NZW females the total cholesterol was lower during the gestation and lactation while the triglyceride level was higher during the gestation and was lowest during lactation when compared to the levels at mating. In growing homozygous WHHLs of both sexes the total cholesterol did not change from 4 to 11 weeks of age. The triglycerides remained unchanged in males but decreased in females at 11 weeks of age. At this age the triglycerides were significantly lower in females than in males. In growing heterozygous WHHLs of both sexes the total cholesterol and triglyceride level decreased with age. At 11 weeks of age the blood lipids were significantly higher in females than in males. The triglyceride levels in homozygous and heterozygous WHHL males and females were comparable at 4 and 6 weeks of age but were significantly lower in heterozygous WHHLs at 11 weeks of age. In growing NZW rabbits of both sexes the blood lipids decreased significantly with age but no sex difference was recorded. The blood lipids in the heterozygous WHHLs of both sexes were higher than in NZWs during the weaning. At 11 weeks of age the blood lipids of the males and triglycerides of the females of both strains were comparable. Only the total cholesterol remained higher in heterozygous WHHL females. The decrease with age in blood lipids in growing rabbits should be kept in mind when designing experiments beginning in animals younger than 3 months of age.

Key words: WHHL rabbit, blood lipids, reproductive performance, hyperlipidemia

INTRODUCTION

The homozygous Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model for human familial hypercholesterolemia. This strain was developed by Watanabe (*Watanabe 1980*) from a male mutant Japanese white rabbit. The mode of inheritance of the trait is recessive. The homozygous animals have a reduced number of functional low density receptors, they exhibit strong hypercholesterolemia and moderate triglyceridemia and spontaneously develop atherosclerosis. Their atherosclerotic lesions are well characterized (*Buja et al. 1983, Rosenfeld et al. 1987 ab, Fischer Hansen 1994*) and are considered to be a close approximation of human lesions. The heterozygous WHHL rabbits have blood lipid levels as other normolipidemic rabbits and develop only minimal spontaneous atherosclerosis which is not observed until the age of approximately 2 years (*Atkinson et*

al. 1989, Esper et al. 1993b, Fischer Hansen et al. 1994). The WHHL rabbits have been used in dietary and medicamental intervention studies (*Rich et al. 1989, Clubb et al. 1990, Lichtenstein & Chobanian 1990, Mao et al. 1991, Fischer Hansen et al. in press*). The intervention often begins just after the weaning period in 6 weeks old rabbits. Despite the fact that the blood lipid levels in grown up WHHL rabbits have been characterized (*Watanabe 1980, Lind et al. 1990, Esper et al. 1993a*), little information on changes in blood lipids in growing WHHLs during the weaning period is available (*Rosenfeld et al. 1987a*). This information might improve the interpretation of possible changes in blood lipids in the early fase of an intervention study. Therefore the aim of this study was to collect information on physiological blood lipid levels in growing WHHL rabbits until 3 months of age. Furthermore, since the WHHL rabbits show a reduced reproductive performance compared to normolipidemic rabbit strains (*Shiomi et al. 1987*) and the information on breeding of the WHHL rabbits is limited, the data characterizing the reproductive performance of our colony and the changes in blood lipids in WHHL and New Zealand White (NZW) females are included in this paper.

MATERIALS AND METHODS

Animals.

The homozygous and heterozygous WHHL rabbits used in this study were obtained from our own breeding colony derived from a parent generation obtained from professor Jansen, University of Leiden, The Netherlands, with permission from dr. Y. Watanabe. The young homozygous WHHL rabbits were derived from mating of homozygous males with heterozygous females. The young heterozygous WHHL rabbits were derived from mating of homozygous males with NZW females or heterozygous WHHL females. All WHHL rabbits with a total cholesterol higher than 10 mmol/l at the end of weaning (6 weeks from birth) were considered homozygous. The young NZW rabbits were breed in our laboratory from parents obtained from Thomae-Pharma-Biberach (Department of Laboratory Animal Service, Biberach, Germany). The microbiological status of all the rabbits was conventional.

Housing, care of animals and clinical observations.

From six weeks of age all the rabbits were housed individually in steel cages under controlled environmental conditions (temperature 18 ± 2°C, relative humidity 55 ± 5%, 12/12 hrs light/dark cycle, air changed 10 times/hr). All the rabbits, except for the pregnant females, were fed 100 g-/rabbit/day of a standard diet Altromin 2113 (Lage, Germany). From the mating until the end of weaning of their offsprings the females received feed *ad libitum* and additionally 10 g oats/animal/day. All the rabbits had free access to tap water. The rabbits were observed at least twice a day for any abnormalities in the clinical condition.

Comparison of reproductive performance.

Homozygous and heterozygous WHHL and NZW females were mated with homozygous WHHL males. The conception rate (%) was calculated by dividing the number of pregnant females by the number of mated females. The weaning rate (%) was calculated for each female by dividing the number of weaned offspring by the litter size at birth.

Assay of plasma lipids.

Blood samples were collected from the heterozygous WHHL and NZW breeding females before mating, 14 days after mating and 14 days after delivery, and from growing rabbits at 4, 6 and 11 weeks of age. All the rabbits except for when they were 4 weeks old were fasted from 14.00 hours the day before blood sampling. The blood samples were collected from the marginal ear vein of unanesthetized animals in tubes containing potassium EDTA, and plasma was isolated after centrifugation at 2000g for 10 minutes. The concentration of plasma cholesterol and plasma triglycerides was determined enzymatically (CHOD-PAP, GPO-PAP, Boehringer Mannheim and UNIMATE, Hoffman La-Roche respectively). Lipoproteins were separated by density gradient ultracentrifugation by the method of *Terpstra et al. (1981)*. The density ranges of the isolated fractions were: very low density lipoprotein (VLDL) $\rho<1.0063$ g/ml, low density lipoprotein (LDL) $1.019<\rho<1.063$, high density lipoprotein (HDL) $1.063<\rho$. The concentration of cholesterol in each fraction was determined as above.

Statistical procedures.

The results of total plasma cholesterol, triglycerides and lipoproteins, data for the mean of litter sizes and weaning rates were analyzed by analysis of variance followed by Duncan's test. The data for conception rates were analyzed by Fisher's exact test. The effects were considered significant for p values less than 0.05. All statistical analyses were performed using Statistical Analysis System (SAS) software (SAS Institute Inc., release 6.03, 1988).

RESULTS

Comparison of reproductive performance.

Table 1 shows the comparison of reproductive performance of homozygous and heterozygous WHHL and NZW females, all of which were mated with homozygous WHHL males. The conception rate of homozygous WHHL females was 14% lower than that of NZW and heterozygous WHHL females but this difference was not statistically significant. The litter size and the weaning rate for homozygous WHHL females was significantly lower than for NZW and heterozygous WHHL females. The litter size and weaning rate were highest in NZW females. However, they were not significantly higher than in heterozygous WHHL females.

Comparison of blood lipids in heterozygous WHHL and NZW breeding females.

Table 2 shows the changes in blood lipids in heterozygous WHHL and NZW females during gestation and lactation. No significant differences in total cholesterol, triglycerides and lipoproteines were recorded between the females from the two rabbit strains at mating, 14 days after mating and 14 days after delivery.

In heterozygous WHHL females the total cholesterol and LDL cholesterol decreased during gestation and reached the lowest value at lactation. At that time the values were significantly lower than at mating. In NZW females total cholesterol was lowest at gestation but not significantly different from that at mating and during lactation. In both heterozygous WHHL and NZW females the VLDL cholesterol was highest and HDL cholesterol was lowest during gestation.

The maximum concentration of total and VLDL triglycerides was recorded during gestation in heterozygous WHHL and NZW females. The lowest LDL triglycerides were recorded during lactation in heterozygous WHHL females while LDL triglycerides did not change significantly in NZW females. The HDL triglycerides did not change significantly in females of any of the strains.

Blood lipids in growing WHHL and NZW rabbits.

Changes in the blood lipids in growing homozygous and heterozygous WHHL and NZW rabbits are shown in Table 3.

In homozygous WHHL rabbits no sex difference was seen in total cholesterol. Age had no effect on total cholesterol apart from a transient lowering at 6 weeks of age for females. In triglycerides a sex difference was present at 11 weeks of age due to a significant decrease with age in females.

In heterozygous WHHLs a sex difference in total cholesterol and triglycerides was present at 11 weeks of age. The total cholesterol and triglyceride concentrations significantly decreased with age in both sexes.

In NZWs no sex difference was recorded in blood lipids with the exception of 6 weeks of age when a transient sex difference in total cholesterol was observed. No sex difference was seen in the concentration of triglycerides. The total cholesterol and triglycerides significantly decreased with age in both sexes.

Strain differences in blood lipids in growing WHHL and NZW rabbits.

Total cholesterol was highest in homozygous WHHL rabbits and lowest in NZW rabbits. A significant difference between the three strains was recorded for females at the three ages and for males at 4 and 6 weeks of age. For 11 weeks old males a significant difference was recorded only between homozygous WHHLs and the two other strains which had comparable cholesterol levels.

Triglycerides in homozygous and heterozygous WHHL males were comparable and significantly higher than in NZW males at 4 and 6 weeks of age. At 11 weeks of age the triglycerides in homozygous WHHL males

Table 1. Reproductive performance of homozygous (HH) and heterozygous (Hh) WHHL females and NZW rabbits.

