DIETARY FISH OIL AND EXPERIMENTAL ATHEROSCLEROSIS

DIETARY FISH OIL AND EXPERIMENTAL ATHEROSCLEROSIS

Visolie consumptie en experimentele atherosclerose

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus Prof. Dr. A.H.G. Rinnooy Kan
en volgens besluit van het College van Dekanen

De openbare verdediging zal plaats vinden op
donderdag 8 juni 1989 om 13.30 uur

door

Johannes Matthijs Hartog
geboren te Rotterdam

PROMOTIECOMMISSIE

PROMOTOREN: Prof. P.G. Hugenholtz
Prof. Dr. W.C. Hülsmann

OVERIGE LEDEN: Dr. P.D. Verdouw
Dr. J.M.J. Lamers

The financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Aan mijn vrouw Marie Louise en dochter Suzanne

CONTENTS

CHAPTER 1

N-3 POLYUNSATURATED FATTY ACIDS, A LITERATURE REVIEW

1.1 Introduction

In the last decades the interest in the role of n-3 fatty acids in several diseases has grown. Low incidences of ischemic heart disease, diabetes, cancer, rheumatism and multiple sclerosis have been found in Arctic populations [1-8]. Fish consuming communities in Japan [9, 10], The Netherlands [11] and United States [12, 13] show also a lower incidence of ischemic heart disease. Several investigators have postulated that dietary n-3 polyunsaturated fatty acids (PUFA's), which are present in the fat of sea fish, may cause the lower incidence of ischemic heart disease [14-17]. Many experimental and human studies have been carried out with purified marine oils [18-32], but the reported results are often difficult to compare because of differences in doses and ratios of eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) used. In this chapter the results of human studies are reviewed. An attempt has been made to clarify some of the differences by ranging the studies according to doses of EPA used. For a review of animal data the reader is referred to references 19, 20, 23, 24, 32.

PUFA's can be divided in two major families: those of the n-6 and those of the n-3 type. The n-6 PUFA's have their first double bond on the sixth carbon atom counting from the methyl end of the fatty acid. The n-3 PUFA's start with their first double bond at the third carbon atom (figure 1).

Vegetable oils contain PUFA's of the n-6 type (linoleic acid; LA, 18:2 n-6), and of n-3 type (alpha-linolenic acid, a-LNA, 18:3 n-3). Some vegetable oils, like primrose oil, are relatively rich in dihomo-gamma-linolenic acid (20:3 n-6, g-LNA). Animal fats often contain some PUFA's of the n-6 type (LA and arachidonic acid; AA 20:4 n-6) and the n-3 type (a-LNA). Fish oils mainly contain PUFA's of the n-3 type (EPA, DHA and docosapentaenoic acid; DPA 22:5 n-3).

Fish do not synthesize the n-3 PUFA's themselves, but they derive their dietary n-3 PUFA's from the consumption of plankton. The marine phytoplankton and zooplankton n-3 PUFA contents depend on the water temperature. The colder the water the more n-3 PUFA's are synthesized by the plankton. It will be clear that there is a wide variation of n-3 PUFA content of fish depending on marine, terrestrial and seasonal circumstances. Another rich source of n-3 fatty acids are, except for fish, the large quantities of fish consuming animals, such as seals.

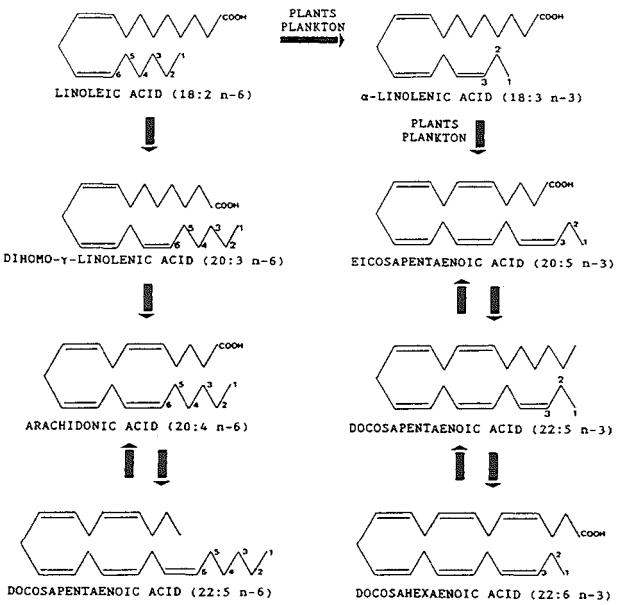

Figure 1. Chemical structures of the polyunsaturated fatty acids of the n-6 and n-3 family.

1.2 Cardiovascular effects of dietary n-3 fatty acids

The American cardiologist Nelson already prescribed a diet with 3 fish meals a week to a large number of post infarction patients 25 years before the epidemiological studies among Eskimos [17], Japanese fishermen [9, 10] and citizens of Zutphen [11] became known. Nelson noted that the incidence of myocardial infarction was decreased among these individuals during the follow up period stretching beyond 16 years [33]. These findings of Nelson did not receive much attention, in contrast with the high interest in later studies. In the following years, various actions of dietary n-3 PUFA's on cardiovascular risk factors have since been described. In the next paragraphs the effects of dietary fish oil on these factors are summarized.

1.2.1 The effects of dietary n-3 fatty acids on plasma cholesterol

There is evidence that the n-3 PUFA's decrease the synthesis of the triglyceride-rich very low density lipoproteins (VLDL) [34, 35]. As VLDL is a precursor particle for low density lipoprotein (LDL) and its surface fragments are incorporated in the high density lipoprotein (HDL), a decrease in VLDL level will be reflected on the plasma levels of LDL and HDL [36-43]. Therefore, a decrease of total cholesterol by dietary fish oil is often seen in all lipoprotein fractions.

Table 1
Effects of dietary n-3 PUFA's on plasma cholesterol (CHOL) and triglyceride (TG) levels in uncontrolled studies in normolipidemic individuals.

REFERENCE	grams EPA/day	PERIOD IN WEEKS	change in %	
			CHOL	TG
Singer et al. (44)	2	2	- 9	-28
Bronsgeest et al. (45)	3	4	0	-39
Lorentz et al. (46)	4	4	- 4	-20
Saynor et al. (47)	4	104	- 5	-41
von Schacky et al. (48)	4	4	- 2	-33
Siess et al. (49)	6	3	- 4	-32
Dyerberg et al. (50)	7	4	-13	-44

Table 2
Effects of dietary n-3 PUFA's on plasma cholesterol (CHOL) and triglyceride (TG) levels in controlled studies in normolipidemic individuals.

REFERENCE	grams EPA/day	PERIOD IN WEEKS	CONTROL	% change from control	
				CHOL	TG
Sanders et al. (51)	2	2	MAIZE+OLIVE	0	-29
Mortensen et al. (52)	2	4	CORN+OLIVE	0	-47
van Houwelingen et al. (53)	2	6	MEAT	0	+ 2
Rogers et al. (54)	2	6	OLIVE	- 5	-54
Nagakawa et al. (55)	2	4	PLACEBO	-15	-25
Fehily et al. (56)	2	12	MEAT+LEAN FISH	+ 1	- 7
Lossoncy et al. (57)	3	3	CHEESE	- 8	-33
Sanders et al. (58)	3	3	LINSEED	- 5	-32
Harris et al. (59)	3	4	COCOA+PEANUT+ EGG	-17	-40*
Illingworth et al. (60)	6	4	COCOA+PEANUT	-24	-43*
Harris et al. (61)	11	4	SAFFLOWER+CORN	-2	-33*

* = control diet balanced for cholesterol content

In the studies of Bang and Dyerberg, the Eskimos consumed 80 mg EPA/kg/day, or 6 g EPA/day [15]. This is probably the maximal dose that can be consumed by a fish diet in humans. In the following overview on the effects of n-3 PUFA's on plasma-lipids we have included only studies with doses of about 2 g EPA/day or more. We define controlled studies as those in which the effects of a fish (oil) diet were compared to those of a control diet by the same or by a parallel group.

Uncontrolled studies reported maximal decreases in plasma cholesterol of normolipidemic individuals up to 13% (table 1), but in controlled studies decreases up to 24% have been described (table 2). From the data of the two tables it appears that there is a dose dependent hypocholesterolemic effect of fish oil. Furthermore, it is clear that when the cholesterol contents of the diets are matched, the cholesterol lowering effects of the n-3 PUFA's are unmasked [59-61]. Many fish diets and fish oil supplements contain high levels of cholesterol. The purified fish oil concentrates, containing low amounts of cholesterol, are more suitable for dietary supplementation.

In hyperlipidemic patients the cholesterol lowering effects of fish oil depend on the type of hyperlipidemia. In an uncontrolled study in type IIA patients there was no effect on plasma cholesterol [64], but in mixed groups the decreases where most marked (24%) in type IV and V hyperlipidemia [62], see table 3.

In the controlled studies (Table 4) in hyperlipidemic patients the effects were maximal (-41%) in type V [66], intermediate (-12%) in type IV and carbohydrate induced hypertriglyceridemic individuals [67, 68], and minimal in the mixed groups and type IIB [66, 69], but in only one study [69] the cholesterol content of the diets was balanced. There seems to be no obvious dose-dependent effect in hyperlipidemic individuals. From these data can be concluded that plasmacholesterol is most affected in individuals with increased plasma triglyceride levels (phenotype IIB, IV and V).

Table 3
Effects of dietary n-3 PUFA's on plasma cholesterol (CHOL) and triglyceride (TG) levels in uncontrolled studies in hyperlipidemic individuals.

REFERENCE	TYPE	grams EPA/day	PERIOD IN WEEKS	change in %	
				CHOL	TG
Singer et al. (62)	IV+V	1	2	-24	-72
Hamazaki et al. (63)	IIA+IIB+IV	2	13	-10	-31
Singer et al. (62)	IV+V	2.2	2	-19	-66
Brox et al. (64)	IIA	2.4	6	- 1	-17
Hay et al. (65)	II+IV	3.5	3	0	-26

Table 4
Effects of dietary n-3 PUFA's on plasma cholesterol (CHOL) and triglyceride (TG) levels in controlled studies in hyperlipidemic individuals.

REFERENCE	TYPE	grams EPA/day	PERIOD IN WEEKS	CONTROL	% change from control	
					CHOL	TG
Simons et al. (66)	IIA+IIB+ IV+V	1.1	12	OLIVE	- 1	-33
Simons et al. (66)	IIA+IIB+ IV+V	2.0	12	OLIVE	- 5	-58
Sanders et al. (67)	IV	2.7	4	CORN+OLIVE	-12	-36
Simons et al. (66)	V	2.9	12	OLIVE	-41	-60
Harris et al. (68)	CHO-induced	9.0	1	CONTROL	-11	-62
Phillipson et al. (69)	IIB	9 - 17	4	SAFFLOWER	- 2	-47*
Phillipson et al. (69)	V	9 - 17	4	SAFFLOWER	-30	-41*

* = control diet balanced for cholesterol content

In a few studies fish oil has been shown to inhibit the increase in plasmacholesterol during dietary cholesterol loading [70, 71], but these results are controversial [72-74]. In these last studies the atherogenic diet contained, however, also considerable amounts of saturated fat [72-74]. Evenso Eskimos consume high amounts of cholesterol without real disturbances of their plasma lipid levels, probably due to the action of n-3 fatty acids [17], but racial differences should be considered too. For instance, black workers on a chicken farm showed no elevated plasmacholesterol despite their high cholesterol intake [75]. The blacks consumed large amounts of chicken eggs and had a high PUFA intake, but the dietary fatty acid composition was not given [75]. It is quite feasable that these eggs were also rich in n-3 PUFA's because the chickens were fed fish flour. From this study we can therefore not conclude that racial influences may protect against cholesterol loading.

There is some evidence for an increased excretion of bile in the faeces during fish oil consumption [76, 77]. A reduction in LDL synthesis has also been reported [60, 78]. In human hepatoma cells incubation with eicosapentaenoic acid inhibited the binding of LDL while the binding of remnants was not affected [79]. However, the effects on the different cholesterol carrying lipoproteins are inconsistent and further research on the lipoprotein metabolism in a well-controlled experimental set-up is needed.

1.2.2 The effects of dietary n-3 fatty acids on plasma triglyceride

N-3 PUFA's lower the synthesis of the VLDL particle by the suppression of hepatic triglyceride synthesis as well as by the apoprotein B100 synthesis [34, 66, 68, 78-83]. An increased VLDL clearance has also been reported [84], but not generally accepted. Postprandial hypertriglyceridemia is decreased significantly, so the VLDL and chylomicron metabolism seems to be affected [47, 68].

In the uncontrolled analysis in normolipidemic individuals (*Table 1*) a maximal decrease in plasma triglyceride level of 44% was found, while in controlled studies (*Table 2*) the decrease was maximally 54%. From these data it seems that the maximal hypotriglyceridemic effect of fish oil is already reached at a dose of about 2 g EPA/day and within 2 weeks [44, 51].

In the uncontrolled studies in type IV and V hyperlipidemic patients a decline in plasma triglyceride of 72% was found, whereas the largest decreases were 31% and 17% in the mixed and type II A group, respectively (*Table 3*). In the controlled studies (*Table 4*) the maximal decrease was 62%. There was no dose dependency for doses higher than 2 g EPA/day and the maximal effect was already observed after 2 weeks [62, 68]. Thus, the overall effects depend on whether the individual is normo- or hyperlipidemic, on the subtype of hyperlipidemia, and on the dose of n-3 PUFA's taken.

1.2.3 The effects of dietary n-3 fatty acids on membrane lipid composition

The fatty acid composition of fat tissue or membrane phospholipids partially reflects the dietary fatty acid intake [28, 85-87]. The half life of fatty acids in adipose tissue is about 2 years or more [88, 89], whereas the turnover in cellular membranes is in the order of magnitude of days [87]. The fatty acid composition of phospholipids may affect membrane function [28, 87]. There is also a competition between the different unsaturated fatty acids for incorporation in membrane phospholipids. The PUFA's compete for the 2-position in membrane phospholipids [20]. Phospholipase A_2 splits off these PUFA's, and AA or EPA are used for the prostanoid production. Therefore, variation in the nature of dietary PUFA's can have an impact on prostaglandin and leukotriene production.

Dyerberg has suggested that n-3 PUFA's possess anti-atheromatous actions [90], and related these actions with the inhibitory effects of n-3 PUFA's on platelet activity [91], see table 5. Several investigators have shown that ischemic heart disease is correlated with a low EPA and DHA content in platelet phospholipids [92-94]. In Japanese and Indians living on Vancouver Island the effects of fish consumption on plasma fatty acid composition was demonstrated [9, 95], see also table 6. Reference data from European immigrants in Minnesota are also given in table 6 [96].