	Mating system (female x male)		
	Hh-WHHL x HH-WHHL	HH-WHHL x HH-WHHL	NZW x HH-WHHL
Total number of mating	149	21	47
Conception rate (%)	81	67	81
Litter size	6.0 ± 2.1	4.3 ± 2.1★	7.2 ± 2.6
Weaning rate (%)	79 ± 29	65 ± 39★	90 ± 16

★: $p<0.05$ compared to two other groups.

Table 2. Changes in blood lipids (mmol/l ± SD) in heterozygous WHHL female and NZW female rabbits during gestation and lactation.

	Heterozygous WHHL females (N=12)			NZW females (N=4)		
	At mating	Gestation[a]	Lactation[b]	At mating	Gestation[a]	Lactation[b]
Total cholesterol	1.51 ± 0.47◊	1.14 ± 0.36	0.92 ± 0.28	1.27 ± 0.30	0.79 ± 0.27	1.18 ± 0.50
VLDL	0.32 ± 0.26	0.44 ± 0.25◊	0.14 ± 0.12	0.13 ± 0.04	0.25 ± 0.08★	0.07 ± 0.08
LDL	0.51 ± 0.28★	0.29 ± 0.14	0.18 ± 0.13	0.28 ± 0.12	0.12 ± 0.10	0.32 ± 0.22
HDL	0.63 ± 0.17	0.34 ± 0.17★	0.70 ± 0.29	0.74 ± 0.08	0.30 ± 0.20★	0.78 ± 0.09
Triglycerides	0.98 ± 0.61	1.47 ± 0.61◊	0.52 ± 0.25	0.52 ± 0.18	0.84 ± 0.15★	0.41 ± 0.13
VLDL	0.37 ± 0.47	0.82 ± 0.41◊	0.24 ± 0.17	0.14 ± 0.09	0.36 ± 0.14★	0.07 ± 0.05
LDL	0.24 ± 0.17◊	0.17 ± 0.07	0.08 ± 0.02	0.10 ± 0.07	0.14 ± 0.10	0.09 ± 0.02
HDL	0.28 ± 0.08	0.31 ± 0.15	0.26 ± 0.12	0.24 ± 0.05	0.24 ± 0.05	0.22 ± 0.04

[a]: 14 days after mating. [b]: 14 days after delivery. ★: $p<0.05$ compared to values at the two other times in the same rabbit strain. ◊: $p<0.05$ compared to the value 14 days after delivery in the same rabbit strain. Mean recovery of cholesterol (%): 95.4 ± 8.3 (SD). Mean recovery of triglycerides (%): 94.6 ± 10.4 (SD).

Tabel 3. Changes in blood lipids (mmol/l ± SD) in WHHL and NZW rabbits from 4th to 11th week of age.

	Number		Age in weeks					
			4[x]		6		11	
Rabbit strain	Males	Females	Males	Females	Males	Females	Males	Females
WHHL homozygous								
Total cholesterol	39	31	24.29 ± 4.65♦	24.88 ± 5.57♦	22.28 ± 4.19♦	22.11 ± 4.55¤♦	23.46 ± 4.03♦	24.01 ± 2.86♦
Triglycerides	39	31	6.11 ± 2.06◊	6.55 ± 2.96◊	5.91 ± 2.01◊	6.06 ± 1.94♦	5.78 ± 2.78★♦	4.61 ± 1.49¤♦
WHHL heterozygous								
Total cholesterol	57	46	6.71 ± 2.13¤◊	7.17 ± 2.66¤	4.54 ± 1.55¤◊	4.22 ± 1.27¤◊	2.44±0.81¤	3.28 ± 1.05★¤◊
Triglycerides	20	19	6.89 ± 3.62¤◊	6.54 ± 2.35¤◊	5.01 ± 1.94¤◊	4.05 ± 1.36¤	0.76±0.32¤◊	1.18 ± 0.49★¤
NZW								
Total cholesterol	21	24	3.55 ± 1.23¤	3.46 ± 0.72¤	2.53 ± 0.41★¤	2.26 ± 0.46¤	1.80 ± 0.32¤	1.68 ± 0.34¤
Triglycerides	21	21	3.69 ± 2.07¤	3.51 ± 1.30¤	1.96 ± 0.68¤	1.92 ± 0.67¤	1.16 ± 0.36¤	1.15 ± 0.25¤

★: $p<0.05$ significant sex difference in the same age group. ¤: $p<0.05$ significantly different from other age groups within the same strain and sex. ◊: $p<0.05$ significantly different from NZW rabbits in the same age and sex. ♦: $p<0.05$ significantly different from heterozygous WHHL and NZW rabbits in the same age and sex.
[x]: Measurement of triglycerides was performed in 25 male and 22 female homozygous WHHL rabbits only in this age group.

were significantly higher than in heterozygous WHHL and NZW males which had comparable triglyceride levels. Triglycerides in homozygous and heterozygous WHHL females were comparable and significantly higher than in NZW females at 4 weeks of age. At 6 weeks of age there was a significant difference in the triglyceride level between the females of the three strains, being highest in homozygous WHHLs and lowest in NZWs. At 11 weeks the triglycerides were significantly higher in homozygous WHHL females than in heterozygous WHHL and NZW females which had comparable triglyceride levels.

DISCUSSION

The comparison of reproductive performance of homozygous and heterozygous WHHL females and NZW females in our colony demonstrated the lowest conception rate, litter size and weaning rate in homozygous WHHL females while there was no difference between the heterozygous WHHL and NZW females. The decreased reproductive performance of homozygous WHHL females was the reason for prefering to mate heterozygous WHHL females with homozygous WHHL males to obtain the homozygous offspring in our colony.

Norido et al. (1993) reported a higher litter size and a lower weaning rate for homozygous WHHL females than in our colony. *Shiomi et al.* (1987) who compared the reproductive performance of homozygous and heterozygous WHHL and NZW females and Japanese White females reported a conception rate and litter size for homozygous WHHL females comparable to those in our colony but a lower weaning rate. The litter size for heterozygous WHHL females reported by *Shiomi et al.* (1987) was identical to ours. The conception and weaning rate for heterozygous WHHL and NZW females were higher in our colony than the reported by *Shiomi et al.* (1987) for heterozygous WHHL and Japanese White females.

Roberts et al. (1974) reported a lower plasma cholesterol in pregnant and lactating normolipidemic females than in non-pregnant and non-lactating. *Kriesten & Murawski* (1981) reported for NZW females that after an increase of plasma cholesterol and a slight decrease in triglycerides in the first week of gestation the blood lipids decreased and reached the lowest level at week 4 of the gestation. During the lactation the blood lipids were still lower than before the gestation. The observed changes in total cholesterol due to gestation and lactation in NZW females in our colony are in accordance with the reports of *Roberts et al.* (1974) and *Kriesten & Murawski* (1981). The changes in total cholesterol in heterozygous WHHL females from our colony are also in accordance with the report of *Roberts et al.* (1993) but the lowest levels of total cholesterol were recorded during the lactation. In contrast to reports of *Kriesten & Murawski* (1981) the highest levels of triglycerides in both heterozygous WHHL and NZW females in our colony were recorded during the gestation and the lowest during lactation.

The information on physiological levels of blood lipids in growing WHHL and NZW rabbits up to 3 months of age is very limited. *Rosenfeld et al.* (1987) presented a figure on temporal patterns of hypercholesterolemia in homozygous WHHL and fat-fed NZW rabbits from 4 to 52 weeks of age. The figure demonstrated that plasma cholesterol in homozygous WHHLs decreased from 4 to 12 weeks of age. *Lind et al.* (1990) who investigated the age and sex related changes in blood lipids of homozygous WHHL rabbits from 10 to 52 weeks of age reported no sex difference in total cholesterol at 10 weeks of age. Furthermore, they did not report statistically significant difference in triglycerides, but the triglycerides values were slightly lower in females than in males. The results from our colony of WHHLs on total cholesterol and triglycerides at age the from 4 to 11 weeks seem comparable to the report of *Lind et al.* (1990) for 10 weeks old homozygous WHHLs.

This was obvious that the homozygous WHHLs had significantly higher plasma cholesterol than the heterozygous WHHLs but the interesting observation in this study was that the homozygous and heterozygous WHHLs had comparable triglycerides during weaning. A significant strain difference in triglyceride levels was first seen at 11 weeks of age when the rabbits were fed standard diet.

The plasma lipids decreased with age in growing heterozygous WHHLs and NZWs. Some of the difference in blood lipids between 4 and 6 weeks of age could be ascribed to the fact that the 4 weeks old offsprings were not fasted before blood sampling as they stayed with their mothers while the 6 weeks old pups were fasted overnight as they were already housed individually. However, the major factor for the significant difference in blood lipids at the weaning and at 11 weeks of age seems to us the difference in the amounts of ingested fats: Suckling pups received up to approximately 13% fat in their mother's milk (*Hagen 1974*) while from 6 weeks of age the rabbits were offered only 3.5% fat in the standard rabbit chow.

Lind et al. (1990) observed a significantly higher total cholesterol in WHHL females than in males first at 22 weeks of age. *Roberts et al.* (1974) reported a higher plasma cholesterol in females than in males for sexually matured normolipidemic rabbits. Our results for 11 weeks old rabbits demonstrated a higher plasma cholesterol in females than in males only in heterozygous WHHLs but on the other hand the 11th week of age may be too early for any sex differences to be established.

CONCLUSION

The reproductive performance of homozygous WHHL females was significantly decreased when compared with heterozygous WHHL and NZW females. In heterozygous WHHL and NZW females the plasma cholesterol decreased during gestation and lactation while the plasma triglycerides increased during gestation and were lowest during lactation when compared to the levels at mating.

At the age from 4 to 11 weeks no changes were observed in homozygous WHHL rabbits in total cholesterol while triglycerides significantly decreased in females. In heterozygous WHHL rabbits and NZW rabbits the blood lipids decreased significantly. Sex differences at 11 weeks

of age were recorded for homozygous WHHLs in triglycerides and for heterozygous WHHLs in total cholesterol and triglycerides while no sex differences in blood lipids were recorded in NZWs. The decrease with age in blood lipids in growing rabbits should be kept in mind when designing experiments beginning in animals younger than 3 months of age.

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III.A.2. Blood lipid changes in homozygous Watanabe heritable hyperlipidemic rabbits from three to six months of life and comparison of physiological blood lipid levels in adult homozygous and heterozygous WHHL and normolipidemic rabbits

SUMMARY

The aim of this study was to examine blood lipids in homozygous WHHL rabbits from 11 to 23 weeks of life and to compare the total cholesterol and triglycerides of adult homozygous WHHL, heterozygous WHHL and normolipidemic rabbits aged at least 6 months. In WHHL males plasma cholesterol did not change significantly from the 11th to 23rd week of age but their triglyceride level at 23 week of age was significantly lower ($p<0.05$) than at the 11th and 19th week of age. No significant changes with age were recorded for females apart from a transient increase in plasma cholesterol and triglycerides at the 19th week of age ($p<0.05$). The females had plasma cholesterol significantly higher than males at the 15th and 19th weeks of age ($p<0.05$) and the triglyceride level was significantly lower than in males at the 11th week of age ($p<0.05$). No sex difference was seen in blood lipids of 23 weeks old and adult homozygous WHHLs. The adult homozygous WHHL rabbits had significantly higher levels of plasma cholesterol and triglycerides compared to heterozygous WHHLs and NZWs due to significantly elevated LDL, VLDL and IDL ($p<0.05$). Blood lipids of adult heterozygous WHHL and normolipidemic rabbits were comparable. In both heterozygous and normolipidemic rabbits the females had a higher plasma cholesterol level than males.

INTRODUCTION

In 1980 Watanabe described a new rabbit strain which developed spontaneous atherosclerosis and exhibited heritable hyperlipidemia due to a recessive genetically conditioned deficiency in low density lipoprotein (LDL) receptor. The homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits were reported to have 8 to 10-fold increased plasma cholesterol and triglyceridemia compared to normolipidemic rabbits. The grown up heterozygous WHHL rabbits were reported to have blood lipid levels as normolipidemic rabbits (*Esper et al. 1993*) or intermediate values between those in normolipidemic rabbits and WHHL homozygots (*Goldstein et al. 1983).*

In 1989 a breeding colony of WHHL rabbits was established in our laboratory from 2 homozygous males and 6 heterozygous females obtained with the permission of Dr. Y.Watanabe from professor Jansen, University of Leiden, Holland. Since then the WHHL rabbits from our colony have been used to monitor the development of spontaneous atherosclerosis in homozygous and heterozygous animals (*III.B.: Hansen et al. 1994*) and for dietary and drug intervention during the first 6 months of life (*III.E., Mortensen et al. 1995, III.F.: Hansen et al. 1995*).

The hyperlipidemia in homozygous WHHL rabbits was reported to diminish with age (*Watanabe 1980, Havel et al. 1982*). Also a sex difference in blood lipids was reported for spontaneously hyperlipidemic rabbits (*Lind et al. 1990*). The age related physiological decrease, if present, and possible sex difference in blood lipids might interfere with the interpretation of the effect of drug or dietary intervention on blood lipids. It has earlier been demonstrated for homozygous WHHL rabbits from our colony that at the age from 4 to 11 weeks no significant changes in total choles-

terol occurred while triglycerides significantly decreased in females at the 11th week of life (*III.A.1*). The aim of this study was 1) to further examine the possible blood lipid changes from the 11th to 23rd week of life in homozygous WHHL rabbits i.e. up to the age of the end of the intervention studies, 2) to compare the concentration of plasma cholesterol and triglycerides in lipoproteins of homozygous WHHL, heterozygous WHHL and NZW rabbits at least 6 months old and 3) to compare the physiological levels of total plasma cholesterol and triglycerides of adult homozygous WHHL, heterozygous WHHL from our colony and normolipidemic rabbits of different strains.

MATERIALS AND METHODS

Housing of the rabbits and clinical observation

For housing conditions and clinical observation see II.E. and III.A.1. The rabbits were fed either 100g/rabbit/day or *ad libitum* of a standard rabbit chow Altromin 2113 (Lage Germany) and had free access to tap water.

Blood samples

Repeated blood samples were collected from 36 male and 31 female homozygous WHHL rabbits obtained from our own breeding colony from 11 weeks until 23 weeks of age at intervals of 4 weeks. Single blood samples were obtained from 4 homozygous WHHL breeding females at minimum 9 months old, 4 NZW breeding females (Thomae-Pharma-Biberach, Department of Laboratory Animal Service, Biberach, Germany) at minimum 9 months old, 21 male and 33 female heterozygous WHHL rabbits 6 to 18.5 months old from our own breeding colony, 16 males and 16 females NZW (Danish Serum Institute, Hvide Steen, Denmark) at minimum 6 months old, from 45 male rabbits Chinchilla (Chbb:CH) (Thomae-Pharma-Biberach, Department of Laboratory Animal Service, Biberach, Germany) approximately 6 months old and from 14 male Russian rabbits at minimum 6 months old (Møllegaard Breeding & Research Centre A/S, LI. Skensved, Denmark). All the rabbits were fasted overnight before blood sampling. Blood samples were collected from the marginal ear vein of unanesthetized rabbits in tubes containing potassium EDTA, and plasma was isolated after centrifugation at 2000g for 10 minutes.

Assay of plasma lipids

For description of measurement of total cholesterol see II.C.1., of total triglycerides see II.C.2. for measurement of total cholesterol and triglycerides in lipoproteins see II.C.3..

Statistical procedures

The results on blood lipids were analysed as described in III.A.1..

RESULTS

Plasma cholesterol did not change significantly in homozygous WHHL males from 11 to 23 weeks of age but their triglyceride at 23 weeks was significantly lower than at 11 and 19 weeks of age. No significant age related changes in plasma lipids were recorded for females from 11 to 23 weeks of age apart from a transient increase in plasma cholesterol and triglycerides at week 19 of age. Females had plasma cholesterol significantly higher than males at 15 and 19 weeks of age and triglycerides significantly lower than males at 11 weeks of age. No sex difference in blood lipids was recorded at 23 weeks of age (Table 1).

The concentration of plasma cholesterol and triglycerides in lipoproteins of these homozygous WHHLs, 4 females and 4 males 6 months old and 4 males and 4 females 17-18.5 months old heterozygous WHHLs and 6 females and 6 males NZWs are shown in Table 2. As no statistically significant difference in blood lipids and their concentrations in lipoproteins was found for heterozygous WHHL females and males of the two age groups their results were pooled. In homozygous WHHL rabbits no significant sex difference in plasma cholesterol and triglycerides in lipoproteins was recorded. About 50% of their total cholesterol and 44% of triglycerides were present in LDL. The homozygous WHHLs had significantly higher levels of plasma cholesterol and triglycerides compared to heterozygous WHHLs and NZWs due to significantly elevated levels of LDL, VLDL and IDL. The HDL cholesterol of homozygous females was significantly lower than in females from the two other strains which had comparable levels of this fraction. The HDL cholesterol of homozygous and heterozygous males was found significantly lower than in NZW males. The concentration of HDL triglycerides in the three normolipidemic rabbit strains were identical from statistical point of view. In heterozygous WHHL rabbits females had significantly higher total, HDL and LDL cholesterol compared to males. In NZW rabbits females had significanlty higher total and IDL cholesterol compared to males. No sex difference was seen in concentration of triglycerides in heterozygous WHHL and NZW rabbits.

In Table 3 the results on blood lipids in adult homozygous and heterozygous WHHL and normolipidemic rabbits of different strains are compiled. The blood lipids of homozygous WHHLs were significantly higher than in heterozygous WHHLs and normolipidemic rabbits. The blood lipids of heterozygous WHHL and normolipidemic rabbits were comparable. In heterozygous WHHLs the females had significantly higher cholesterol than the males. In normolipidemic rabbits the plasma cholesterol of NZW females was significantly higher than the mean plasma cholesterol for all males. Furtheremore, a strain difference was seen in male plasma cholesterol.

Table 1. Changes in blood lipids (mean ± SD) in homozygous WHHL rabbits from 11 to 23 weeks of age.