Table 5
Polyunsaturated fatty acid composition of platelet phospholipids.

REFERENCE	POPULATION	mole % of fatty acids				
		18:2 n-6	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Dyerberg et al. (91)	eskimos	3.9	8.5	8.0	3.3	5.8
	danes	8.2	22.1	0.5	1.0	1.5
Prisco et al. (92)	healthy volunteers	5.9	27.1	0.7	2.1	2.3
	angina pectoris	5.6	27.1	0.6	2.1	2.2
	myocardial infarction	5.3	25.6	0.6	2.0	2.2
Wood et al. (93)	controls	4.2	26.8	0.4	1.5	1.7
	active angina	3.8	24.3	0.3	1.3	1.5
Kristensen et al. (94)	angina pectoris	13.9	14.6	0.3	-	0.7

Table 6
Polyunsaturated fatty acid composition of plasma lipids

REFERENCE	POPULATION	mole % of fatty acids				
		18:2 n-6	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Hirai et al. (9)	japanese fishermen	27.7	6.8	3.8	-	7.1
	japanese farmers	30.4	5.8	2.3	-	4.5
Hamazaki et al. (63)	japanese hemodialysis patients	31.5	3.8	1.2	-	1.9
Bates et al. (95)	europeans	21.4	11.4	1.0	0.9	3.5
	mixed race	30.7	7.5	1.4	0.6	3.0
	indians	28.8	4.8	0.5	0.5	2.8
	fish eating indians	24.2	4.7	2.6	0.8	6.0
Holman et al. (96)	european immigrants	19.6	11.7	1.1	0.7	2.2

From these data it appears that not the decrease in AA content is important [94], but the increased EPA and DHA contents are important for the protection against ischemic heart disease. Dyerberg has proposed that the presence of EPA and the low content of AA leads to a decreased production of thromboxane A_2 and synthesis of inactive thromboxane A_3 [17]. However, Goodnight and Hornstra suggest that cyclo-oxygenase is inhibited by the poorly metabolized substrate EPA, resulting in a decreased thromboxane production [20, 97]. It has also been demonstrated that only

Table 7
Polyunsaturated fatty acid composition of platelet lipids (TL) or phosphocholine (PC) after fish(oil) consumption in uncontrolled trials.

REFERENCE	LIPID	grams EPA/ day	PERIOD IN WEEKS	18:2 n-6	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Driss et al. (104)	PC	0.15	4	13.3	16.8	0.8	0.8	1.4
			BEFORE	12.3	16.1	0.9	1.0	2.1
Hirai et al. (105)	TL	1.4	4	-	36.3	4.7*	-	3.0
			BEFORE	-	39.1	1.8	-	3.1
Sanders et al. (58)	TL	1.8	3	7.4	22.7*	4.2*	3.3*	4.3
		0.9	3	8.2	23.0*	3.2*	2.6*	3.3
		0.5	3	8.4	25.2*	2.3*	2.6*	3.1
			BEFORE	7.8	28.9	0.9	2.0	2.4
Ahmed et al. (106)	PC	1.8	2	7.9*	9.6*	2.9*	1.1*	1.8*
			BEFORE	10.2	14.1	0.3	0.5	0.7
Sanders et al. (107)	TL	1.8	6	7.2	24.4*	3.2*	3.2	4.6*
			BEFORE	7.4	28.8	0.6	3.1	2.6
Sanders et al. (108)	TL	1.8	6	7.2	24.4*	3.2*	3.2	4.6*
			BEFORE	7.3	28.8	0.6	3.0	2.6
Thorngren et al. (109)	PC	2.5	6	6.2*	15.6*	2.0*	1.1*	2.5*
			BEFORE	7.0	18.3	0.6	0.9	1.5
Thorngren et al. (110)	PC	2.5	11	5.9*	16.9	1.6*	1.2*	2.7*
			BEFORE	7.1	18.3	0.4	0.9	1.7
Kristensen et al. (94)	TL	2.7	1	12.8	11.8*	3.9*	-	2.6*
			BEFORE	13.7	14.8	0.3	-	0.9
von	TL	3.8	4	6.0	20.0	4.2*	-	5.4*
Schacky et al. (48)		1.9	4	6.0	23.0	2.5*	-	4.0*
		0.9	4	6.0	25.0	2.2*	-	3.5*
			BEFORE	6.0	26.0	0.2	-	2.6
Lorenz et al. (46)	TL	4.4	4	6.4*	14.2*	4.5*	-	5.2*
			BEFORE	7.8	19.0	0.3	-	2.0
Sanders et al. (51)	TL	9.0	1	3.0*	15.0*	5.1*	1.2*	6.0*
			BEFORE	6.4	25.5	1.0	1.0	1.5

* = $p < 0.05$ versus before

a small amount of thromboxane A_3 is produced after fish oil consumption [98, 99]. In vitro studies have shown that n-3 PUFA's are rapidly incorporated in various types of cells [100-102] and inhibit the prostanoid production [28, 100-102]. It was shown that there is competition between AA and EPA for cyclo-oxygenase [102]. For an review of the effects of n-3 PUFA's on myocardial function see chapter 3.

Regarding human dietary studies on platelet phospholipid fatty acid composition, it is shown that incorporation of EPA is not detectable for doses of less than 0.5 g EPA/day [103, 104]. The incorporation of n-3 PUFA's in the platelet membrane in uncontrolled trials is clearly dose dependent (table 7). The maximal EPA and DHA contents are 4.5% and 5.2%, respectively.

Table 8
Polyunsaturated fatty acid composition of platelet lipids (TL) or phosphocholine (PC) after fish(oil) consumption in controlled trials.

REFERENCE			LIPID	grams EPA/day CONTROL	PERIOD IN WEEKS	18:2 n-6	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Brox	et al.	(111)	PC	2.0	6	8.6*	11.2*	2.4*	1.1	2.4*
				CORN	6	10.5	13.8	0.7	0.9	1.5
Nagakawa	et al.	(55)	TL	2.0	4	-	16.8	4.0*	-	-
				PLACEBO	2	-	15.0	2.4	-	-
Brox	et al.	(64)	TL	2.4	6	-	7.5*	2.7*	-	-
				CONTROL	6	-	10.0	1.3	-	-
Kristensen	et al.	(94)	TL	2.7	12	12.8	11.8	3.9*	-	2.6*
				CORN	16	13.3	13.7	0.2	-	0.8
Sanders	et al.	(58)	TL	3.0	2	7.0	23.0*	4.1*	-	4.0*
				LINSEED	2	7.6	30.9	1.2	-	2.9
Sanders	et al.	(112)	TL	3.5	2	-	-	4.1*	-	3.9*
				LINSEED	2	-	-	1.2	-	2.9
Goodnight	et al.	(113)	TL	5.0	4	4.0*	20.2*	6.1*	-	3.7*
				AMERICAN DIET	4	7.5	27.6	0.1	-	1.1

* = $p < 0.05$ versus control

The controlled studies on platelet fatty acid composition (*Table 8*) show maximal contents EPA and DHA of 6.1% and 4.0%, respectively. When we compare these data with those found in Eskimos (*Table 5*: 8.0% and 5.8%, respectively), it can be concluded that fish oil supplementation to patients yields roughly the same changes in PUFA composition of platelets.

1.2.4 The effects of dietary n-3 fatty acids on prostaglandin synthesis

The prostaglandins (PG's) can be synthesized from different PUFA's: PG_1-series (g-LNA), PG_2-series (AA) and PG_3-series (EPA). The synthesis of PG's is dependent on the local production of the precursor fatty acids: g-LNA, AA and EPA. These precursor fatty acids are incorporated preferentially into the membrane phospholipids at the 2-position and can be mobilized by phospholipase A_2. The various PUFA's compete for cyclo-oxygenase, the key enzyme of PG synthesis (*Figure 2*). For the platelet function thromboxane (TXA) and prostacyclin (PGI) are of relevance. The PG's are very labile substances and therefore act locally. The availability of the various precursor fatty acids depends on the dietary fatty acids, that influences the PUFA composition of the membrane phospholipids.

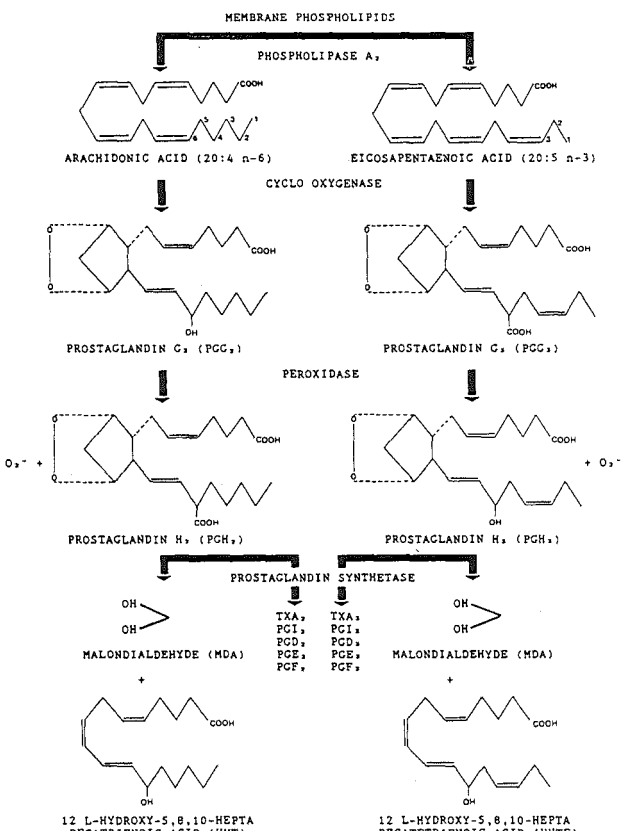

Figure 2. Prostaglandin synthesis of the 2 and 3-series.

The biological role of the 1-series of the PG's is not clear at present. EPA is a poor substrate for cyclo-oxygenase so only minor amounts of the 3-series are formed after fish (oil) consumption [20, 97-99, 114]. However, after long term administration of fish oil (3.8 g EPA/day) almost 30% of the total PGI production is of the 3-series [48].

TXA_3 has only a weak proaggregatory action [90, 115], while PGI_3 is a potent antiaggregator and vasodilator [116, 117]. The TXA production is reduced [46, 48-50, 103, 109] or unchanged [105] after fish oil consumption. Also in controlled studies the TXA production was decreased [51, 64, 104, 111] after fish (oil) consumption. Several factors could explain this effect:

1. Phospholipid AA content is decreased, because of the exchange for n-3 PUFA's [48].
2. After platelet stimulation less AA is released in the presence of n-3 PUFA's, probably by an inhibition of phospholipase A_2 [118].
3. AA and EPA compete for cyclo-oxygenase [20, 97, 117, 120].

The unstable product TXA and its stable metabolites are shown in figure 3. The effects of fish oil on TXA_2 generation have been described after doses as low as 0.15 g EPA/day [104]. This is, however, difficult to comprehend because of the low incorporation of EPA into the membrane phospholipids at such a low dose [104].

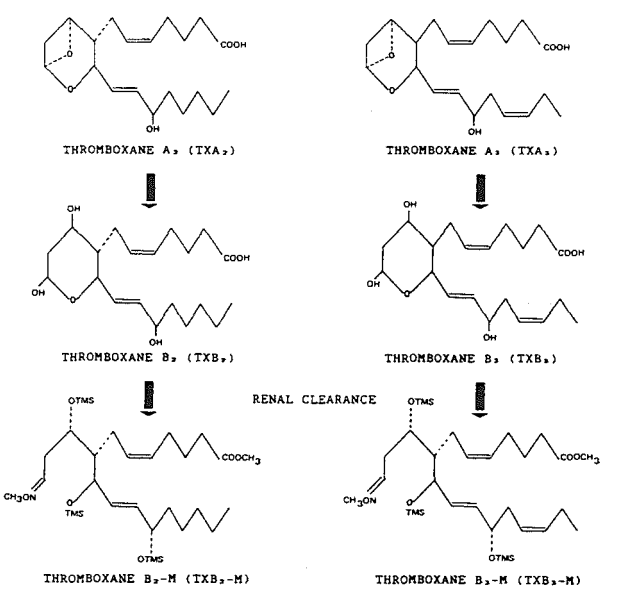

Figure 3. Thromboxane metabolism.

The PGI production takes place mainly in the endothelium although monocytes produce some too [121]. It is possible to estimate the overall PGI synthesis by analysis of the urine. However, this analysis is very complicated and therefore acurate analysis has been carried out in only a few studies [99, 114, 122]. The metabolites of the labile PGI are shown in figure 4. The PGI production seems to be unaffected [48, 111] or moderately increased [114] after fish (oil) consumption. It seems that there is no competition between AA and EPA for the PGI production, as the PGI_2 production is not affected and the PGI_3 production is added to the total PGI production. Like PGI_2, PGI_3 has significant anti-aggregatory and vasodilatory properties [116, 117]. This leads to a modification of the TXA/PGI ratio resulting in less platelet aggregation [48, 123].

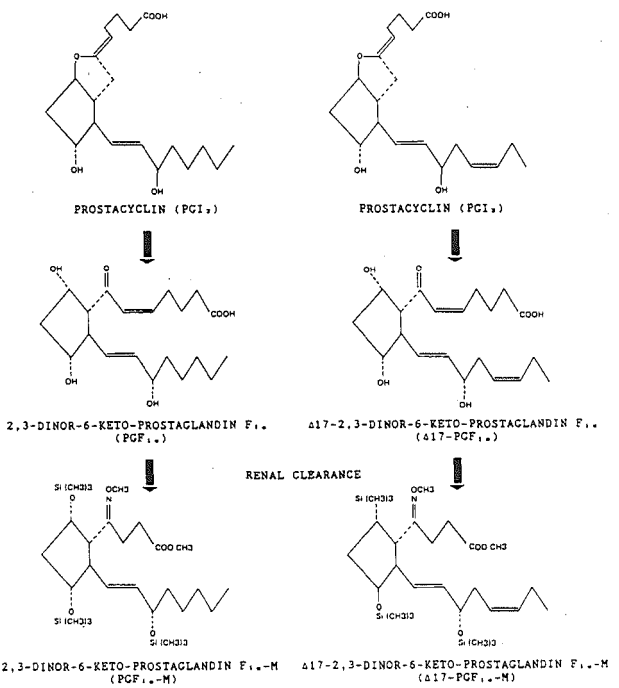

Figure 4. Prostacyclin metabolism.

The effects of fish (oil) consumption on other PG's have not been fully elucidated. In Eskimo's the urinary excretion of PGE and PGF_{2alfa} metabolites were not different [124]. Also in a volunteer study no effect of dietary cod liver oil supplements was found on urinary PGE and PGF_{1alfa} metabolites [46]. In animal experiments different effects on PGD_2, PGE_2 and PGF_{2alfa} have been shown while also production of the 3-series has been reported.

It must be noted that the PG plasmalevels can be influenced by the bloodsampling methods. Therefore the 24 hour urinary excretion of the PG metabolites is a more reliable technique which reflects the in vivo situation.

1.2.5 The effects of dietary n-3 fatty acids on platelet function

Platelets play a key role in the development of mural thrombosis, intimal proliferation and atherosclerosis as they adhere to damaged endothelium or denudated blood vessels [125]. The platelets are activated by the adherence to the vessel wall which leads to TXA production and release of several compounds, such as adenosine diphosphate, serotonin, adrenalin, beta-thromboglobulin, platelet factor 4, leukotrienes and growth factors. Subsequently a thrombus is formed which is then stabilized by fibrin. Smooth muscle cells will proliferate and migrate into the thrombus after which it is transformed into an atherosclerotic lesion. It has been shown that ischemic heart disease is associated with increased thrombotic tendency of blood [126, 127].

Table 9
Effects of fish(oil) consumption on platelet aggregation induced by low doses of collagen (COLL), adenosine diphosphate (ADP), adrenalin (ADR), thrombin (THR) and arachidonic acid (AA) in platelet rich plasma in uncontrolled human trials.

REFERENCE	grams EPA/day	PERIOD IN WEEKS	COLL	ADP	ADR	THR	AA
Velando et al. (103)	0.05	8	-				=
Hirai et al. (105)	1.4	4	-	-			
Sanders et al. (58)	1.7	3	=				
Ahmed et al. (106)	1.8	2				-	
Sanders et al. (108)	1.8	6		+			
Nagakawa et al. (55)	2	4	-	-	-		
Bradlow et al. (128)	1-4	1-3	-	-	-		-
Thorngren et al. (109)	2-3	6	-	-			
Thorngren et al. (110)	2-3	11	-	-			
Kristensen et al. (94)	2.7	1	-	=	-		
Lorenz et al. (46)	3.5	4	-	-			
Terano et al. (129)	3.6	4	-	-	-		
von Schacky et al. (48)	3.8	4	-				
Siess et al. (49)	6	3	-	-	=		=
Dyerberg et al. (50)	6.3	4	-	-			

= : unchanged; - : decreased; + : increased.

Table 10
Effects of fish(oil) consumption on platelet aggregation induced by low doses of collagen (COLL), adenosine diphosphate (ADP), adrenalin (ADR), thrombin (THR) and arachidonic acid (AA) in platelet rich plasma in controlled human trials.

REFERENCE	grams EPA/day	PERIOD IN WEEKS	CONTROL	COLL	ADP	ADR	THR	AA
Driss et al. (104)	0.15	4	PLACEBO	-	-	-		
Sanders et al. (51)	1.7	2	OLIVE+MAIZE	=	=			
Mortensen et al. (52)	2	4	OLIVE+CORN	=	=			
Nagakawa et al. (55)	2	4	PLACEBO	-	-	-		
Brox et al. (111)	2	6	CORN	=				=
Brox et al. (64)	2.4	6	CONTROL	=			=	
von Schacky et al. (48)	3.8	4	LOW FISH	-				
Goodnight et al. (113)	5	4	AMERICAN	=	-	=	=	=

: unchanged; - : decreased.

Tables 9 and 10 show the effects of fish oil consumption on platelet aggregation induced by different stimuli in platelet rich plasma. In nearly all uncontrolled studies platelet aggregation is inhibited by fish (oil) consumption, except for AA induced aggregation. This is in agreement with the competition of AA and EPA for cyclo-oxygenase [20, 97, 117-120].

The effects of fish (oil) consumption on platelet aggregation in the controlled studies is less impressive, because the vegetable oils used as control also decrease the platelet aggregation [51, 52, 64, 111].

The above described studies on platelet function were performed in platelet rich plasma. Thus, prior to aggregation measurements the isolation procedures must take place, which may interfere with the outcome of the measurements. Therefore, the assessment of aggregation in whole blood is preferred. Two whole blood methods have been developed. One method is based on the adherence of platelets to a glass column [130]. With this method platelet reactivity was found to be decreased after n-3 fatty acid consumption [131, 132]. The second method is based on changes in the impedance of blood during platelet aggegration [133, 134]. However, no data on the effects of fish oil on this elegant method are available.

The in vitro studies of platelet function do not necessarily reflect the in vivo platelet activity. Techniques which better reflect platelet function in vivo should become available. The platelet survival time measured by ^{111}Indium labelling was increased after fish oil administration [65, 129]. The platelet specific proteins beta-thromboglobulin (BTG) and platelet factor 4 (PF_4) plasma levels are indicators of the in vivo platelet activity. The BTG plasma levels were shown to decrease [65, 122]

or to be unaffected [64] after fish oil consumption. The PF_4 plasma levels were not affected by the fish oil diets [122], so from the behaviour of the concentrations of this protein one would conclude that fish oil has no effect on platelet activity. The blood sampling method does not exclude platelet activation sothat the aforementioned data on platelet specific proteins should be considered with care. Therefore, the urinary excretion of these compounds is a more reliable indicator for the platelet activity in vivo.

1.2.6 The effects of dietary n-3 fatty acids on hemorheology

Dietary n-3 PUFA's are incorporated into the erythrocyt membrane phospholipids, probably during the erythrocyte development [48]. These changes in membrane phospholipid composition lead to increased erythrocyte deformability [129, 135, 136], and consequently to a decrease of whole blood viscosity [53, 129, 135-140]. However, in a few studies blood viscosity was not affected [63, 66]. It is tempting to attribute the enhanced erythrocyte deformability to an increased membrane fluidity. However, compensatory mechanisms in membrane composition with respect to cholesterol, phospholipid and protein content may occur sothat in some cases the fluidity is not changed significantly after fish oil consumption [87, 138].

1.2.7 The effects of dietary n-3 fatty acids on hemostasis

In traditionally-living Greenland Eskimos the tendency to bleeding is increased and bleeding times are significantly prolonged [91]. In a Nordic historical document published in 1450 it was mentioned that when the Eskimos were injured the "blood almost did not stop flowing" [141]. Frequent massive nose bleedings have also been reported [142].

In nearly all uncontrolled studies the cutaneous bleeding time was increased after fish (oil) consumption [46, 47, 50, 58, 106, 108-110, 122], except for one study [55]. In half of the controlled studies the bleeding time was prolonged after fish (oil) consumption [52, 53, 59, 113, 143], whereas in the other studies it was not significantly different from the control diet [54, 64, 66, 94, 111].

Following endothelial injury thrombin is released, which activates blood platelets [144]. The activated platelets then stimulate blood coagulation. The blood coagulation is controlled by a number of natural anticoagulants like antithrombin III (AT III). Finally, the clot can still be dissolved by fibrinolysis. In Greenland Eskimos the prothrombin time (PT) and the activated partial thromboplastin time (APTT) are not different from Danes [91], yet the Eskimos had a higher fibrinogen content and AT III activity [91, 145]. In most human fish (oil) trials PT was not affected [55, 110, 146], but in one study the prothrombin time was increased [129]. APTT was not affected in any of the dietary studies [52, 55, 110, 129, 146]. The fibrinogen content was unchanged [52, 55, 66, 108, 110, 146]. The AT III activity was increased [52],

unchanged [145, 146] or decreased [108] in the human fish (oil) trials. Two studies on the effect of dietary fish oil on fibrinolysis have been published. In the first study using the lysis time of a diluted fibrin clot, no effect was found [108]. In the second study an increased fibrinolytic activity was found with enhanced levels of tissue plasminogen activator and reduced levels of inhibitors of plasminogen activator [147].

1.2.8 The effects of dietary n-3 fatty acids on arterial blood pressure

From animal experiments is known that diets rich in saturated fatty acids are associated with increased blood pressure [148]. This increased blood pressure can be lowered by increasing the P/S ratio and LA content of the diet [148]. In man a negative relationship has been demonstrated between adipose tissue LA content and arterial blood pressure [149, 150].

The role of dietary n-3 PUFA's on arterial blood pressure is yet not clear. The blood pressure in Eskimos is not different from that of Danes (J. Dyerberg, personal communication). However, Japanese with high fish consumption have a lower mean blood pressure than the average Japanese [10].

The majority of evidence suggests that systolic arterial blood pressure, in both normotensive as well as hypertensive individuals, is more sensitive to modulation by fish oil than diastolic arterial blood pressure [44, 52, 54, 62, 63, 105, 108, 151-153].

1.2.9 The effects of dietary n-3 fatty acids on thrombosis, atherosclerosis and ischemia

In patients undergoing coronary angioplasty the incidence of restenosis was lower in patients which had taken fish oil supplements [154-156], but fish oil was ineffective when supplementation was started only shortly before the angioplasty [157]. It has been suggested that fish oil exerts this beneficial action by reducing thromboembolic events and intimal proliferation. In studies on rats with an aortic loop model fish oil lowered the tendency to thrombosis [98, 158].

Arntzenius et al. provided angiographic evidence that the progression of atherosclerosis can be retarded by a diet with an increased P/S ratio and at least one fish meal per week [159], probably by the diet-induced changes in lipoprotein metabolism. However, the amount of dietary n-3 PUFA's was too low to assume that these could have contributed to the changes in lipoprotein metabolism. It must be noted that this study lacked a proper control group. In a controlled trial with dietary restrictions and lipid lowering drugs regression of coronary artery atherosclerosis has been demonstrated [160]. This study awaits further coformation because in man regression of atherosclerosis is difficult to assess when only angiographic evidence is used [160]. Fish oil diets retarded the development of atherosclerosis in the pig [74, 161], dog [162] and nonhuman primate [163], although in one study the athero-

sclerotic process in the pig was not affected by the fish oil supplement [164]. In atherosclerotic rabbits, fish oil has been shown to accelerate the development of atherosclerosis [97].

Epidemiological data indicate a protective effect of fish consumption against ischemic heart disease [11-13]. Nelson's first prospective study in patients with coronary heart disease showed a protective effect of a diet rich in polyunsaturated fatty acids and fish [33]. In patients suffering from angina pectoris fish oil lowered the incidence of anginal attacks [47, 165], but in another study fish oil was not superior to a vegetable oil supplement [95]. In peripheral vascular disease fish oil showed several beneficial effects although the studies were too short lasting to have an effect on the clinical symptoms [122, 147]. Dietary supplementation of fish oil exerted a protective effect on experimental cerebral infarction in cats [166] and myocardial infarction in dogs [167]. The effects of fish oil diets on the incidence of ischemia-induced arrhythmias in rats are still controversial [168, 169].

1.3 Adverse effects of dietary n-3 fatty acids

The most common unappreciated effects of fish oil supplement intake are the smell and taste of the oils. However, with a capsulated supplement this effect is bypassed. The intake of high amounts of fish oil concentrates can lead to gastro-intestinal discomfort like nausea, diarrhea and obstipation. Because of environmental pollution, fish in coastal waters may have accumulated mercury and chlorinated carbons. Consumption of these fish twice a week has not yet lead to any harm [170]. In the refined fish oil concentrates these toxins are removed.

Fish oil has been used for decennia as a vitamin A and D supplement. Intake of too high amounts of vitamin A and D can lead to hypervitaminosis. Therefore, the intake of vitamin A and D free fish oil is advised. As mentioned before, the purified fish oil concentrates are also cholesterol free.

High n-3 PUFA intake can lead to anti-oxidant deficiency. The products of chemical reactions and free-radical induced auto-oxidation can cause membrane damage. Lipid peroxidation and vitamin E deficiency can lead to yellow fat disease and mulberry heart disease [171-173]. Fish protect themselves from body fat peroxidation by storage of selenium and vitamin E. Fish oil concentrates should therefore be supplemented with natural antioxidants like vitamin E and C, and selenium.

Some scepticism about the use of fish oil extracts also stems from the fact that the extracts contain rather high levels of long chain monoenes like cetoleic acid (22:1 n-11), an isomer of erucic acid (22:1 n-9), and gadoleic acid (20:1 n-9). Erucic acid has been shown to cause transient myocardial lipidosis and fibrosis in experimental animal studies [174, 175]. Nowadays purified fish oil extracts are available that are low in cetoleic and gadoleic acid. The inhibitory effect on blood coagulation which is beneficial against ischemic heart disease can lead to increased bleeding. In

Eskimos the increased incidence of apoplexy is worrisome [6, 7], but in other fish consuming communities the incidence of cerebrovascular accidents is rather low [10, 176]. In the Western World the combination of anticoagulants and fish oil supplements should not be advised.

REFERENCES

1. Rabinowitch JM. Clinical and other observations on Canadian Eskimos in the Eastern Arctic. Can Med Assoc J 1937; 34: 343-8.
2. Stefansson O. Cancer: disease of civilization. Hill & Wang, New York, 1960.
3. Gottman AW. A report of 103 autopsies on Alaskan natives. AMA Arch Pathol 1960; 70: 117-24.
4. Sagild U, Littauer J, Sand Jespersen C, Andersen S. Epidemiological studies in Greenland 1962-1964: I Diabetes mellitus in Eskimos. Acta Med Scand 1966; 179: 29.
5. Sinclair HM. Nutrition and atherosclerosis. Symp Zool Soc London, 1968; 21: 275-88.
6. Arthaud B. Cause of death in 339 Alaskan natives as determined by autopsy. AMA Arch Pathol 1970; 90: 433-8.
7. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland. Acta Med Scand 1980; 208: 401-6.
8. Malaurie J. The last Izings of Thale. Jonathan Cope, London, 1982.
9. Hirai A, Hamazaki T, Terano T, Nishikawa T, Tamura Y, Kumagai, Sajiki J. Eicosapentaenoic acid and platelet function in Japanese. Lancet 1980; II: 1132-3.
10. Kagawa Y, Nishizawa M, Suzuki M, Miyataka T, Hamamoto T, Goto K, Montonaga E, Izumikawa H, Hirata H, Ebitara A. Eicosapolyenoic acid of serum lipids of Japanese islanders with low incidence of cardiovascular diseases. J Nutr Sci Vitaminol 1982; 28: 441-53.
11. Kromhout D, Bosschieter EB, de Lezenne Coulander C. The inverse relation between fish consumption and 20 year mortality from coronary heart disease. N Engl J Med 1985; 312: 1205-8.
12. Shekelle RB, Missell LV, Paul O, Shyrock AM, Stamler J. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985; 313: 820.
13. Norell SE, Ahlbom A, Feychting M, Pedersen NL. Fish consumption and mortality from coronary heart disease. Br Med J 1986; 293: 426.
14. Sinclair HM. Essential fatty acids and their relation to pyridoxine. Biochem Soc Symp 1952; 9: 80-99.
15. Bang HO, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. Am J Clin Nutr 1980; 33: 2657-61.
16. Sinclair HM. The importance of fish in the prevention of chronic degenerative diseases. In: Noelle H (ed). Nahrung aus dem Meer. Berlin, Springer Verlag, 1981; 201-10.
17. Dyerberg J, Bang HO. A hypothesis on the development of acute myocardial infarction in Greenlanders. Scand J Clin Lab Invest 1982; 42 (S161): 7-13.