Blood lipids	Number	Age in weeks			
		11	15	19	23
Total cholesterol					
Males	36	23.5 ± 4.0	23.1 ± 4.5	24.9 ± 5.9	24.4 ± 5.2
Females	31	24.0 ± 2.9	25.7 ± 5.1†	28.0 ± 5.1★†	25.7 ± 5.0
Triglycerides					
Males	36	5.78 ± 2.78†	4.87 ± 1.81	5.61 ± 2.03	4.42 ± 1.77◊
Females	31	4.61 ± 1.49	4.49 ± 1.17	5.21 ± 1.27♦	3.94 ± 0.93

★ $p<0.05$ different from the level at 11 weeks of age within the same sex.
◊ $p<0.05$ different from the levels at 11 and 19 weeks of age within the same sex.
♦ $p<0.05$ different from the levels at other ages within the same sex.
† $p<0.05$ sex difference in the same age.

Table 2. Concentration of cholesterol and triglycerides (mmol/l ± SD) in lipoproteins in homozygous and heterozygous WHHL and NZW rabbits.

	Homozygous WHHL		Heterozygous WHHL		NZW	
	Females	Males	Females	Males	Females	Males
Total cholesterol	25.7 ± 5.0♦	24.5 ± 5.3♦	1.15 ± 0.35†	0.71 ± 0.28	1.34 ± 0.36†	0.88 ± 0.29
HDL	0.31 ± 0.16♦	0.32 ± 0.25	0.51 ± 0.11†	0.28 ± 0.12	0.66 ± 0.22	0.62 ± 0.11♦
LDL	12.4 ± 3.36♦	13.4 ±4.83♦	0.41 ± 0.31†	0.12 ± 0.06	0.41 ± 0.32	0.20 ± 0.22
IDL	4.91 ± 1.73♦	4.50 ± 2.07♦	0.11 ± 0.09	0.11 ± 0.06	0.17 ± 0.06†	0.07 ± 0.07
VLDL	6.36 ± 2.14♦	5.67 ± 3.12♦	0.13 ± 0.18	0.20 ± 0.18	0.12 ± 0.09	0.05 ± 0.01
Number of rabbits	31	34	8	8	6	6
Triglycerides	3.95 ± 0.89♦	4.59 ± 1.82♦	0.73 ± 0.22	1.10 ± 0.58	0.47 ± 0.19	0.62 ± 0.10
HDL	0.21 ± 0.39	0.25 ± 0.45	0.27 ± 0.04	0.25 ± 0.12	0.28 ± 0.05	0.40 ± 0.10
LDL	1.71 ± 0.38♦	2.07 ± 0.42♦	0.19 ± 0.12	0.11 ± 0.04	0.08 ± 0.02	0.09 ± 0.07
IDL	0.76 ± 0.26♦	0.86 ± 0.42♦	0.05 ± 0.04	0.07 ± 0.02	0.05 ± 0.02	0.06 ± 0.04
VLDL	1.30 ± 0.69♦	1.54 ± 1.23◊	0.22 ± 0.09	0.66 ± 0.45	0.16 ± 0.25	0.09 ± 0.03
Number of rabbits	30	34	6	7	6	6

♦ $p<0.05$ different from values in two other strains
† $p<0.05$ sex difference within the same strain
◊ $p<0.05$ different from value in NZW
Mean recover of cholesterol (% ± SD): 97.50 ± 7.41
Mean recover of triglycerides (% ± SD): 102.95 ± 12.67

Table 3. Total cholesterol and triglycerides (mmol/l ± SD) in grown up homozygous and heterozygous WHHL and normolipidemic rabbits.

Rabbit strain	Total cholesterol	Number	Triglycerides	Number
Homozygous WHHL				
Females	26.3 ± 5.1♦	35	4.00 ± 1.04♦	35
Males	24.4 ± 5.2♦	35	4.54 ± 1.81♦	35
Heterozygous WHHL				
Females	1.64 ± 0.66†	33	0.73 ± 0.51	20
Males	0.62 ± 0.22	21	1.10 ± 0.58	7
Normolipidemic rabbits				
Females NZW	1.44 ± 0.67†[a]	20	0.78 ± 0.26	20
Males NZW	1.37 ± 0.36[b]	16	0.99 ± 0.50	16
Males Chinchilla (Chbb:CH)	1.14 ± 0.32[b]	45	0.76 ± 0.37	45
Males Russian	0.75 ± 0.17[b]	14	0.35 ± 0.08[b]	14
Males total	1.12 ± 0.36	75	0.73 ± 0.42	75

♦ $p<0.05$ different from heterozygous WHHL and normolipidemic rabbits of the same sex.
† $p<0.05$ sex difference.
†[a] $p<0.05$ sex difference compared to total mean for males
[b] $p<0.05$ different from values in other strains

DISCUSSION

Changes in plasma cholesterol in homozygous WHHL rabbits

Previous reports have demonstrated an age dependent decrease in blood lipid levels in spontaneously hyperlipidemic rabbits (*Watanabe 1980, Lind et al. 1990*). However, the decrease either does not occur (*Watanabe 1980, nota bene* that the sex of animals was not stated) or is minimal in males (*Lind et al. 1990*) during the first 6 months of life. Thus, the lack of significant changes in plasma cholesterol of homozygous WHHL rabbits of both sexes from our breeding colony from 11 to 23 weeks of age is in accordance with the previous reports. Concerning the sex difference in plasma cholesterol, *Lind et al. (1990)* demonstrated in spontaneously hyperlipidemic rabbits a higher plasma cholesterol in females than in males from the 22nd to the 52nd week of life. In our colony, no sex difference in the plasma cholesterol levels was recorded in 23 weeks old and adult homozygous WHHL rabbits (Table 1 and 3). However, a higher female plasma cholesterol level was recorded at the 15th and 19th week of age. Thus, it may be concluded that the sex difference in plasma cholesterol was transcient.

Changes in plasma triglycerides in homozygous WHHL rabbits

Watanabe (1980) demonstrated for homozygous WHHL rabbits that their plasma triglyceride values were markedly lower at 6 months of age than at 1 months of age. *Lind et al.* (1990) demonstrated a marked decrease in male triglyceride levels from 10 to 16 weeks of age and a lack of significant changes from 16 to 40 weeks of age. For female triglyceride levels, *Lind et al.* (1990) reported a slight decrease from 10 to 16 weeks of

age followed at first by an increase with the maximum value at 28 weeks of age and then a decrease up to 40 weeks of age. In the present study, the female triglycerides significantly but transiently increased at 19 weeks of age while the triglycerides in the males significantly decreased at 23 weeks when the values are compared to those at 11 and 19 weeks of age. Thus our findings on triglyceride changes in males are comparable to those reported by *Watanabe* (1980).

Blood lipid levels in lipoproteins in homozygous WHHL rabbits

The concentration of blood lipids in the lipoproteins of homozygous WHHl rabbits from our colony are in accordance with previous reports (*Havel et al. 1982, Goldstein et al. 1983*). Our results confirmed that the hyperlipidemia is mainly associated with increased LDL levels. Furthermore, these rabbits also have significantly increased levels of VLDL and IDL whereas the HDL levels are relatively reduced.

Comparison of data for homozygous WHHL rabbits with those of heterozygous WHHL and normolipidemic rabbits

The comparison of blood lipid levels in adult homozygous WHHL, heterozygous WHHL and normolipidemic rabbits revealed significantly higher plasma lipid values in homozygous WHHL animals. The values of plasma cholesterol were 20-fold and of triglycerides 3-to 8-fold higher than in heterozygous and normolipidemic rabbits. Thus the plasma cholesterol level in the homozygous WHHLs in our colony is higher than the reported by *Watanabe* (1980). Our data also showed lack of a statistically significant difference in blood lipids between heterozygous WHHL and normolipidemic rabbits. This is in accordance with the report of *Esper et al.* (1993). The significantly higher plasma cholesterol in heterozygous WHHL and normolipidemic rabbit females than in males is furthermore, in accordance with the report of *Roberts et al.* (1974) for normolipidemic rabbits.

In conclusion, the determinations of blood lipids in homozygous WHHL rabbits from our colony from 11 weeks up to 23 weeks of age demonstrated only transient changes in blood lipid levels. However, these transient changes should be kept in mind when designing intervention studies in the first 6 months of life of homozygous WHHL rabbits. The use of an equal number of females and males in all experimental groups and a higher number of animals per group is recommended as it may facilitate the interpretation of the effects of an intervention on blood lipid levels. This may prevent a possible misinterpretation of the obtained data. Furthermore, the present study confirmed that the major carrier of plasma lipids in homozygous WHHL rabbits from our colony was LDL, that plasma lipids of adult homozygous WHHL rabbits were significantly higher than those of heterozygous WHHL and normolipidemic rabbits. Finally, the blood lipid levels of heterozygous rabbits are comparable to those in normolipidemic rabbits.

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III.B., III.E. & III.F. in the thesis.