18. Stansby ME. Nutritional properties of fish oils. World Rev Nutr Dietetics 1969; 11: 46-105.
19. Illingworth DR, Connor WE. Present status of polyunsaturated fats in the prevention of cardiovascular disease. In: Nutrition and Food Science (3), Santos W, Lopes M, Barbosa JJ, Chaves D (eds), New York, Plenum Press, 1980; 365-78.
20. Goodnight SH, Harris WS, Connor WE, Illingworth DR. Polyunsaturated fatty acids, hyperlipidemia and thrombosis. Arteriosclerosis 1982; 2: 87-113.
21. Sanders TAB. The importance of eicosapentaenoic and docosahexaenoic acids. In: Padley FB, Podmore J (eds). The role of fats in nutrition. Chichester, Ellis Horwood, 1985; 101-18.
22. Stansby ME. Medical effects of fish or fish oil in the diet. NWAFC Processed Report 1985; 17.
23. Herold PM, Kinsella JE. Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am J Clin Nutr 1986; 43: 566-98.
24. Norum KR, Drevon CA. Dietary n-3 fatty acids and cardiovascular diseases. Arteriosclerosis 1986; 6: 352-5.
25. Dyerberg J. Linoleate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr Rev 1986; 44: 125-34.
26. Lands WEM. Renewed questions about polyunsaturated fatty acids. Nutr Rev 1986; 44: 189-95.
27. Zeller FP, Spears C. Fish oil: effectiveness as a dietary supplement in the prevention of heart disease. Drug Intell Clin Pharm 1987; 21: 584-9.
28. Lamers JMJ, Hartog JM, Verdouw PD, Hulsmann WC. Dietary fatty acids and myocardial function. Basic Res Cardiol 1987; 82 (S1): 209-21.
29. Nestel PJ. Polyunsaturated fatty acids (n-3,n-6). Am J Clin Nutr 1987; 45: 1161-7.
30. Schacky von C. Prophylaxis of atherosclerosis with marine omega-3 fatty acids. A comprehensive strategy. Ann Int Med 1987; 107: 890-9.
31. Editorial. Fish oil. Lancet 1988; I: 1081-3.
32. Leaf A, Weber PC. Cardiovascular effects of n-3 fatty acids. N Engl J Med 1988; 318: 549-57.
33. Nelson AM. Diet therapy in coronary disease effects on mortality of high protein high seafood, fat controlled diet. Geriatrics 1972; 27: 103-16.
34. Nestel PJ, Connor WE, Reardon MF, Connor S, Wong S, Boston R. Suppression by diets rich in fish oil of very low density lipoprotein production in man. J Clin Invest 1984; 74: 82-9.
35. Nestel PJ. Dietary effects on alipoprotein metabolism.a In: Fidge NH, Nestel PJ (eds). Atherosclerosis VII. Amsterdam, Elsevier Science Publishers BV, 1986.
36. Groot PHE, Hartog JM, Scheek LM, Verdouw PD, Lamers JMJ. The effects of diets supplemented with lard fat or mackerel oil on plasma lipoprotein lipid concentrations in domestic swine (submitted).
37. Erkelens DW. Samenstelling van lipoproteinen. In: Havekes L, Krans HMJ, Vermeer BJ (eds). Hyperlipidemien. Leiden, Boerhaave Commissie voor post-academisch onderwijs in de geneeskunde, 1985; 9-19.

38. Havekes L. Metabolisme van lipoproteinen. In: Havekes L, Krans HMJ, Vermeer BJ, (eds). Hyperlipidemien. Leiden, Boerhaave commissie voor post-academisch onderwijs in de geneeskunde, 1985; 21-9.
39. Jansen H. De rol van lipoproteine en hepatische lipase. In: Havekes L, Krans HMJ, Vermeer BJ, (eds). Hyperlipidemieen. Leiden, Boerhaave commissie voor postacademisch onderwijs in de geneeskunde, 1985; 45-50.
40. Hulsmann WC. Pathofysiologie van het vet transport. In: Frenkel M, Bernards JA, van Gool J, Hulsmann WC, Stoelinga GBA, (eds). Pathofysiologie van de mens. Utrecht, Wetenschappelijke uitgeverij Bunger, 1982; 250-9.
41. Erkelens DW. Pathofysiologie van lipidenmetabolisme. In: Arntzenius AC (ed). Atherosclerose en hypertensie. Pathogenese, epidemiologie en preventie. Utrecht, Wetenschappelijke uitgeverij Bunger, 1985; 27-40.
42. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986; 232: 34-47.
43. Havekes L, Gevers Leuven JA, Frants RR. Erfelijke aspecten van het lipoproteine metabolisme. Hart Bull 1988; 19: 103-10.
44. Singer P, Wirth M, Voigt S, Richter-Heinrich E, Godicke W, Berger J, Naumann E, Listing J, Hartradt W, Taube C. Blood pressure- and lipid-lowering effect of mackerel and herring diet in patients with mild essential hypertension. Atherosclerosis 1985; 56: 223-35.
45. Bronsgeest-Schoute HC, van Gent CM, Luten JB, Ruiter A. The effect of various intakes of omega-3 fatty acids on the blood lipid composition in healthy human subjects. Am J Clin Nutr 1981; 34: 1752-7.
46. Lorenz R, Spengler U, Fischer S, Duhm J, Weber PC. Platelet function, thromboxane formation and blood pressure control during supplementation of the Western diet with cod liver oil. Circulation 1983; 67: 504-11.
47. Saynor R, Verel D, Gillott T. The long-term effect of dietary supplementation with fish lipid concentrate on serum lipids, bleeding time, platelets and angina. Atherosclerosis 1984; 50: 3-10.
48. Schacky von C, Fischer S, Weber PC. Long term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. J Clin Invest 1985; 76: 1626-31.
49. Siess W, Roth P, Scherer B, Kurzmann I, Bohlig B, Weber PC. Platelet-membrane fatty acids, platelet aggregation, and thrombaxane formation during a mackerel diet. Lancet 1980; I: 441-4.
50. Dyerberg J. Platelet-vessel wall interaction: influence of diet. Phil Trans R Soc London 1981; 294: 373-81.
51. Sanders TAB, Hochland MC. A comparison of the influence on plasmalipids and platelet function of supplements of omega-3 and omega-6 polyunsaturated fatty acids. Br J Nutr 1983; 50: 521-9.
52. Mortensen JZ, Schmidt EB, Nielsen AH, Dyerberg J. The effect of n-6 and n-3 polyunsaturated fatty acids on hemostasis, blood lipids and blood pressure. Thromb Haemostas 1983; 50: 543-6.
53. Houwelingen van R, Nordoy A, Beek van der E, Houtsmuller U, Metz de M, Hornstra G. Effect of a moderate fish intake on blood pressure, bleeding time, hematology, and clinical chemistry in healthy males. Am J Clin Nutr 1987: 46: 424-36.

54. Rogers S, James KS, Batland BK, Etherington MO, O'Brien JR, Jones JG. Effects of a fish oil supplement on serum lipids, blood pressure, bleeding time, haemostatic and rheological variables. A double blind randomised controlled trial in healthy volunteers. Atherosclerosis 1987; 63: 137-43.
55. Nagakawa Y, Orimo H, Harasawa M, Morita I, Yashiro K, Murota S. Effect of eicosapentaenoic acid on the platelet aggregation and composition of fatty acid in man. A double blind study. Atherosclerosis 1983; 47: 71-5.
56. Fehily AM, Barr ML, Philips KM, Deadman NM. The effect of fatty fish on plasma lipid and lipoprotein concentrations. Am J Clin Nutr 1983; 38: 349-51.
57. Lossoncy von TO, Ruiter A, Bronsgeest-Schoute HC, van Gent CM, Hermans RJJ. The effect of a fish diet on serum lipids in healthy human subjects. Am J Clin Nutr 1978; 31: 1340-6.
58. Sanders TAB, Roshani F. The influence of different types of omega-3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. Clin Sc 1983; 64: 91-9.
59. Harris WS, Connor WE. The effects of salmon oil upon plasma lipids, lipoproteins, and triglyceride clearance. Trans Assoc Am Physicians 1980; 93: 148-55.
60. Illingworth DR, Harris WC, Connor WE. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. Arteriosclerosis 1984; 4: 270-5.
61. Harris WS, Connor WE, McMurry MP. The comparative reductions of the plasma lipids and lipoproteins by dietary polyunsatured fats: salmon oil versus vegetable oils. Metabolism 1983; 32: 179-84.
62. Singer P, Wirth M, Berger I, Voigt S, Gerike U, Godicke W, Koberle U, Heine H. Influence on serum lipids, lipoproteins and blood pressure of mackerel and herring diet in patients with type IV and V hyperlipoproteinemia. Atherosclerosis 1985; 56: 111-8.
63. Hamazaki T, Nakazawa R, Tateno S, Shishido H, Isoda K, Hattori Y, Yoshida T, Fujita T, Yano S, Kumagai A. Effects of fish oil rich in eicosapentaenoic acid on serum lipid in hyperlipidemic hemodialysis patients. Kidney Int 1984: 26; 81-4.
64. Brox JH, Killie JE, Osterud B, Holme S, Nordoy A. Effects of cod liver oil on platelets and coagulation in familial hypecholesterolemia (type IIA). Acta Med Scand 1983; 213: 137-44.
65. Hay CRM, Durber AP, Saynor R. Effect of fish oil on platelet kinetics in patients with ischaemic heart disease. Lancet 1982; I: 1269-72.
66. Simons LA, Hickie JB, Balasubramaniam S. On the effects of dietary n-3 fatty acids (Maxepa) on plasma lipids and lipoproteins in patients with hyperlipidemia. Atherosclerosis 1985; 54: 75-88.
67. Sanders TAB, Sullivan DR, Reeve J, Thompson GR. Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. Arterioscleroris 1985; 5: 459-65.
68. Harris WS, Connor WE, Inkeles SB, Illingworth DR. Dietary omega-3 fatty acids prevent carbohydrate-induced hypertriglyceridemia. Metabolism 1984; 33:1016-9.

69. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR. Reduction of plasmalipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. N Engl J Med 1985; 312: 1210-6.
70. Nestel PJ. Fish oil attenuates the cholesterol induced rise in lipoprotein cholesterol. Am J Clin Nutr 1986; 43: 752-7.
71. Parks JS, Martin JA, Sonbert BL, Bullock BC. Alteration of high density liprotein subfractions of nonhuman primates fed fish oil diets. Selective lowering of HDL subfractions of intermediate size and density. Arteriosclerosis 1987; 7: 71-79.
72. Hill EG, Lundberg WO, Titus JC. Experimental atherosclerosis in swine. I. A comparison of menhaden oil supplements in tallow and coconut oil diets. Mayo Clin Proc 1971; 46: 613-20.
73. Hill EG, Lundberg WO, Titus JC. Experimental atherosclerosis in swine. II. Effects of methionine and menhaden oil on an atherogenic diet containing tallow and cholesterol. Mayo Clin Proc 1971; 46: 621-5.
74. Weiner BH, Ockene JS, Levine PH, Cuenoud HF, Fisher M, Johnson BF, Daoud AS, Jarmolych J, Hosmer D, Johnson MH, Natale A, Vaudreuil C, Hoogasian JJ. Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. N Engl J Med 1986; 315: 841-6.
75. Vorster HH, Silvis N, Venter CS, van Ryssen JJ, Huisman H, van Eeden TS, Walker ARP. Serum cholesterol, lipoproteins, and plasma coagulation factors in South African blacks on a high-egg but low-fat intake. Am J Clin Nutr 1987; 46: 52-7.
76. Connor WE, Lin DS, Harris WS. A comparison of dietary omega-3 fatty acids in humans: effects upon plasmalipids, lipoproteins and sterol balance. Arteriosclerosis 1981; 1: 363A.
77. Balasubramaniam S, Simons LA, Chang S, Hickie JB. Reduction in plasma cholesterol and increase in biliary cholesterol by a diet rich in n-3 fatty acids in the rat. J Lipid Res 1985; 26: 684-9.
78. Illingworth DR, Harris WS, Connor WE. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. Arteriosclerosis 1984; 4: 270-5.
79. Wong S, Nestel PJ. Eicosapentaenoic acid inhibits the secretion of triacylglycerol and of apoprotein B and the binding of LDL in Hep G2 cells. Atherosclerosis 1987; 64: 139-46.
80. Yang YT, Williams MA. Comparison of C_{18}-, C_{20}-, and C_{22}- unsaturated fatty acids in reducing fatty acid synthesis in isolated rat hepatocytes. Biochim Biophys Acta 1978; 531: 133-40.
81. Paul R, Ramesha CS, Ganguly J. On the mechanism of hypocholesterolemic effects of polyunsaturated lipids. Adv Lipid Res 1980; 17: 155-70.
82. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL. The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochim Biophys Acta 1984; 792: 103-9.
83. Wong S, Reardon M, Nestel PJ. Reduced triglycerides formation from long-chain polyenoic fatty acids in rat hepatocytes. Metabolism 1985; 34: 900-5.
84. Harris WS, Connor WE, Illingworth DR, Foster DM. The mechanism of the hypotriglyceridemic effect of dietary omega-3 fatty acids in man. Clin Res 1984; 32: 560A.