III.B. ATHEROSCLEROSIS IN WATANABE HERITABLE HYPERLIPIDEMIC RABBITS. EVALUATION BY MACROSCOPIC, MICROSCOPIC AND BIOCHEMICAL METHODS AND COMPARISON OF ATHEROSCLEROSIS VARIABLES

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Atherosclerosis in Watanabe heritable hyperlipidaemic rabbits

Evaluation by macroscopic, microscopic and biochemical methods and comparison of atherosclerosis variables

BIRGIT FISCHER HANSEN,[1] ALICJA MORTENSEN,[2] JØRGEN FISCHER HANSEN,[3] PER IBSEN,[2] HENRIK FRANDSEN,[2] and BØRGE G. NORDESTGAARD[4]

[1]Department of Pathology and [3]Cardiology, Hvidovre Hospital, Hvidovre, Denmark
[2]Institute of Toxicology, National Food Agency, Søborg, Denmark and
[4]Department of Clinical Biochemistry and Medicine B, Rigshospitalet, Copenhagen, Denmark

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The spontaneous development of atherosclerotic disease in 38 homozygous and 34 heterozygous Watanabe heritable hyperlipidaemic rabbits was evaluated by qualitative and quantitative light microscopy in aorta, coronary, pulmonary and renal arteries, by naked eye and macroscopic morphometric estimation of aortic atherosclerosis extent and by biochemical analysis of aortic cholesterol content. No noteworthy atherosclerosis was demonstrated within 19 months in heterozygous rabbits. In homozygous rabbits, atherosclerotic lesions were seen from the age of 4 months and progressed with age. All 19-month-old rabbits had severe atherosclerotic disease. As much as 64% of the variation in atherosclerosis extent/severity could be explained by serum cholesterol and age. A highly significant correlation between the various methods for quantitation of atherosclerosis extent and/or severity was demonstrated, suggesting that quantitative microscopy, macroscopic morphometry and determination of aortic cholesterol content may be equally valid as a measure of atherosclerosis in WHHL rabbits and are therefore interchangeable.

Key words: Atherosclerosis; WHHL rabbits; morphology; quantitation; biochemical analysis.

Birgit Fischer Hansen, Department of Pathology 134, Hvidovre Hospital, Kettegård Allé 30, DK-2650 Hvidovre, Denmark.

III.C. THE INFLUENCE OF DIETARY OLIVE OIL AND MARGARINE ON AORTIC CHOLESTEROL ACCUMULATION IN CHOLESTEROL-FED RABBITS MAINTAINED AT SIMILAR PLASMA CHOLESTEROL LEVEL

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The influence of dietary olive oil and margarine on aortic cholesterol accumulation in cholesterol-fed rabbits maintained at similar plasma cholesterol level

Alicja Mortensen[a], Per L. Espensen[b], Birgit Fischer Hansen[c] and Per Ibsen[a]

[a]*National Food Agency of Denmark, Institute of Toxicology, Mørkøj Bygade 19, DK-2860 Søborg,* [b]*Rigshospitalet, Clinical Chemical Department, Blegdamsvej 9, DK-2800 Copenhagen,* [c]*Hvidovre Hospital, Institute of Pathology, Kettegård Allé 30, DK-2560 Hvidovre (Denmark)*

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Summary

The present study compares the atherogenicity of a standard diet and diets with 10% olive oil or 10% margarine added, in rabbits maintained at a mean plasma cholesterol level of about 20 mM for 13 weeks. Each group consisted of 15 animals. The distribution of cholesterol in plasma between VLDL, IDL, LDL and HDL was similar in the 3 groups. The thoracic aortic cholesterol accumulation was 16.6 ± 1.6, 11.4 ± 1.0 ($P < 0.05$) and 12.6 ± 1.7 ($P > 0.05$) nmol/mg wet weight for the group receiving standard diet, diet with 10% olive oil added and diet with 10% margarine added, respectively. There was no significant difference between groups in the occurrence of the atherosclerotic changes in the proximal and distal parts of coronary arteries, abdominal aorta and renal arteries. The occurrence of atherosclerotic changes in the pulmonary arteries was equal in the groups receiving standard diet and diet with 10% margarine added while it was significantly lower ($P < 0.05$) in the group receiving diet with 10% olive oil added. The atherosclerotic changes at the aortic orifice of coronary arteries were quanticated morphometrically and were most severe in the group on the standard diet. The results indicate a comparable atherogenic effect of 10% olive oil or margarine addition to standard diet on development of atherosclerosis in rabbits maintained at a similar plasma cholesterol level. The study also suggests that supplementation of olive oil or margarine to standard rabbit diet leads to lower cholesterol accumulation in the thoracic aorta compared with standard diet, an effect not modulated by changes in plasma cholesterol concentrations.

Key words: Aortic cholesterol; Atherosclerosis; Olive oil; Margarine; *trans* fatty acids; Hydrogenated fat; Rabbits

III.D. EXTRAVASCULAR LIPID DEPOSITION AND MORPHOLOGY OF ATHEROSCLEROSIS IN HETEROZYGOUS WHHL RABBITS FED VEGETABLE (N-6) AND MARINE (N-3) OILS

Mortensen A, Hansen FB, Frandsen H, Hansen FJ, Andersen AS, Høy C-E & Meyer O.

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Extravascular lipid deposition and morphology of atherosclerosis in heterozygous WHHL rabbits fed vegetable (n-6) and marine (n-3) oils

by *Alicja Mortensen*[1], *Birgit Fischer Hansen*[2], *Henrik Frandsen*[1], *Jørgen Fischer Hansen*[3], *Peder S. Andersen*[4], *Carl-Erik Høy*[4] & *Otto Meyer*[1]

[1]National Food Agency of Denmark, Institute of Toxicology, Mørkhøj Bygade 19, DK-2860 Søborg, [2]Department of Pathology and [3]Cardiology, Hvidovre Hospital, Kettegård Allé 30. DK-2560 Hvidovre, [4]Department of Biochemistry and Nutrition and Center for Food Research, Technical University of Denmark, DK-2800 Lyngby.

III. E. EFFECT OF OLIVE OIL AND FISH OIL ON BLOOD LIPIDS AND AORTIC ATHEROSCLEROSIS IN HOMOZYGOUS WATANABE HERITABLE HYPERLIPIDEMIC (WHHL) RABBITS

Alicja Mortensen[1], Birgit Fischer Hansen[2], Jørgen Fischer Hansen[3], Henrik Frandsen[1], Elzbieta Bartnikowska[4], Peder S. Andersen[5], Lone S. Bertelsen[5]

[1] National Food Agency, Institute of Toxicology, Mørkhøy Bygade 19, DK-2860 Søborg, Denmark, [2] Department of Pathology and of [3] Cardiology, Hvidovre Hospital, DK-2650 Hvidovre, Denmark, [4] Department of Human Nutrition, Warsaw Agriculture University, Warsaw, Poland, [5] Department of Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Lyngby, Denmark

Atherosclerosis 1995, 115 (Suppl): S19 (Abstract based on this study)

PART IV

GENERAL DISCUSSION AND CONCLUSION

IV.A. COMPARISON OF THE RABBIT MODELS USED IN THIS PROJECT AND THEIR POSITION IN ATHEROSCLEROSIS RESEARCH

In the present work three rabbit models were used: the cholesterol-fed normolipidemic rabbit, the cholesterol-fed heterozygous WHHL rabbit and the homozygous WHHL rabbit.

IV.A.1. Blood lipids and spontaneous atherosclerosis

Physiological blood lipid levels in heterozygous WHHL rabbits are comparable to those of normolipidemic rabbits while homozygous WHHLs exhibit pronounced hyperlipidemia due to an inherited deficiency in the LDL receptor. The levels of plasma cholesterol and triglycerides of homozygous WHHL rabbits are significantly higher than in heterozygous WHHLs and NZWs due to significantly elevated levels of LDL, VLDL and IDL. During the first 6 months of life of homozygous WHHL rabbits of both sexes the plasma cholesterol and the triglycerides do not change significantly but transient fluctuation in their concentrations have been recorded. The blood lipids of heterozygous WHHL and NZW rabbits decreased significantly from the 4th to 11th week of age (*III.A.1&2*).

In normolipidemic rabbits spontaneous aortic lesions of various morphologic appearance have been described (*Haust & More 1965, Schenk et al. 1966*) but spontaneous aortic atherosclerosis is a rare phenomenon (*Butler & Clubb 1989*). In heterozygous WHHL rabbits spontaneous atherosclerosis is of little significance until the age of approximately two years (*III.B.: Hansen et al. 1994, Atkinson et al. 1989*) but the presence of atherosclerotic intimal lesions of different type have been reported in 3 years old heterozygous WHHLs (*Esper et al. 1993*). In homozygous WHHL rabbits spontaneous atherosclerosis develops at an early age, e.g. fatty streaks or fibrous plaques with occasionally foam cells were seen already in the aortas of 4 months old rabbits from our colony (*III.B.: Hansen et al. 1994*).

IV.A.2. Position of rabbit models in atherosclerosis research

The normolipidemic rabbit has been widely used in atherosclerosis research as it more readily than other laboratory species develops aortic lesions after cholesterol feeding, the so called experimental atherosclerosis. In this regard the normolipidemic rabbit has been superior to other laboratory animal species used in atherosclerosis research. The homozygous WHHL rabbit has been applied in atherosclerosis research due to its unique

characteristic - the spontaneous hyperlipidemia and atherosclerosis. The position of heterozygous WHHL rabbit among the laboratory animal models for atherosclerosis research, remains to be established. *Atkinson et al. (1989)* proposed that the heterozygous WHHL rabbit might be a more relevant model than its homozygous sibling since humans with heterozygous familial hypercholesterolemia outnumber those with homozygous familial hypercholesterolemia. Furthermore, he reported that the lesions in 1% cholesterol-fed WHHL heterozygotes morphologically resembled those in WHHL homozygotes and humans. This indicated the superiority of the heterozygous WHHL rabbit over the cholesterol-fed normolipidemic rabbit concerning the request of morphological resemblance between the atherosclerotic lesions in the animal model and in humans. *Esper et al. (1993 a, b)* suggested that the older heterozygous WHHL rabbit is superior to the cholesterol-fed rabbit model. This suggestion was based on the facts that in heterozygous WHHL rabbits 1) the age and gender dependent changes in blood lipids were demonstrated and 2) the atherosclerotic lesions of 3 years old animals were comparable to the human atherosclerotic lesions from morphological point of view. The studies on the heterozygous WHHL rabbit in our laboratory (III.D.) demonstrated that 1% cholesterol-fed heterozygous WHHL rabbit was not suitable for dietary intervention studies due to a disturbed lipid metabolism manifested by a massive hypercholesterolemia which could mask any effect of a test compound on the blood lipid levels and by extravascular lipid deposition in various organs. In addition, the atherosclerotic lesions in 1% cholesterol fed heterozygous WHHL rabbit were comparable to the lesions in the normolipidemic cholesterol-fed rabbits receiving lower cholesterol doses and in 6 months old homozygous WHHL rabbits. The latter observation suggests that the normolipidemic rabbit fed low cholesterol doses like in III.C. *(Mortensen et al. 1992)* may be a better model than 1% cholesterol-fed heterozygous WHHL rabbit. This is due to the fact that it develops atherosclerotic lesions of similar morphology without extreme hypercholesterolemia which can affect the outcome of any study. However, a comparison of atherosclerotic lesions in heterozygous WHHL and normolipidemic rabbits fed identical cholesterol doses at levels lower than 1% was not conducted in the present project. Therefore, it cannot be excluded that the atherosclerotic lesions due to lower cholesterol doses in heterozygous WHHLs would be of closer resemblance to atherosclerotic lesions in homozygous WHHLs than those in normolipidemic rabbits fed identical cholesterol doses. The suggestion of *Esper et al. (1993 a, b)* of using older heterozygous WHHL rabbit as a model in atherosclerosis research does not seem advantageous from a practical point of view. The price of a 2-3 years old rabbit is higher than of a rabbit which is not less than 6 months old due to the maintenance cost. The purchase of a sufficient number of 2-3 years old animals may be difficult or at least time demanding. Furthermore, the use of animals with already existing spontaneous atherosclerotic lesions demands more animals per experimental group to ensure the detection of any differences in the aortic and/or coronary atherosclerosis due to the intervention. The dietary or medicamental prevention studies in this model may provide false results because lesions already existing probably cannot be prevented. If heterozygous WHHL was chosen for studies of prevention of spontaneous

atherosclerosis the younger subjects without detectable atherosclerotic lesions should probably be used and the larger than traditionally experimental groups should probably be employed in order to ensure the correct interpretation of the results. Furthermore, the intervention should end approximately at the time when 100% of the nontreated animals was expected to have developed spontaneous changes. However, this would mean an increase use of animals and an experimental period of about 2-3 years. On the other hand the heterozygous WHHL rabbit/cholesterol-fed heterozygous WHHL rabbit could be expected to be more sensitive than normolipidemic rabbit/cholesterol-fed normolipidemic rabbit to atherogenic effect of different environmental factors due to its heterozygous genetic background for spontaneous atherosclerosis. However, no reports on this subject are, to the author's knowledge available at present.

The position of the cholesterol-fed rabbit based on normolipidemic strains in atherosclerosis research is well established in spite of some differences in physiological lipid metabolism between the normolipidemic rabbit and human and in spite of the different origin of hypercholesterolemia in cholesterol-fed rabbit and man (I.D.). Because of these differences, the atherosclerosis in cholesterol-fed rabbit should always be regarded as a model of human disease and never as an analogous disease process. The normolipidemic rabbit is very sensitive to dietary cholesterol. Like humans can be classified as hypo- or hyper-responders with regard to the cholesterolemic response to dietary modifications, the rabbits are classified as hypo- or hyper-responders to dietary cholesterol. Even if strains of rabbit hypo- and hyper-responders have been developed in some laboratories (*Loose-Mitchell et al. 1991, Overturf & Loose-Mitchell 1992, Thiery et al. 1995*), the dietary or medicamental intervention or screening of lipid lowering compounds is routinely performed in groups comprising both kinds of cholesterolemic responders. This is the reason for occasional large interindividual variations in the plasma cholesterol and in the severity and extent of aortic atherosclerosis in the same treatment group despite the fact that all the rabbits are treated with the same cholesterol dose. Even a controlled individual cholesterol dose regimen which diminishes the differences in plasma cholesterol does not reduce the interindividual variation in severity and extent of aortic atherosclerosis (Fig. 7, p. 134). The interindividual variation in response to cholesterol feeding should be kept in mind when taking a decision on the necessary number of animals per experimental group. The cholesterol dose or the level of a plasma cholesterol maintained at certain concentration during the study are of crucial importance in experiments in cholesterol-fed rabbits as a too severe or too low experimental hypercholesterolemia may mask the effects of intervention on plasma lipids and aortic atherosclerosis in this model. Also the duration of the exposure to exogenous cholesterol should be carefully chosen as a too short or too long exposure may affect the outcome of the study. In contrast to the mentioned factors, the age of animals at the start of the study is a secondary issue because of a very limited occurrence of any spontaneous atherosclerotic lesions.

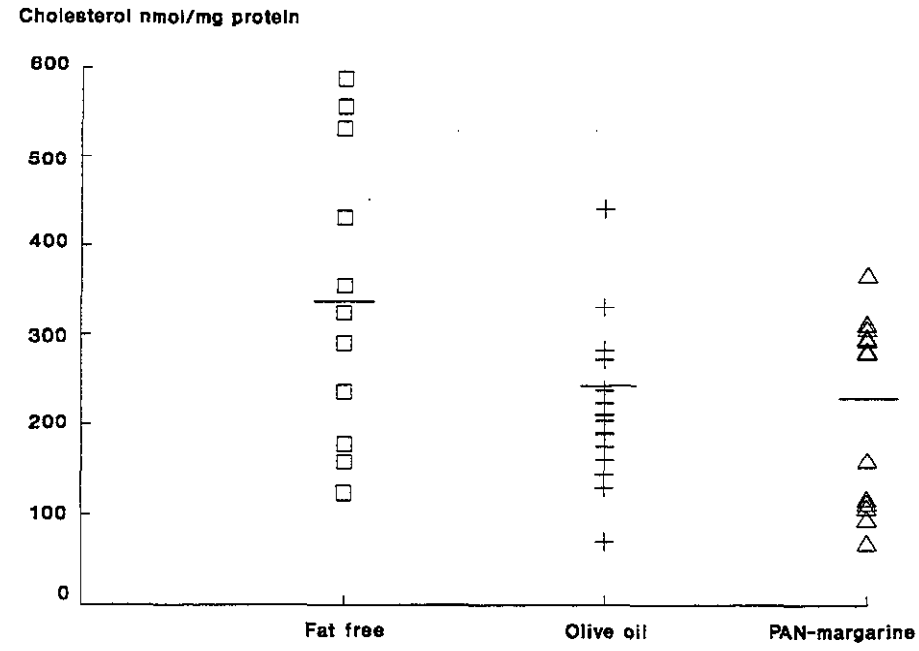

Fig.7. Individual cholesterol accumulation in inner proximal layer of thoracic aorta of the rabbits from III.C. fed standard diet added cholesterol ("fat free"), standard diet added cholesterol and 10% olive oil, standard diet added cholesterol and 10% PAN-margarine and maintained on mean plasma cholesterol of 20.1, 22.5 and 20.5 mmol/l respectively due to controled individual cholesterol dose regimen. —— marks the mean level of accumulated cholesterol in each group.