85. Dayton S, Hashimoto S, Pearce ML. Adipose tissue linoleic acid as a criterion of adherence to a modified diet. J Lipid Res 1967; 8: 508-10.
86. King ME, Stavens BW, Spector AA. Diet-induced changes in plasma-membrane fatty acid composition effect physical properties detected with a spin-label probe. Biochemistry 1977; 16: 5280-5.
87. Stubbs CD, Smith AD. The modification of mammalian polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984; 779: 89-137.
88. Hirsch J, Farquhar JW, Ahrens EN Jr, Peterson ML, Stoffel W. Studies in adipose tissue in man. A microtechnique for sampling and analysis. Am J Clin Nutr 1960; 8: 449-511.
89. Dayton S, Hashimoto S, Dixon WP, Pearce ML. Composition of lipids in human serum and adipose tissue during prolonged feeding of a diet high in unsaturated fat. J Lipid Res 1966; 7: 103-111.
90. Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet 1978; II: 117-9.
91. Dyerberg J, Bang HO. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet 1979; II: 433-5.
92. Prisco D, Rogasi PG, Matucci M, Abbata R, Gensini GF, Serneri GGN. Increased thromboxane A_2 generation and altered membrane fatty acid composition in platelets from patients with active angina pectoris. Thromb Res 1986; 44: 101-12.
93. Wood DA, Riemersma RA, Butler S, Thomson M, Macintyre C, Elton RA, Oliver MF. Linoleic and eicosapentaenoic acids in adipose tissue and platelets and risk of coronary heart disease. Lancet 1987; I: 177-83.
94. Kristensen SD, Schmidt EB, Andersen HR, Dyerberg J. Fish oil in angina pectoris. Atherosclerosis 1987; 64: 13-9.
95. Bates C, van Dam C. Plasma essential fatty acids in pure and mixed race American Indians on and off a diet exceptionally rich in salmon. Prostaglandin Leukotriene Med 1985; 17: 77-84.
96. Holman RT, Smythe L, Johnson S. Effect of sex and age on fatty acid composition of human serum lipids. Am J Clin Nutr 1979; 32: 2390-9.
97. Hornstra G, Haddeman E, Kloeze J, Verschuren PM. Dietary-fat-induced changes in the formation of prostanoids of the 2 and 3 series in relation to arterial thrombosis (rat) and atherosclerosis (rabbit). Adv Prostaglandin Thromboxane Leukotriene Res 1983; 12: 193-202.
98. Hornstra G, Haddeman F, Hoor ten F. Fish oils, protaglandins, and arterial thrombosis. Lancet 1979; II: 1080.
99. Fischer S, Weber PC. Thromboxane A_3 (TXA_3) is formed in human platelets after dietary eicosapentaenoic acid (C20:5 omega-3). Biochem Biophys Res Comm 1983; 116: 1091-9.
100. Morita I, Takahash R, Saito Y, Murota S. Effects of eicosapentaenoic acid on arachidonic acid metabolism in cultured vascular cells and platelets: species difference. Thromb Res 1983; 31: 211-7.
101. Morita I, Saito Y, Chang WC, Murota S. Effects of purified eicosapentaenoic acid on arachidonic acid metabolism in cultured murine aortic smooth muscle cells, vessel walls and platelets. Lipids 1983; 18: 42-9.

102. Spector AA, Kaduce TL, Figard PH, Norton KC, Hoak JC, Czervionke RL. Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. J Lipid Res 1983; 24: 1595-604.
103. Velando B, Lagarde M, Guichardant M, Dechavanne M, Beylot M, Sautot G, Berthezene F. Decrease of platelet activity after intake of small amounts of eicosapentaenoic acid in diabetics. Thromb Haemostas 1982; 48: 344.
104. Driss F, Vericel E, Lagarde M, Dechavanne M, Darcet Ph. Inhibition of platelet aggregation and thromboxane synthesis after intake of small amounts of eicosapentaenoic acid. Thromb Res 1984; 36: 389-96.
105. Hirai A, Terano T, Hamazaki T, Sajiki J, Kondo S, Ozawa A, Fujita T, Miyamoto T, Tamura Y, Kumagai A. The effects of the oral administration of fish oil concentrate on the release and the metabolism of [^{14}C] arachidonic acid and [^{14}C] eicosapentaenoic acid by human platelets. Thromb Res 1982; 28: 285-98.
106. Ahmed AA, Holub BJ. Alteration and recovery of bleeding times, platelet aggregation and fatty acid composition of composition of individual phospholipids in platelets of human subjects receiving a supplement of cod liver oil. Lipids 1984; 19: 617-24.
107. Sanders TAB, Naismith DJ, Haines AP, Vickers M. Cod liver oil, platelet fatty acids, and bleeding time. Lancet 1980; I:1189.
108. Sanders TAB, Vickers M, Haines AP. Effect on blood lipids and haemostasis of a supplement of cod-liver oil, rich in eicosapentaenoic and docosahexaenoic acids, in healthy young men. Clin Sci 1981; 61: 317-24.
109. Thorngren M, Shafi S, Born GVR. Delay in primary heamostasis produced by a fish diet without change in local thromboxane A_2. Br J Haematol 1984; 58: 567-78.
110. Thorngren M, Gustafson A. Effects of 11-week increase in dietary eicosapentaenoic acid on bleeding time, lipids, and platelet aggregation. Lancet 1981; II: 1190-3.
111. Brox JH, Killie JE, Gunnes S, Nordoy A. The effect of cod liver oil and corn oil on platelets and vessel wall in man. Thromb Haemostas 1981; 46: 604-11.
112. Sanders TAB, Younger KM. The effect of dietary supplements of omega-3 polyunsaturated fatty acids on the fatty acid composition of platelets and plasma choline phosphoglycerides. Br J Nutr 1981; 45: 613-6.
113. Goodnight SH, Harris WS, Connor WE. The effects of dietary omega-3 fatty acids on platelet composition and function in man: a prospective, controlled study. Blood 1981; 58: 880-5.
114. Fischer S, Weber PC. Prostaglandin I_3 is formed in vivo in man after dietary eicosapentaenoic acid. Nature 1984; 307: 165-8.
115. Siess W, Siegel FL, Lapetina EG. Dihomogammalinolenic acid, but not eicosapentaenoic acid, activates washed human platelets. Biochim Biophys Acta 1984; 801: 265-76.
116. Nidy EG, Johnson RA. Synthesis of prostaglandin I_3 (PGI_3). Tetrahedron Lett 1978; 27: 2375-8.
117. Needleman P, Raz A, Minkes MS, Ferrendelli JA, Sprecher H. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. Proc Natl Acad Sci USA 1979; 76: 944-8.

118. von Schacky C, Siess W, Fischer S, Weber PC. A comparative study of eicosapentaenoic acid metabolism by human platelets -8 in vivo and in vitro. J Lipid Res 1985; 26: 457-64.
119. Lands WEM, Le Tellier PR, Rome LH, Vanderhoek JY. Inhibition of prostaglandin biosynthesis. Adv Biosci 1973; 9: 15-28.
120. Strasser TH, Fischer S, Weber PC. Leukotriene B_5 is formed in human neutrophils after dietary supplementation with eicosapentaenoic acid. Proc Natl Acad Sci USA 1985; 82: 1540-3.
121. Lee TH, Hoover RL, Williams JD, Sperling RI, Ravalese J, Spur BW, Robinson DR, Gorey EJ, Lewis RA, Austen KF. Effects of dietary enrichment with eicosapentaenoic and docosahexaenoic aicds on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. N Engl J Med 1985; 312: 1217-24.
122. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 1983; 220: 568-75.
123. Moncada S, Salmon JA. Leukocytes and tissue injury: the use of eicosapentaenoic acid in the control of white cell activation. Wiener Klin Wochenschr 1986; 98: 104-106.
124. Mullane KM, Salmon JA, Kraemer R. Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. Fed Proc 1987; 46: 2422-33.
125. Ross R. The pathogenesis of atherosclerosis - an update. N Engl J Med 1986; 314: 488-500.
126. Taylor TG, Gibney MJ, Morgan JB. Haemostatic function and polyunsaturated fatty acids. Lancet 1979; II: 1378.
127. FitzGerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. N Engl J Med 1986; 315: 938-9.
128. Bradlow BA, Chetty N, van der Westhuyzen J, Mendelsohn D, Gibson JE. The effects of a mixed fish diet on platelet function, fatty acids and serum lipids. Thromb Res 1983; 29: 561-8.
129. Terano T, Hirai A, Hamazaki T, Kobayashi S, Fujita T, Tamura Y, Kumagai A. Effect of oral administration of highly purified eicosapentaenoic acid on platelet function, blood viscosity and red cell deformability in healthy human subjects. Atherosclerosis 1983; 46: 321-31.
130. Hellem AJ. The adhesiveness of human blood platelets in vitro. Scand J Clin Invest 1960; 12: S51.
131. Harris WS, Connor WE, Goodnight SH jr. Dietary fish oils, plasma lipids and platelets in man. Prog Lip Res 1981; 20: 75-9.
132. FitzGerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 1984; 310: 1065-8.
133. Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behaviour in blood. J Pharmacol Meth 1980; 3: 135-58.
134. Ingerman-Wojenski CM, Silver MJ. A quick method for screening platelet dysfunctions using the whole blood lumi-aggregometer. Thromb Haemostas 1984; 51: 154-6.
135. Cartwright IJ, Pockley AG, Galloway JH, Greaves M, Preston FE. The effects of dietary omega-3 polyunsaturated fatty acids on erythrocyte membrane

phospholipids, erythrocyte deformability and bloodviscosity in healthy volunteers. Atherosclerosis 1985; 55: 267-81.
136. Kobayashi S, Hamazaki T, Hirai A, Tamura Y, Kumagai A. Epidemiologic and clinical studies of the effect of eicosapentaenoic acid (EPA C20:5 n-3) on bloodviscosity. Clin Hemorheol 1985; 5: 493-505.
137. Woodcock BE, Smith E, Lambert WH, Morris Jones W, Galloway JH, Greaves M, Preston FE. Beneficial effect of fish oil on bloodviscosity in peripheral vascular disease. Br Med J 1984; 288: 592-4.
138. Popp-Snijders C. Omega-3 polyunsaturated fatty acids and erythrocyte membranes. A study in healthy and diabetic subjects. Academic thesis. Free University, Amsterdam, The Netherlands 1985.
139. Kobayashi S, Hirai A, Terano T, Hamazaki T, Tamura Y, Kumagai A. Reduction in bloodviscosity by eicosapentaenoic acid. Lancet 1981; II: 197.
140. Miller ME, Anagnostou AA, Ley B, Marshall P, Steiner M. Effect of fish oil concentrates on hemorheological and hemostatic aspects of diabetes mellitus: a preliminary study. Thromb Res 1987; 47: 201-14.
141. Gad F. Gronlands historie I. Kobnhavn 1967; 111.
142. Freuchen P. Om sunhed stilstanden blandt polareskimoerne. Ungeskr Laeg 1915; 77: 1089-1108.
143. Kremer JM, Bigauvette J, Michalek AV, Timchalk MA, Lininger L, Rynes RI, Huyck C, Zieminsky J, Bartholomew LE. Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. Lancet 1985; I: 184-7.
144. Hornstra G. Platelet-vessel wall interaction: role of blood- clotting. Phil Trans R Soc Lond B 1981; 294: 344-71.
145. Stoffersen E, Jorgensen KA, Dyerberg J. Antitrombin III and dietary intake of polyunsaturated fatty acids. Scand J Clin Lab Invest 1982; 42: 83-6.
146. Saynor R, Verel D. Effect of a marine oil high in eicosapentaenoic acid on blood lipids and coagulation. IRCS Med Sci 1980; 8: 378-9.
147. Barcelli U, Glas-Greenwalt P, Pollack VE. Enhancing effect of dietary supplement with omega-3 fatty acids on plasma fibrinolysis in normal subjects. Thromb Res 1985; 39: 307-12.
148. Iacono JM, Judd JT, Marshal MW, Canary JJ, Dougherty RM, Mackin JF, Weinland BT. The role of dietary essential fatty acids and prostaglandins in reducing bloodpressure. Prog Lip Res 1981; 20: 349-64.
149. Oster P, Arab L, Schellenberg B, Heuck CC, Mordasini R, Schlierf G. Bloodpressure and adipose tissue linoleic acid. Res Exp Med 1979; 175: 287-91.
150. Singer P, Voigt S, Godicke W. Inverse relationship between linoleic acid in serum and in adipose tissue of patients with essential hypertension. Prostaglandins Leukotrienes Med 1982; 9: 603-13.
151. Singer P, Jaeger W, Wirth M, Voigt S, Naumann E, Zimontkowski S, Hajdu I, Goedicke W. Lipid and bloodpressure-lowering effect of mackerel diet in man. Atherosclerosis 1983; 49: 99-108.
152. Singer P, Jaeger W, Voight S, Thiel H. Defective desaturation and elongation of n-6 and n-3 fatty acids in hypertensive patients. Prostaglandins Leukotrienes Med 1984; 15: 159-65.

153. Singer P. Neue Gesichtspunkte bei der Behandlung von arterieller Hypertonie und Hyperlipoproteinamien mit mehrfach ungesattigten Fettsauren. Akt Ernahr 1986; 11: 29-39.
154. Hornstra G. Dietary lipids, platelet function, and arterial thrombosis in animals and man. Proc Nutr Soc 1985; 44: 371-8.
155. Black KL, Culp B, Madison D, Randall OS, Lands WEM. The protective effect of dietary fish oil on cerebral infarction. Prostaglan Med 1979; 5: 257-68.
156. Culp BR, Lands WEM, Lucchesi BR, Pitt R, Romson J. The effects of dietary supplementation of fish oil on experimental myocardial infarction. Prostaglandins 1980; 20: 1021-31.
157. Gudbjarnason S, Oskarsdottir G, Doell B, Hallgrimson J. Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 1978; 25: 130-44.
158. Benediktsdottir WA, Gudbjarnason S. Arachidonic and docosahexaenoic acid content of sarcolemmal phospholipids in relation to ventricular fibrillation in rats. J Mol Cell Cardiol 1986; 18: 90A.
159. Hill EG, Lundberg WO, Titus JL. Experimental atherosclerosis in swine. I. A comparison of menhaden oil supplements in tallow and coconut-oil diets. Mayo Clin Proc 1971; 46: 613-20.
160. Landymore RW, MacAulay M, Sheridan B, Cameron C. Comparison of cod-liver oil and aspirin-dipyridamole for the prevention of intimal hyperplasia in autologous vein grafts. Ann Thorac Surg 1986; 41: 54-7.
161. Davis HR, Bridenstine RT, Vesselinovitch D, Wissler RW. Fish oil inhibits development of atherosclerosis in rhesus monkeys. Arteriosclerosis 1987; 7: 441-9.
162. Hill EG, Lundberg WO, Titus JL. Experimental atherosclerosis in swine. II. Effects of methionine and menhaden oil on an atherogenic diet containing tallow and cholesterol. Mayo Clin Proc 1971; 46: 621-5.
163. Verheugt FWA, Schouten JA, Eeltink JC, Roos JP. Omega-3 polyunsaturated fatty acids in the treatment of angina pectoris: effect on objective signs of exercise induced myocardial ischemia. Curr Ther Res 1986; 39: 208-13.
164. Dehmer GJ, Popma JJ, van den Berg EK, Eichhorn EJ, Prewitt JB, Campbell WB, Jennings L, Willerson JT, Schmitz JM. Reduction in the rate of early restenosis after angioplasty by a diet supplemented with n-3 fatty acids. N Engl J Med 1988; 319: 733-40.
165. Schmitz JM, van den Berg EK, Prewitt JP, Mallay CR, Willerson JT, Dehmer GJ. Dietary supplementation with n-3 fatty acids may reduce the rate of restenosis after coronary angioplasty. Clin Res 1987; 35 (1): 6A.
166. Slack JD, Pinkerton CA, van Tassel J. Can oral fish oil supplement minimize restenosis after percutaneous transluminal coronary angioplasty? J Am Coll Cardiol 1987; 9: 64A.
167. Grigg CE, Kay T, Manolas EG, Hunt D, Valentine PA. Does max-EPA lower the risk of restenosis after PTCA? A prospective randomized trial. Circulation 1987; 76 (IV): 214.
168. Arntzenius AC, Kromhout D, Barth JD, Reiber JHC, Bruschke AVG, Buis B, van Gent CM, Kempen-Voogd N, Strikwerda S, van der Velde EA. Diet, lipoproteins and the progression of coronary atherosclerosis. N Engl J Med 1985; 312: 851-3.

169. Blankenhorn DH, Nessim SA, Johnson RL, Sanmarco ME, Azen SP, Cashin-Hempill L. Beneficial effects of combined cholestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass graft. JAMA 1987; 257: 3233-40.
170. Luten JB. De voedingswaarde van spoorelementen uit vis, schaal- en schelpdieren. Voeding 1985; 46: 410-5.
171. Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM. The influence of dietary mackerel oil on the condition of organs and blood lipid composition in the young growing pig. Am J Clin Nutr 1978; 31: 2159-66.
172. Danse LHJC. A pathogenetic study of yellow fat disease. Academic thesis, University of Utrecht, The Netherlands,1978.
173. Van Vleet JF, Ferrans VJ. Ultrastructure of hyaline microthrombi in myocardial capillaries of pigs with spontaneous "mulberry heart disease". Am J Vet Res 1977; 38: 2077-80.
174. Beare-Rogers JL. Docosenoic acids in dietary fats. Prog Chem Fats other Lipids 1977; 15: 29-56.
175. Speijers GJA. Toxische effecten van raapolie, erucazuur en linoleenzuur bij de rat. Academic thesis, University of Utrecht, 1983.
176. Hirai A, Terano T, Saito H, Tamura Y, Toshida S. Clinical and epidemiological studies of eicosapentaenoic acid in Japan. In: Lands WEM (ed). Polyunsaturated fatty acids and eicosanoids. Champaign, Ill: American Oil Chemists' Society 1987; 9-24.

CHAPTER 2

STUDY OBJECTIVES

2.1 Introduction

In the review of (most human) fish oil studies in chapter 1 it was shown that the data are not all confirmative and only a few studies were well-controlled. However, the safety of the needed experimental high doses of fish oil requires further investigation.

The general aims of this thesis were to investigate the effects of high doses of dietary fish oil on: 1. plasmalipids, 2.membrane phospholipid fatty acid composition, 3. membrane function, 4. sensitivity of membranes to lipid peroxidation, 5. morphology of various tissues 6. myocardial function during normoxia, and after ischemia and reperfusion, 7. platelet function 8. prostanoid synthesis, 9. platelet vessel wall interaction, and 10. rate of regression of atherosclerotic lesions.

For the optimal control of our studies we performed the experiments in an animal model. The pig was chosen because its anatomy, physiology and lipid metabolism are similar to that of man. Swine are also known to develop spontaneously atherosclerotic lesions, which are similar to the atherosclerotic lesions in man.

2.2 Chapter outline

The effects of dietary monoenes and polyunsaturated fatty acids (n-3 and n-6) on physiological and pathophysiological aspects of cardiac function are described in the review of chapter 3. In chapters 4, 5 and 6 the effects of different doses (daily 0.3 - 0.6 g EPA/kg body weight) of purified fish oil are reported on lipoprotein metabolism, cardiac membrane phospholipid fatty acid composition, and cardiovascular performance in the pig.

The effects of a codliver oil diet on catecholamine stress in rats is described in chapter 7. In chapters 8, 9 and 10 the effects of different doses of purified fish oil in swine on platelet and cardiac membrane phospholipid fatty acid composition, prostanoids, lipid-peroxidation, and cardiac function and arrhythmias during recurrent brief coronary artery occlusions and reperfusions are evaluated.

In chapter 11 the effects of fish oil on intimal thickening in a chronic model of partial coronary artery constriction in the pig is depicted. After reviewing briefly some aspects of the atherosclerotic process and providing a rationale for the

interference of fish oil in this process (chapter 12), a study on the effects of fish oil on the regression of atherosclerosis has been described in chapter 13.

In the epicrisis (chapter 14) the main conclusions of this thesis are described in the light of the possible benefit of the consumption of fish oil supplements by patients with ischemic heart disease.

CHAPTER 3

Dietary fatty acids and myocardial function

J. M. J. Lamers[1], J. M. Hartog[2], P. D. Verdouw[2] and W. C. Hülsmann[1]

Departments of Biochemistry I[1] and Thoraxcenter[2], Medical Faculty, Erasmus University Rotterdam, Rotterdam, The Netherlands

Summary

It is widely recognized that dietary polyunsaturated fatty acids (PUFA's) and cholesterol can profoundly influence the development of atherosclerotic plaques in coronary vessels, which may lead to myocardial infarction. The possibility that dietary fatty acids may also directly influence cardiac function has received less attention. We therefore reviewed the evidence of the effects of dietary fatty acids, in particular n-3 and n-6 PUFA's, on myocardial phospholipid fatty acid composition and cardiovascular performance. Heart organelles appear to incorporate uncommon fatty acids like 22:1 and trans- 18:1. Diets enriched with 22:1 induce myocardial lipidosis. N-9, n-6 and n-3 families compete among membrane C20 and C22 acids. Several studies have dealt with the relation between diet-induced changes of cardiac membrane (sarcolemma, sarcoplasmic reticulum and mitochondria) phospholipids and membrane function. In view of the variety of diets used and of the membrane functions studied, the results do not permit equivocal interpretation. Several investigators have reported an altered stress response of the heart due to a change of PUFA's in the diet. In rats fed with a low 18:2n-6/18:3n-3 ratio combined with relatively low amounts of saturated fatty acids, a high incidence of myocardial lesions has been observed. Pigs are less sensitive but more susceptible to the development of vitamin E deficiency, when the dietary PUFA content is high. Increased contractility and coronary flow rate have been reported for Langendorff-perfused hearts of rats fed 18:2n-6-rich diets. The effects on coronary flow rate are possibly related to alterations in eicosanoid synthesis, which may also contribute to the reduction by n-6 or n-3 PUFA's in infarct size, magnitude of recovery of function and suppression of reperfusion arrhythmias following release of a coronary artery ligation. On the other hand, increased peroxidation of membrane lipids, due to their high content of n-3 PUFA, may be deleterious.

Key words: dietary polyunsaturated fatty acids; heart function; membrane function; eicosanoid synthesis; lipid peroxidation.

Introduction

Since 1952, the plasma cholesterol lowering effect of dietary n-6 and n-3 PUFA's has been demonstrated repeatedly (23, 27, 35, 56). Epidemiological as well as primary and secondary prevention studies of ischaemic heart disease have revealed the protective effects of diets enriched with n-6 or n-3 PUFA's. In particular the n-3 PUFA's, also protect against thromboembolic events (23, 27, 35, 56) by inhibition of platelet aggregation. Hypertension, another risk factor in the development of coronary heart disease, may be favourably affected by n-6 PUFA's (67, 74), but here the data on the effects of n-3 PUFA's are conflicting (35).

Since Burr and Burr (12) described that n-6 PUFA's prevented abnormal increase of transepidermal water loss and decreased growth disturbances, the former are considered to be essential for growing rats. In spite of supplementation with n-3 PUFA, the symptoms still developed (48). Hence, as far as this is concerned, there is no compensatory role for n-3 PUFA's.

Dietary n-6 and n-3 PUFA's are metabolically competitive. Desaturation and elongation, incorporation into membrane phospholipids, conversion into eicosanoids, oxidative breakdown,

storage in tissue triglyceride and incorporation into circulating lipoproteins may all be modified by competition between fatty acids of different chain length and different unsaturated fatty acid families. The 3 major unsaturated fatty acid families are of the n-9, n-6 and n-3 type. The 6-, 5- and 4-desaturase, handling the further desaturation of these acids, are principal regulatory enzymes in the biosynthesis of endogenous PUFA's (Fig. 1). The activity of these enzymes is not

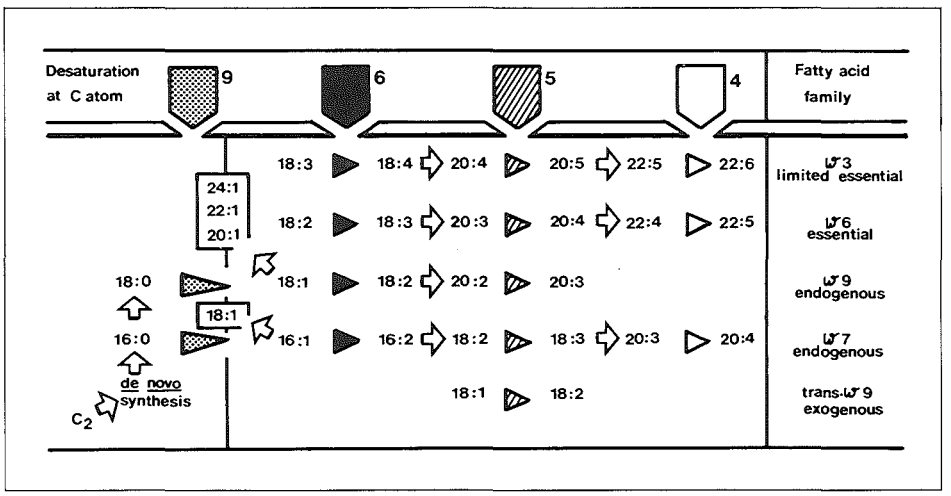

Fig. 1. Main pathways of PUFA biosynthesis and further elongation and desaturation of dietary fatty acids.

only modified by hormones but also by dietary PUFA's (10). Competitive actions, increasing with the number of double bonds, of fatty acids on 6-desaturase are also evident between 18:1n-9, 18:2n-6 and 18:3n-3 acids (72). A number of n-6 PUFA's, as arachidonic acid (20:4n-6) and docosapentanoic acid (22:5n-6), inhibit the desaturation of linoleic acid (18:2n-6) to γ-linolenic acid (18:3n-6). Oxidative desaturation of 18:2n-6 is inhibited by n-3 PUFA's. It has also been shown that n-3 PUFA's of C20 and C22 chain length compete powerfully with the 5-desaturation of C20 fatty acids (69, 72). Competition between 20:5n-3 and 20:4n-6 for incorporation into cardiac phospholipids is present after addition of labelled PUFA's to the perfusion medium of Langendorff-perfused rat hearts (26).

In this review we only summarize the direct effects of dietary fatty acids, in particular the PUFA's, on myocardial membrane biochemistry, function and pathophysiology. We will not discuss the possibile benefits of balancing the relative amount of dietary n-3 and n-6 fatty acids with respect to their essentiality. Such investigations comparing high-fat diets with low-fat diets have also not been considered.

Some attemps have been made to define the mechanism of the cardiovascular action of dietary fatty acids. So far four mechanisms have been envisaged: 1) the conversion of n-6 and n-3 fatty acids to eicosanoids (17, 20, 33, 36, 49, 74), 2) the contribution of PUFA's to membrane structure fluidity and membrane protein interactions (1, 14, 15, 20, 25, 30, 48, 53, 69, 72), 3) the reduced capacity of the heart to oxidize docosenoic acid (22:1) leading to transient cardiac lipidosis and slow development of fibrosis (9, 58), 4) the PUFA-induced increased need for antioxidants to prevent yellow fat disease (18, 23, 31, 35, 64).

Flow of dietary fatty acids into heart phospholipids

Changes in fatty acid composition of membrane phospholipids reflect the composition of the diet, although not strictly (48, 69, 72). Metabolic effects distort this relationship, as is evident from the competition between n-9, n-6 and n-3 fatty acids for the desaturation system (Figs. 1 and 2). There is also competition for incorporation of fatty acid into the phospholipid molecule during de novo synthesis in the endoplasmic reticulum or reacylation processes within the organelles and sarcolemma membrane. Some fatty acids, 20:4n-6 and 20:5n-3, are precursors for eicosanoid synthesis, which independently influence membrane fatty acid composition (Fig. 2). In the cell, the phospholipids phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are rapidly converted into the phospholipids phosphatidylcholine (PC) and phosphatidylinnositol-4, 5- biphosphate (PIP_2), respectively. These processes are involved in hormone-mediated cell responses. In heart, β-, and α_1-adrenergic receptor stimulation, respectively, initiate these phospholipid responses, but the conversion rates of PE and PI conversion are extremely low (11, 57).

Regulation of the fatty acyl group composition of membranes occurs by enzymes within the endoplasmic reticulum, the mitochondria, sarcoplasmic reticulum or sarcolemma. In the endoplasmic reticulum de novo synthesis occurs, while fine adjustment takes place in the mitochondria, sarcoplasmic reticulum and sarcolemma. Lysophospholipid formation, reacylation, transacylation and headgroup exchange, responsible for this fine tuning, play an important role in the dietary fatty acid and excessive β-adrenergic stimulation-induced adjustment of membrane fatty acyl group composition (see below).

The influence of dietary fatty acids on heart tissue fatty acid patterns has been studied extensively (Table 1). The used diets contained large amounts of 18:2n-6 (SFO), saturated fatty acids (LF, CNO and SKF), monoenes 18:1n-9 (OO) both 18:2n-6 and 18:3n-3 (SO), 18:3n-3 (LO), both 20:5n-3 and 22:6n-3 (FO), trans-18:1n-9 (PHCO and PHPO) and monoenes 20:1 and 22:1 (HEAR and PHFO). Most studies have been carried out with rats (Table 1), but lack uniformity in fatty acid composition of basal and dietary food, tissue subfractionation and separation of

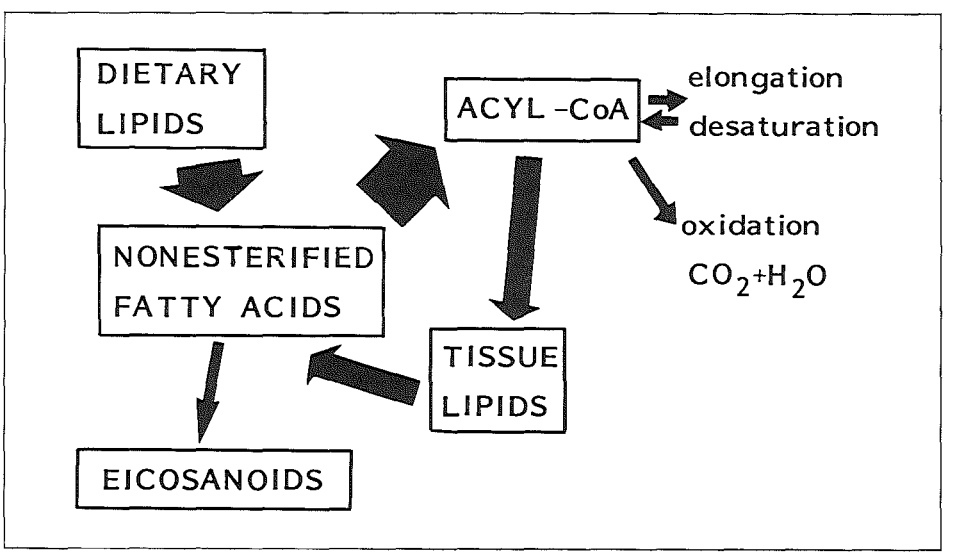

Fig. 2. Metabolic effects that distort parallelism between dietary and tissue lipid fatty composition.