The homozygous WHHL rabbit has a well established position in atherosclerosis research as an animal model of human FH because several similarities between the disease process in these rabbits and in humans have been established (I.D.4.). The disease in the homozygous WHHL rabbit can be regarded as analogous to this particular human disease. Therefore this animal model seems excellent for development and testing of causal treatment e.g. gene therapies of FH. Additionally, the results of conventional symptomatic intervention (e.g new lipid lowering compounds) or other nongenetic intervention (e.g. modulation of dietary factors) in this model seem to be directly relevant for patients with FH. Furthermore, these results may be of importance for the prevention and treatment of atherosclerosis in patients who have two functional LDL receptor genes but who suffer from other forms of endogenous hypercholesterolemia which is aggravated by exogenous factors such as a high intake of fat, cholesterol or calories. In this regard the homozygous WHHL rabbit is not a better model than the cholesterol-fed rabbit as the etiology of disease in both animal models is different than in this category of human patients who develop atherosclerosis. It has been suggested that treatment directed at counter-acting high LDL levels has beneficial effects in reducing atherosclerosis

while treatment directed toward the modification of conventional risk factors (e.g. reducing plasma cholesterol and platelet adhesion) or delaying certain mediators of tissue injury does not prevent or reduce atherosclerosis in homozygous WHHL rabbits *(Buja et al. 1990)*. This suggestion means an important handicap for the model and a limitation of its use in atherosclerosis research. However, the results of two intervention studies in homozygous WHHL rabbits included in this project demonstrate the opposite. The calcium-channel antagonist -(-)anipamil delayed the development of atherosclerosis in homozygous WHHL rabbits (III.F.). Also the treatment with fish oil with a high content of n-3 polyunsaturated fatty acids decreased the blood lipid levels and the development of aortic atherosclerosis compared to olive oil treatment (III.E.). It seems that in the homozygous WHHL rabbit model the age of animals at the start of the intervention is crucial for detection of its positive or negative effects as the atherosclerotic changes once started cannot be prevented but may be delayed. The degree of aortic atherosclerosis in this model depends on the total cholesterol and age (III.B.). Therefore the intervention should begin before the atherosclerotic lesions are detectable by light microscopy. Furthermore, the homozygous WHHL rabbit might not be suitable for studies on regression of atherosclerosis after withdrawal of an exogenous atherogenic factor, because the effect of the withdrawal may be masked by the effect of endogenous atherogenic factor, the genetic defect, which is always present in the animal. This however should be verified by further investigations. Other important issues which should be kept in mind while designing the studies in homozygous WHHL rabbits are the decrease with age in blood lipids in older animals observed in other laboratories (*Watanabe 1980, Lind et al. 1990*) and a possible occurrence of transient fluctuations in blood lipid levels up to 6 months of life (III.A.1&2) as well as the inter-individual variation in plasma cholesterol and the degree of aortic atherosclerosis (III.B.).

IV.A.3. Comparison of cholesterol-fed and homozygous WHHL rabbit models

The comparison of the cholesterol-fed and homozygous WHHL rabbit models (Table, p. 136) emphasizes the major difference between them - the nature of atherosclerosis. However, the morphologic and biochemical studies on the development of atherosclerosis from other laboratories indicate that key processes involved in lesion initiation and progression are the same in both models in spite of the different origin of hypercholesterolemia *(Rosenfeld et al. 1987 a, b)*. As presented in the Table, both models have specific characteristics which point to some specific rules for the application of the models and for the study design. For instance, homozy-

Table: Similarities and differences between the cholesterol-fed and homozygous WHHL rabbit models.

	Cholesterol-fed rabbit		Homozygous WHHL rabbit
-	Hypercholesterolemia and atherosclerosis due to exogenous cholesterol = experimental atherosclerosis.	-	Hypercholesterolemia and atherosclerosis due to genetic defect = spontaneous disease.
-	Interindividual variation in hypercholesterolemic response to exogenous (dietary) cholesterol.	-	Interindividual variation in plasma cholesterol.
-	Interindividual variation in degree of experimental atherosclerosis.	-	Interindividual variation in degree of spontaneous atherosclerosis.
-	Major carrier of plasma cholesterol is β-VLDL.	-	Major carrier of plasma cholesterol is LDL
-	The earliest detectable events in experimental atherosclerosis are increased monocyte adherence and their subendothelial migration followed by formation of fatty streaks composed primarily of macrophage-derived foam cells.	-	The earliest detectable events in spontaneous atherosclerosis are increased monocyte adherence and their subendothelial migration followed by formation of fatty streaks composed primarily of macrophage-derived foam cells.
-	Morphology of atherosclerotic lesions due to high cholesterol doses is different from that in humans. However, lower cholesterol doses can result in changes which may resemble human atherosclerotic lesions.	-	Morphology of advanced atherosclerotic lesions is considered a close resemblance of human atherosclerotic lesions.
-	Experimental disease progresses only as long as the animal is exposed to the exogenous cholesterol.	-	Spontaneous disease progresses with age.
-	Atherogenic factor can be retracted.	-	Atherogenic factor always present.
-	Age of animals at the start of the experiment is of little importance. Instead, the level of cholesterol dose or level of aimed plasma cholesterol concentration during the study are of crucial importance for the sensitivity of the animal model.	-	Age of animals at the start of experiment is of crucial importance for the sensitivity of the animal model.
-	Too short or too long exposure to exogenous cholesterol may give rise to a false result of the study.	-	Too short or too long duration of the study may give rise to a false result. The factor which seems important for determination of the duration of the study is the optimal animal age with regard to differentiation of atherosclerotic lesions between untreated control and treated groups.

gous WHHL rabbits should enter the intervention study just after the weaning, before the spontaneous atherosclerotic lesions are established. The cholesterol-fed rabbit model offers posibility to use older animals at the start of cholesterol feeding without risking that spontaneous atherosclerosis would interfere with the interpretation of the experimental results. The distinctive features of both models suggest they are not merely substitutes for each other but two different models based on the same animal species which may supplement each other. Examples of published contradictory results on the effect of the calcium antagonist verapamil, the compound probucol or L-arginine on experimental atherosclerosis in cholesterol-fed NZW rabbits or spontaneous atherosclerosis in WHHL rabbits (*Tilton et al. 1985, Buja et al. 1990, Cooke et al. 1992, Mortensen et al. 1995*) could support this suggestion. However, an additional reason for the divergence in the results in the two models could be that some of the rules concerning study design due to the distinctive features of the models (e.g. too high hypercholesterolemia in cholesterol-fed rabbit, age at the start of the study in homozygous WHHL rabbits) were unintentionally disregarded while designing some of the studies mentioned above. This leads to another important issue, which should be emphasized, and which concerns the homozygous WHHL rabbit. The mean plasma cholesterol level may vary between the different breeding colonies depending on the range of plasma cholesterol level that has been maintained in breeding animals and on the fat content and composition of breeding diet used in the colony. Keeping in mind that the degree of aortic atherosclerosis in this model depends on the level of total cholesterol and age (III.B.) one could expect that the first detectable atherosclerotic changes may occur at different age in rabbits from different breeding colonies. This means that the optimal age for the start and termination of the study may be different for homozygous WHHL rabbits from separate breeding colonies. Therefore a detailed knowledge of development and progress of spontaneous atherosclerosis in the line of homozygous WHHL rabbit applied for the study is essential for the efficient experimental design. Furthermore, the data from historical controls from the same line of homozygous WHHL rabbits and kept under comparable conditions may contribute to the interpretation of experimental data. Thus the possibility of keeping own breeding colony with well characterized atherosclerosis parameters is of a great advantage even now when the homozygous WHHL rabbit has become commercially available. Additionally, home-breeding permits to chose animals with comparable plasma cholesterol levels for a study which means a reduction of interindividual variation of this parameter.

IV.B. CONCLUSION

In the present work, the 1% cholesterol-fed heterozygous WHHL rabbit was found not suitable for dietary intervention studies due to a disturbed lipid metabolism manifested by a massive hypercholesterolemia which could mask any effect of a test compound on the blood lipid levels and by extravascular lipid deposition in various organs. The cholesterol-fed normolipidemic and homozygous WHHL rabbits seem both useful and equally important animal models in atherosclerosis research except for a particular application like testing of specific therapies for FH for which the homozygous WHHL is unquestionably the model of choice. When the human atherosclerosis due to hypercholesterolemias caused by other factors than in FH is concerned, both models can be used but the differences in etiology between the disease in the laboratory animal models and in humans should be considered. The cholesterol-fed and the homozygous WHHL rabbits have their specific characteristics which determine the principles for the study design and the domain of their application. Therefore the two models should be regarded as supportive for each other, rather than interchangeable. Although key processes in the initiation and progression of atherosclerotic lesions are similar in the cholesterol-fed and homozygous WHHL rabbits the hypercholesterolemia is of different origin. Therefore the testing of new therapies, new compounds or dietary modifications in both models, instead of only one, may provide more information on the mechanism of their antiatherogenic or atherogenic action.

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SUMMARY

Laboratory animal models play an important role in atherosclerosis research (chapter I.C.1.-3.). One of the most popular laboratory animal species in this field of research is the rabbit (chapter I.C.4.). The rabbit fulfils most of the criteria for an animal model for human atherosclerosis (chapter I.D.1). In this project the following rabbit models were established, used to compare the atherogenic effect of chosen dietary fats, and evaluated: 1) the cholesterol-fed normolipidemic rabbit, 2) the 1% cholesterol-fed heterozygous Watanabe heritable hyperlipidemic (WHHL) rabbit and 3) the homozygous WHHL rabbit (chapter I.D.3.). The reproductive performance in homozygous WHHL females was significantly decreased when compared to heterozygous WHHL or New Zealand (NZW) females (chapter III.A.1.). Physiological blood lipid levels in the heterozygous WHHL and in the normolipidemic rabbits were comparable. The blood lipid levels in homozygous WHHL rabbits were significantly higher than in heterozygous WHHL and normolipidemic NZW rabbits due to significantly elevated levels of low density lipoproteins (LDL), very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL). During the first months of life of homozygous WHHL rabbits of both sexes the plasma cholesterol and triglycerides did not change significantly but transient fluctuations in their concentrations were recorded. The blood lipids of heterozygous WHHL and NZW rabbits decreased significantly from the 4th to the 11th week of age (chapter III.A.1 & 2.). In homozygous WHHL rabbit spontaneous atherosclerosis develops at an early age, e.g. the atherosclerotic lesions detectable by light microscopy were demonstrated already in the aorta of some 4 months old rabbits. No noteworthy atherosclerosis was demonstrated within the first 19 months in heterozygous WHHL rabbits (chapter III.B.). The atherosclerotic lesions in cholesterol-fed normolipidemic rabbits maintained at a plasma cholesterol level of approximately 20 mmol/l during 93 days were composed of loosely textured connective tissue with varying amounts of lipid-laden macrophages. The atherogenic effect of margarine (hydrogenated fat) and olive oil was comparable in this rabbit model (chapter III.C.). The atherosclerotic lesions in 1% cholesterol-fed heterozygous WHHL rabbits were fibrous plaques (i.e. discrete fibrous thickenings) and fibrous plaques with a few foam cells. The morphology of these lesions was similar to lesions in cholesterol-fed normolipidemic rabbits from the study III.C. and to lesions in 6 months old homozygous WHHL rabbits from III.B. The study demonstrated that the 1% cholesterol fed rabbit was not a suitable model for dietary studies due to a disturbed lipid metabolism manifested as a massive hypercholesterolemia which could mask any effect of a test compound on the blood lipid levels and by extravascular lipid deposition in various organs. Irrespective the limitation in the use of this model it was shown that the marine oil seemed to protect against the development of atherosclerosis in the pulmonary arteries and prevented lipid accumulation in the myocardium, but was associated with a severe lipid infiltration in the