Table 1. Studies on the relation between dietary fats and cardiac membrane phospholipid composition

Authors and reference	Species	Diets
Egwin and Kummerov (24)	rat	PHSO, CO
Szuha and McCarl (71)	rat	MM
Ruiter et al (64)	pig	FO, OO
Gudbjarnason et al (30)	rat	FO
Dewailly et al (21)	rat	LEAR, HEAR, PNO
Clandinin and Robblee (60)	rat	SO, LEAR, HEAR
Kramer (44)	rat	LEAR, HEAR, OO, SO, CO
Bellenand et al (6)	rat	SFO, CNO, HEAR
Yasuda et al (78)	rat	synthetic
De Deckere (20)	rat	SFO, LF, PHCNO
Tahin et al (72)	rat	SO, SFO, LF, FO
Iritana and Fugikawa (40)	rat	FO, CO
Awad and Chattopadhyay (4)	rat	CO, synthetic
Menon and Dhopeshwarkar (54)	rat	HCO, synthetic
Charnock et al (13)	rat	SKF, SFO
Arens et al (2)	pig	SFO, SO, LO,OO, LF
Montfoort et al (55)	rat	FO
Hartog et al (32)	pig	FO, LF
Takamura et al (73)	rat	FO, CO
Swanson and Kinsella (70)	rat	FO

Abbreviations: PHSO, partially hydrogenated soybean oil; CO, corn oil; MM, mother milk; FO, fish oil; OO, olive oil; LEAR, low erucic acid rapeseed oil; HEAR, high erucic acid rapeseed oil; SO, soybean oil; PNO, peanut oil; PHFO, partially hydrogenated fish oil; SFO, sunflower oil; LF, lard dat; PHCNO, partially hydrogenated coconut oil; CNO, coconut oil; SKF, sheep kidney fat; PHCO, hydrogenated corn oil; LO, linseed oil.

phospholipid classes. Nevertheless, the results on phospholipid extracts from whole hearts provide valuable data on e. g. competition between n-9, n-6 and n-3 fatty acids. Low but adequate amounts of dietary 18:2n-6, decreases its membrane content. The 20:4n-6 membrane content is relatively insensitive to low amounts of dietary 18:2n-6 while the C_{20} and C_{22} n-3 fatty acid membrane contents increase despite the presence of low amounts of 18:3n-3 in the diet (2, 13, 20, 53, 72). Dietary C20 and C22 n-3 fatty acids drastically reduce myocardial 20:4n-6 levels by competition with 18:2n-6 for the 6-desaturase (13, 24, 30, 32, 40, 55, 70, 72, 73). None of these dietary interventions affect total saturated fatty acid and PUFA content of cardiac membranes. Because membrane n-6 fatty acids are replaced by n-3 fatty acids, an 18:2n-6-rich diet slightly decreases the double bond index (DBI) of cardiac membranes (13, 20) whereas a large increase occurs with n-3 PUFA-rich diets (13, 24, 30, 32, 40, 55, 70, 72, 73). The changes in fatty acid composition of heart membranes usually begin within a few weeks after starting the diet (72).

Although changes in dietary monoene content exert little effect on tissue fatty acid patterns, 18:1n-9 participates in the substitution of dietary PUFA's (4, 5, 13, 30, 32, 60, 70, 72). Dietary 18:1n-9 and 22:1n-9 are reflected in myocardial fatty acid patterns (21, 38, 44, 64, 72, 78), which may be caused by the abundant presence of the phospholipid cardiolipin (CL) in the mitochondria, as cardiolipin fatty acid composition is most sensitive to dietary docosanoenes (21, 38, 39, 72, 78). Rats fed rapeseed oil, which contains the monoene 22:1n-9, showed a persistent rise in 22:1n-9 in CL but not in mitochondrial PC or PE (21, 38, 39, 78). The dissimilar effects of dietary fat composition on mitochondrial PC and PE on the one hand, and of CL on the other hand is noteworthy in view of other biosynthetic differences between these phospholipids. Mitochon-

drial PC and PE are mainly synthesized in the endoplasmic reticulum, whereas CL is exclusively of mitochondrial origin (39). Other uncommon fatty acids like elaidic acid (trans-18:1n-9) are incorporated into heart phospholipids (3, 38, 54, 72). The fatty acid trans-18:2n-6 is, however, not incorporated (72).

For the reasons outlined above it is likely that the membranes of sarcolemma, sarcoplasmic reticulum and mitochondria have different phospholipid fatty acid compositions and dietary responses (1, 4, 21, 38, 39, 53, 60, 72). A related problem is whether the diet-induced changes reflect only fatty acid changes or overall phospholipid composition modifications of cardiac membranes. In PE, for example, dietary fish oil induces a shift in n-6 and n-3 fatty acid distribution which is usually more pronounced than in PC (60, 55, 70). Mitochondrial CL contains relatively the largest amount of 18:2n-6, so that a reduced dietary 18:2n-6 content is more effective (30, 38, 39, 44, 70). The sarcolemmal sphingomyelin fraction contains mostly saturated and monoene fatty acids so that dietary PUFA's are not expected to produce any change (3, 28, 44, 64, 78). In spite of the absence of the saturated fatty acids 22:0 and 24:1 in diets enriched with 20:5n-3 or 22:6n-3, the 22:0 content decreases but that of 24:1 decreases in myocardial sarcolemmal sphingomyelin of pigs (unpublished data from this laboratory). Dietary fatty acids cause in general much smaller shifts in phospholipid class than in fatty acid composition (3, 55, 60, 64, 70).

The molecular species of individual phospholipid classes in phospholipid mixtures extracted from whole heart, pure organelles and sarcolemma (Fig. 3) has been studied by phospholipase A_2 breakdown of a PE fraction of total phospholipids of rat heart (55), by conversion to monoacetyldiacylglycerols by phospholipase C/acetic anhydride pyrridine treatment and further separation on $AgNO_3$-thin layer chromatography (78) and separation by HPLC (73). Fatty acids in the PC and PE fraction show positional specificity with preferential location of saturated fatty acids and monoenes at the 1-position and polyunsaturated fatty acids at the 2-position of the glycerol moiety (28, 55, 73, 78). In CL those fatty acids are randomly distributed between 1- and 2-positions (39). Feeding rats with fish oil affects primarily the fatty acid composition on the 2-position of PC and PE fractions of total heart phospholipids (55, 73). Canine sarcolemmal

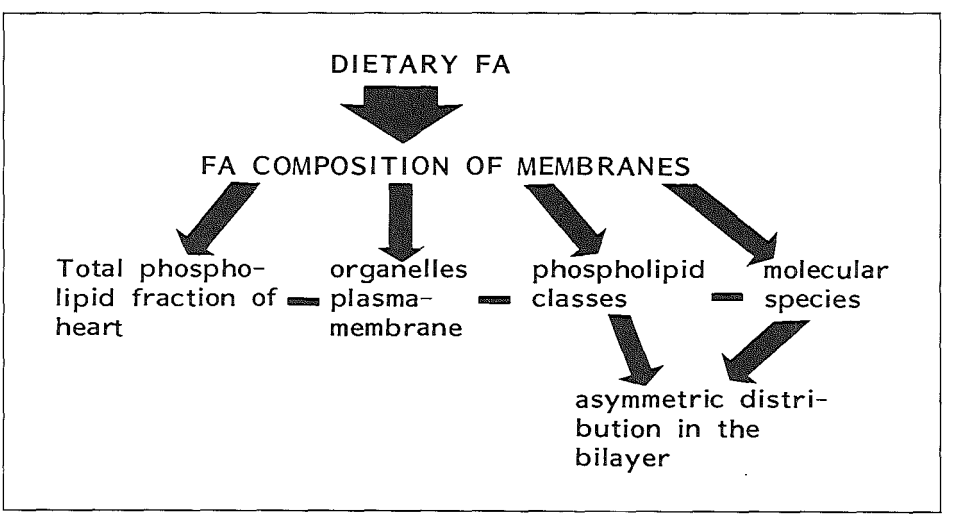

Fig. 3. Dietary fatty acids potentially influence various characteristics of the membrane phospholipid-bilayer.

phospholipids contain 40% plasmalogens which have a molecular species composition different from the overall composition (28). Whether these molecular species respond differently to dietary PUFA's is not known.

It is now well established that proteins and phospholipids have an asymmetrical distribution over biomembranes. PE, phosphatidylserine (PS) and PI are predominantly localized at the cytosolic side of the plasma membrane bilayer, whereas PC and sphingomyelin are predominantly localized at the surface (63, 69). The asymmetry partially serves a metabolic purpose because the substrate for cytosolic and membrane-bound enzymes must be at the appropriate leaflet of the bilayer. This stresses the importance of knowledge about diet-induced changes in fatty acid composition in all phospholipid classes (Fig. 3).

Membrane function

The consequences of extensive alterations in the fatty acid composition (of phospholipids) in the functions of the heart membranes is a key problem. It is generally assumed that the selectivity of many enzymatic and transport functions of membranes require the structural complexity of the protein molecules, whereas the phospholipids play only a supporting role. Although specific functions of phospholipids in membrane-linked processes have been described, their role is considered to be of secondary importance (25, 69). They provide the insulating barrier that limits the transfer of ions (Ca^{2+}, Na^+ and K^+) across the membrane, especially by means of the apolar region provided by the paraffinic chains of their constituent fatty acids. Nevertheless, evidence has been obtained that the permeability of membranes of cells other than myocytes can be markedly influenced by the fatty acid composition of the phospholipids and in particular their degree of unsaturation (37, 46, 63, 69). We measured Ca^{2+} permeability and Na^+/Ca^{2+} exchange activity in heart sarcolemmal membranes from pigs fed either mackerel oil or lard fat (47). The diet induced a marked change in membrane DBI (mackerel oil: 1.85 and lard fat: 1.28, cf. [33, 47]) but not in Ca^{2+} movements in sarcolemmal membrane (47). Discrepancies were also noted in the response of cardiac sarcolemma Na^+/K^+ ATPase to 18:2n-6-rich diets (1, 22) which excludes extrapolation to possible changes in membrane function in vivo.

The DBI is important for changes in fluidity of the membrane core (25, 69). Abeywardena et al. described the rotational diffusion of a diphenylhexatriene probe as a fluidity marker (1). They showed that in rats on a 18:2n-6-rich diet the fluidity of heart sarcoplasmic reticulum but not the Ca^{2+} pump activity was altered. On the other hand a different break in the Arrhenius plot of mitochondrial succinate dehydrogenase suggested a change in membrane fluidity (53). Before the change in fluidity of the membrane bilayer can be attributed to modified levels of unsaturation, several other factors have to be considered. Whether changes occur in membrane composition as the neutral triglyceride content, the phospholipid/protein ratio, the phospholipid class distribution, and the cholesterol/phospholipid ratio should also be known. In most studies, these characteristics have not been assayed (Table 1). For example, an increase in the cholesterol/phospholipid ratio tends to increase the order of lipid motion in the bilayer (79). We (47) found in the heart sarcolemma of mackerel oil fed pigs not only a marked increase of the DBI (from 1.28 to 1.85), but also in the cholesterol/phospholipid ratio (from 0.38 to 0.64), while the phospholipid: protein ratio decreased from 0.94 to 0.69. The latter finding confirms our earlier observations in total heart phospholipid fraction of hearts rats fed with cod liver oil (55).

Heart sarcolemma from mackerel oil fed pigs had increased 5'-nucleotidase and Ca^{2+} pump activity, while adenylate cyclase was more sensitive to isoproterenol stimulation (47). However, it is not clear whether these enzymatic changes were the consequence of increased DBI or cholesterol content of the membrane as e. g. a high cholesterol/phospholipid ratio has been shown to reduce cardiac sarcolemmal Ca^{2+} pumping ATPase activity (59) and to increase rat lung membrane adenylate cyclase β-receptor sensitivity (65).

We also have to take into account the response of the myocardial membrane function to hormonal and neurotransmitter stimuli, such as α_1-adrenergic and muscarinic stimulation of PI breakdown and β-adrenergic activation of PE-N-methylation. For example, the α_1-receptor regulated phospholipase C may have a preference for a particular molecular species of PIP_2. It may also be that a particular diglyceride species most powerfully activates the Ca^{2+}-dependent C-kinase. At present no information concerning this matter is available.

Eicosanoid synthesis

Eicosanoids are formed from 20:4n-6, a fatty acid which is produced by action of 6-desaturase on 18:2n-6. It is first cleaved from membrane phospholipids by phospholipase A_2. The cyclo-oxygenase and lipoxygenase enzyme systems subsequently give various types of so-called leucotrienes (e. g. LTC_4, LTD_4, LTB_4) and prostaglandins (e. g. PGI_2, TXA_2, PGE_2 and PGF_2). The eicosanoids are not stored in tissues and their release from organs is therefore indicative of de novo synthesis. The precise sites and regulation of the rate of eicosanoid synthesis in the heart are not clear, as both the coronary vasculature and myocytes contribute to myocardial prostaglandin synthesis (36, 41, 66). The major 20:4n-6 metabolite released from the isolated perfused heart (endothelial cells and myocytes) is PGI_2 (20, 66, 74) while platelets are a major site for TXA_2 production. PGI_2 and TXA_2 play opposite roles in the regulation of platelet aggregability, vasoconstriction and perhaps arrhythmogenesis (27, 66, 41). Hence, the ratio of their concentrations may be more important. Feeding rats with 18:2n-6-rich diets increased production of the prostaglandins PGI_2, PGE_2 and PGF_2 in Langendorff-perfused myocardium (20, 36, 74). Cardiac membrane phospholipids from rats fed with 18:2n-6-rich diet do not contain more 20:4n-6 (2, 13, 20, 53, 72). Therefore, in order to explain the increased prostaglandin production, compartmentalization of 20:4n-6 or alteration of membrane-bound phospholipase A_2 should be considered. In animals fed with fish oil a change in eicosanoid production is most likely due to the altered membrane fatty acid composition because of a displacement of 20:4n-6 by 20:5n-3 within the phospholipids. The fatty acid 20:5n-3 inhibits formation and antagonizes actions of 20:4n-6-derived prostaglandins (Fig. 4). The 20:5n-3 is a competitive inhibitor and a poor substrate for cyclo-oxygenase (23, 27, 35, 56, 67). Small quantities of TXA_3 and PGI_3 (weak agonists) are also formed. We measured by radio-immunoassays the stable endproducts of PGI_2 (6-keto-$PGI_{1\alpha}$) and of TXA_2 (TXB_2) in the coronary vein of mackerel oil fed pigs (33). Baseline values were reduced by 29% and 59%, respectively, compared to that of lard fat animals. Likewise, the TXA_2/PGI_2 balance was changed (55% reduced). Production of small amounts of the weak agonists TXA_3 and PGI_3 may also be expected. Concentrations of TXA_2 and PGI_2 in the coronary vein during reperfusion following coronary artery occlusion were also lower in the mackerel oil than in the lard fat fed pigs. The more vigorous hyperaemic response in the mackerel oil fed animals observed under these circumstances could therefore be related to altered prostaglandin production (33).