liver, and was more atherogenic than the vegetable oil based on the morphological appearance of the aortic and coronary atherosclerosis (chapter III.D.). The comparison of olive oil and fish oil effect on blood lipids and spontaneous atherosclerosis in homozygous WHHL rabbits indicated a hypolipidemic effect and lower atherogenicity of fish oil, as lower plasma lipids and a lesser degree of aortic atherosclerosis were recorded in the fish oil treated animals when compared to olive oil treated animals (chapter III.E.). The calcium antagonist (-)-anipamil delayed the development of atherosclerosis in homozygous WHHL rabbits when compared to untreated controls (chapter III.F.). The position of the heterozygous WHHL rabbit among the laboratory animal models for atherosclerosis research remains to be clarified. The position of the cholesterol-fed normolipidemic rabbit and the homozygous WHHL rabbit is well established in atherosclerosis research (chapter IV.A.2). Both models have their specific characteristics which determine the domain of their application and suggest that they should be regarded as supportive for each other, rather than interchangeable. Although key processes in the initiation and progression of atherosclerotic lesions are similar in the cholesterol-fed and homozygous WHHL rabbits the hypercholesterolemia is of different origin (chapter IV.A.3). Therefore testing of new therapies, new compounds or dietary modifications in both models, instead of only one, may provide more information on the mechanism of their antiatherogenic or atherogenic action (chapter IV.B.).

SAMMENDRAG

Dyremodeller baseret på laboratoriedyr spiller en vigtig rolle i atheroskleroseforskningen (kapitel I.C.1.-3.). En af de mest anvendte dyrearter på dette forskningsområde er kaninen (kapitel I.C.4.). Kaninen opfylder de fleste krav, som stilles til en dyremodel for human atherosklerose (kapitel I.D.1). I dette projekt blev følgende kaninmodeller etableret, anvendt til at sammenligne den atherogene effekt af udvalgte fedtstoffer, og evalueret: 1) Den kolesterolfodrede normolipidæmisk kanin, 2) den heterozygote Watanabe Heritable Hyperlipidemic (WHHL) kanin fodret 1% kolesterol, og 3) den homozygote WHHL kanin (kapitel III.D.3.). Reproduktionsevnen hos homozygote WHHL hunkaniner var signifikant nedsat sammenlignet med reproduktionsevnen hos heterozygote WHHL hunkaniner eller New Zealand White (NZW) hunkaniner (kapitel III.A.1.). Det fysiologiske niveau af blodlipider hos heterozygote WHHL og normolipidæmiske kaniner var sammenligneligt. Det fysiologiske niveau af blodlipider hos homozygote WHHL kaniner var signifikant højere end hos heterozygote WHHL og normolipidæmiske NZW kaniner på grund af et signifikant forhøjede niveauer af low density lipiproteiner (LDL), very low density lipoproteiner (VLDL), og intermediate density lipoproteiner (IDL). Koncentrationen af plasmakolesterol og plasmatriglycerid ændrede sig ikke signifikant i de første levemåneder hos homozygote WHHL kaniner af begge køn, men forbigående ændringer af koncentrationen blev observeret. Hos heterozygote WHHL kaniner og NZW kaniner blev en signifikant nedgang i koncentrationen af blodlipider observeret fra 4. til 11. leveuge (kapitler III.A.1. & 2.). Den spontane atherosklerose hos homozygote WHHL kaniner udvikles i en tidlig alder. F.eks. blev lysmikroskopiske atherosklerotiske forandringer allerede observeret hos nogle 4 måneder gamle dyr. Hos heterozygote WHHL kaniner blev ingen bemærkelsesværdige spontane atherosklerotiske forandringer observeret i de første 19 levemåneder (kapitel III.B.). De atherosklerotiske forandringer hos kolesterolfodrede, normolipidæmiske kaniner med en fast plasma kolesterolkoncentration på 20 mmol/l i løbet af 93 dage, bestod af fibrøse plaques med få skumceller. Den atherogene effekt af margarine (hydrogeneret fedt) og olivenolie var sammenlignelig i denne kaninmodel (kapitel III.C.). De atherosklerotiske forandringer hos heterozygote WHHL kaniner givet foder med 1% kolesterol var fibrøse plaques uden eller med få skumceller. Disse forandringer lignede forandringerne hos de kolesterolfodrede normolipidæmiske kaniner fra undersøgelsen i kapitel III.C. og forandringer hos 6 måneder gamle homozygote WHHL kaniner fra undersøgelsen i kapitel III.B.. Resultater fra forsøget i kapitel III.D. viste, at den heterozygote WHHL kanin givet foder med 1% kolesterol ikke var en velegnet model til at undersøge kostens effekt på eksperimentel atherosklerose på grund af et forstyrret fedtstofskifte. Dette sås som en svær grad af hyperkolesterolæmi og ekstravaskulære fedtaflejringer i diverse organer. Den svære hyperkolesterolæmi kan tilsløre alle effekter af teststofferne på koncentrationen af blodlipider. Uanset begrænsningerne af denne model har undersøgelsen vist, at

den anvendte marine olie tilsyneladende beskyttede mod udviklingen af atherosklerose i lungearterierne og forhindrede aflejring af fedt i myocardiet, men var associeret med svære fedtinfiltrationer i leveren. Marine olie var mere atherogen end den vegetabilske olie vurderet ud fra den morfologiske billede af atherosklerose i aortaen og koronararterierne (kapitel III.D.). En sammenligning af effekten af fiskeolie og olivenolie på spontan atherosklerose hos homozygote WHHL kaniner viste en hypolipidæmisk effekt og mindre udtalt atherogen effekt af fiskeolien, idet der sås et lavere niveau af blodlipider og en mindre grad af atherosklerose i aorta hos dyrene behandlet med fiskeolie (kapitel III.E.). Kalciumantagonisten (-)-anipamil forsinkede udviklingen af atherosklerose hos homozygote WHHL kaniner sammenlignet med en ubehandlet kontrolgruppe (kapitel III.F.). Anvendeligheden af den heterozygote WHHL kanin i forhold til andre dyremodeller i atheroskleroseforskningen skal afklares. Den kolesterolfodrede normolipidæmiske kanin og den homozygote WHHL kanin er anerkendte dyremodeller i atheroskleroseforskningen (kapitel IV.A.2.). Begge modeller har deres specifikke egenskaber, som bestemmer deres anvendelsesmuligheder, og som antyder, at de best supplerer og ikke erstatter hinanden. Skønt nøgleprocesserne i initieringen og udviklingen af atheroskleroticke læsioner er sammenlignelige i den kolesterolfodrede kanin og den homozygote WHHL kanin er hyperkolesterolæmien af forskellig oprindelse (kapitel IV.A.3.). Derfor kan afprøvning af nye terapier, nye stoffer eller kostændringer i begge modeller i stedet for kun i én af disse bidrage med flere oplysninger om deres antiatherogene eller atherogene virkninger (kapitel IV.B.).

LIST OF ABBREVIATIONS

(All the abbreviations are also explained in the text where they are introduced.)

AI	Area of intima
ApoB-100	Apolipoprotein B-100
BHT	Butylated hydroxytoluene
bw	Body weight
CETP	Cholestryl ester transfer protein
CHD	Coronary heart disease
EDTA	Ethylenediaminetetra acetic acid disodium salt
FCH	Familial combined hyperlipidemia
FH	Familial hypercholesterolemia
HDL	High density lipoprotein
HP	Hepatic lipase
IDL	Intermediate density lipoprotein
IEL	Internal elastic lamella
LCAT	Lecithin cholesteryl acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
meq	Miliequivalent
MUFA	Monounsaturated fatty acid(s)
NZW rabbit	New Zealand White rabbit
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acid(s)
Ratio I/M	Ratio intima/media
SFA	Saturated fatty acid(s)
SR	Scavenger receptor
VLDL	Very low density lipoprotein
WHHL rabbit	Watanabe heritable hyperlipidemic rabbit

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Ballerup, February 1996 Alicja Mortensen