Coronary flow rate and contractility

Elevated coronary blood flow and contractility have been measured in Langendorff-perfused hearts of rats which had been on 18:2n-6-rich diets (20, 36). On the other hand, no effects of this diet on tension in isolated papillary muscle could be demonstrated (14). That an increased PGI_2 production might be responsible for the effect of a 18:2n-6-rich diet on flow is supported by use of the prostaglandin synthesis inhibitors aspirin and indomethacin (20, 36). The mechanisms by which an 18:2n-6 rich diet might affect heart contractility are largely unknown, although changes in Na^+/K^+ ATPase have been implicated (20). One must, however, be careful before extrapolating observations obtained on myocardial performance in in vitro experiments to the in

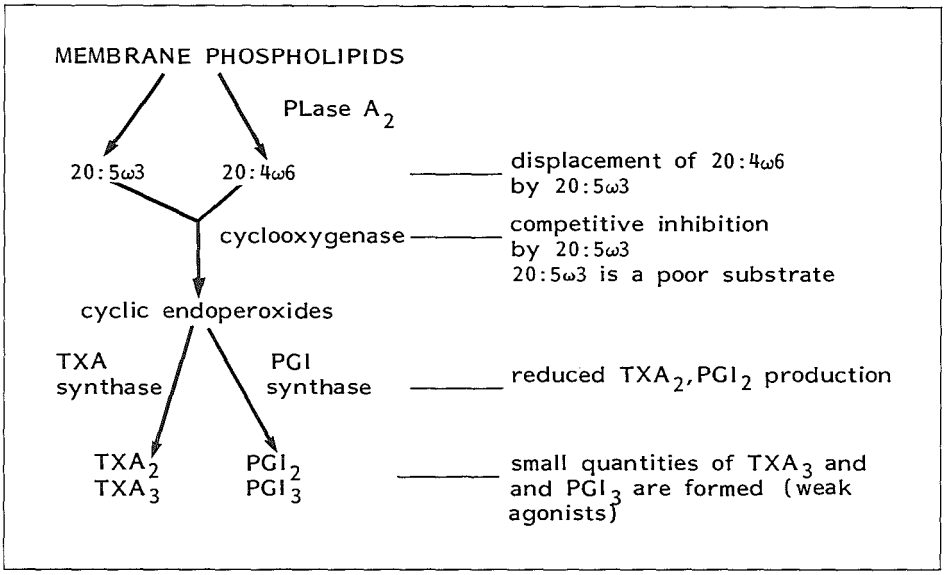

Fig. 4. Antagonism by n-3 PUFA's of formation and action of n-6-derived prostaglandins.

situ situation because of low TXA_2 production in platelet-free perfused hearts. Indeed, we found no changes in in situ determined parameters of cardiac function of mackerel oil fed pigs, despite marked alterations in prostaglandin levels (32, 33). Monoene-rich diets, known to induce focal myocardial fibrotic lesions due to transient lipidosis, did not influence myocardial contractility of rat hearts (22).

Cardiotoxicity of high doses of catecholamines

Rona and coworkers first described an infarct-like necrosis in rats after administration of isoproterenol (see 62 for review). Since then, this has become a standard model for the study of protection against catecholamine-induced myocardial insult. Isoproterenol proved to be more toxic than epinephrine and norepinephrine, but this difference is related to their diverse effects on the coronary vasculature. Several factors such as relative hypoxia, formation of toxic oxidation products, microcirculatory effects and myocardial Ca^{2+} overload have been implicated in the development of cardiac necrosis (62). Increased mortality in rats, fed with cod liver oil was found after chronic isoproterenol treatment (30). By the same group, a decreased mortality was found with 20:4n-6-rich diets (29). Protection by dietary 18:2n-6 against catecholamine-induced necrosis has also been reported (16). We did not find an effect on mortality during catecholamine stress of cod liver oil fed rats, although ST-segment elevation in diethylether-induced anaesthetized cod liver oil fed rats was more pronounced (55). Protection by cod liver oil feeding against fatal ventricular fibrillation in old rats subjected to subcutaneous injection of 1 mg/kg of isoproterenol has also been reported (7). Because of the discrepancies in results and the lack of knowledge about the cause of death (damage), a causal relation between dietary PUFA composition and the sensitivity of the heart to stress cannot be given. Moreover, the use of cod liver oil may be inappropriate for studying the effect of dietary fish oil because of the high content

of vitamin D and monoene fatty acid cetoleic acid and the low content of vitamin E. The possible role of endogenous antioxidant shortage caused by dietary PUFA's in the response of the heart to stress is discussed below.

In the catecholamine stress experiments the phospholipids undergo partial replacement of n-6 by n-3 PUFA's similar to that with dietary fish oil (30, 55). Perhaps the Ca^{2+} overload, induced by overstimulation of the heart β-adrenoceptors, activates tissue phospholipase A_2 thereby favouring the removal of 2-positional fatty acids from the membrane phospholipids rapidly followed by reacylation.

Heart lesions due to a dietary fatty acid imbalance

Rats fed with vegetable oils or partially hydrogenated marine oils appear to be more prone to myocardial lesions than other species such as pigs, monkeys and mice (5, 8, 9, 15, 51, 58). Lesions, detected by histometric analysis, exhibit microvascular alterations (oedematous swelling and loosening of blood vessels) and myocardial necrosis (granular degradation of myocardial fibres, vacuolization of muscle fibres and sarcolysis, mononuclear cell infiltration). This type of necrosis, was first reported for male rats fed with high 22:1n-9 rapeseed oil for 5-7 weeks by Roine et al. in 1960 (61). Necrosis was not related to dietary 22:1n-9 intake, but the importance of alterations in the fatty acid composition of the diets has been demonstrated (8, 15). In a well-controlled study (8)in which 18:1n-9, 18:2n-6 and 18:3n-3 were gradually altered over a wide range by mixing several fat types (SFO, SO, LO, OO, LF, Table 1), the incidence of lesions was higher when dietary intake of 18:2n-6/18:3n-3 was less than 2—3 at constant 18:1n-9 content (50%). This has been confirmed by others (see [15] for review) employing mixtures of CO, LEAR, LF, SO (Table 1) and cocoabutter. The number of lesions correlated positively with 18:3n-3, 18:1n-9, 22:1n-9 content and negatively with 16:0, 18:0 or 18:2n-6. Pigs are less sensitive to a dietary fat imbalance, but yellow fat disease, due to vitamin E deficiency, is seen more frequently in this species (8). So far all studies relating dietary fat intake and the occurrence of myocardial lesions were purely descriptive (8, 15, 51). The mechanism by which an imbalance in dietary fatty acids produces the lesions is completely unknown.

Arrhythmias, recovery of function and infarct size after coronary artery ligation

A lower incidence of arrhythmias and mortality of rats on 18:2n-6 rich diets after coronary artery ligation has been described (49, 52), but the same diet did not affect the incidence of aconitine and $CaCl_2$-induced arrhythmias in rats (50). Dietary supplementation with fish oil also showed a reduction in the incidence of arrhythmias and infarct size after ligation of a coronary artery in dogs (7). However, we could not observe any difference in the incidence of ventricular arrhythmias and recovery of regional myocardial function during and after multiple coronary artery occlusions in anaesthetized pigs, which had been fed either mackerel oil or lard fat for 8 to 16 weeks (33, 34). The mechanism by which dietary n-6 or n-3 PUFA's might exert protection can only be speculated upon but an altered membrane phospholipid composition, which modifies eicosanoid production has been suggested (17, 49). However, since eicosanoids might be beneficial as well as deleterious it is difficult to define their role, if any, during myocardial ischaemia.

Lipid peroxidation

Membrane PUFA's are very susceptible to O_2 free radicals ($O_2^{\cdot-}$ and $OH^{\cdot}$) and organic free radicals are formed from these. The divinyl methane structure within the PUFA chains is particularly prone to abstraction of the allylic hydrogen, resulting in formation of fairly stable lipid-

free radicals. In the presence of O_2, these lipid-free radicals initiate a subsequent chain of autooxidation reactions. O_2, having free radical-like properties, is far more soluble within the nonpolar lipid phase of the membrane (see review in ref [68]). Cells contain a broad spectrum of antioxidants and free radical-controlling enzymes such as superoxide dismutase and catalase. The antioxidant vitamin E is largely found in association with membrane lipids and this localization probably serves as a local defence mechanism against PUFA peroxidation. Evidence is growing that free radicals play a pivotal role in the extension of myocardial damage during reperfusion following a period of ischaemia (42, 68, 77). It has indeed been shown that superoxide dismutase and catalase protected porcine hearts subjected to one hour of normothermic regional ischaemia, followed by one hour of global hypothermic arrest and one hour of normothermic reperfusion (19). A significant amount of thiobarbituric acid reactive material (malondialdehyde, MDA) appeared in the perfusate, demonstrating free radical-mediated peroxidation of PUFA containing 3 or more double bonds which was prevented by addition of superoxide dismutase and catalase. In addition there was an improvement in the contractile recovery of the heart (19). Because of their susceptibility to lipid peroxidation, harmful effects of dietary PUFA may be expected during myocardial ischaemia and reperfusion.

The first symptoms of vitamin E deficiency are a disorder of fat depots: adipose, liver cell degeneration, inflammation, fibrosis and accumulation of lipofuscin pigment (18). Although yellow fat disease can occur in rats, pigs are especially sensitive. Cardiomyopathy is frequently observed in animals with selenium-vitamin E deficiency (75). This deficiency (in pigs) is also termed "mulberry heart disease" because of a reddish purple gross appearance of the heart. Pigs on cod liver oil, mackerel oil- or 18:3 enriched diets developed mild symptoms of vitamin E deficiency (8, 18, 64). None of the symptoms were present in pigs fed mackerel oil supplemented with vitamin E and selenium (32, 33). We observed no increase in venous plasma MDA levels during the dietary period. However, the MDA production induced by the addition of Fe^{2+}, ADP and dihydrofumarate to isolated cardiac sarcolemmal membranes obtained from mackerel oil fed animals was increased by about 2 fold (unpublished observations from our laboratory). This result is in qualitative agreement with studies of liver microsomes isolated from rats fed herring oil (31). In the latter investigation it was also shown that dietary supplementation of vitamin E markedly reduced the in vitro induced MDA formation (31). In coronary venous blood of mackerel oil-fed pigs, collected immediately after a period of ischaemia, we found a much higher MDA production than in lard fat-fed pigs, but no difference in the short-term recovery of myocardial function (33, 34). This might indicate that in pigs the enhancement of lipid peroxidation due to a PUFA diet is not critical in the development of contractile failure after myocardial infarction.

References

1. Abeywardena MY, McMurchie EJ, Russell GR, Sawyer WH, Charnock JS (1984) Response of rat heart membranes and associated iontransporting ATPases to dietary lipid. Biochim Biophys Acta 776: 48—59
2. Arens M, Könker S, Werner G, Petersen U (1986) Physiological effect of various mixtures of oleic, linoleic and linolenic acids on growing pigs. 5. Influence on the lipids of the heart muscle. Fette-Seifen-Anstrichmittel 86: 47—50
3. Awad AB, Chattopadhyay JP (1983) Alteration of rat heart sarcolemma lipid composition by dietary elaidic acid. J Nutr 113: 913—920
4. Awad AB, Chattopadhyay JP (1983) Effect of dietary fats on the lipid composition and enzyme activities of rat cardiac sarcolemma. J Nutr 113: 1878—1884
5. Beare-Rogers JL (1977) Docosenoic acids in dietary fats. Prog Chem Fats Lipids 15: 29—56
6. Bellenand JF, Baloutch G, Ong N, Lecerf J (1980) Effects of coconut oil on heart lipids and on fatty acid utilization in rapeseed oil. Lipids 15: 938—943

7. Benediktsdòttir VE, Gudbjarnason S (1986) Arachidonic acid and docosahexanoic acid content of sarcolemmal phospholipids in relation to ventricular fibrillation in rats. J Mol Cell Cardiol 18: 90 (abstract)
8. Bijster GM, Vles RO (1984) Physiological effect of various mixtures of oleic, linoleic and linolenic acids on growing pigs: 6. Histomorphometric investigation of heart, liver, kidney and adipose tissue. Fette-Seifen-Anstrichmittel 86: 89—94
9. Bremer J, Norum KR (1982) Metabolism of very long-chain monounsaturated fatty acids (22:1) and the adaptation to their presence in the diet. J Lipid Res 23, 243—256
10. Brenner RR (1982) Nutritional and hormonal factors including desaturation of essential fatty acids. Prog Lipid Res 20, 41—47
11. Brown JH, Buxton IL, Brunton LL (1985) α_1-adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res 57, 532—537
12. Burr GO, Burr MM (1929) A new deficiency disease by rapid exclusion of fat from the diet. J Biol Chem 82, 345—367
13. Charnock JS, Dryden WF, McMurchie EJ, Abeywardena MY, Rusell GR (1983) Differences in the fatty acid composition of atrial and ventricular phospholipids of rat heart following standard and lipid-supplemented diets. Comp Biochem Physiol 75B, 47—52
14. Charnock JS, McLennan PL, Abeywardena MY, Dryden WF (1985) Diet and cardiac arrhythmia: Effects of lipids on age-related changes in myocardial function in the rat. Ann Nutr Metabol 39, 306—318
15. Clandinin MT (1978) The role of dietary long chain fatty acids in mitochondrial structure and function. Effects in rat cardiac mitochondrial respiration. J Nutr 108, 273—281
16. Crandall DL, Griffith DR, Beitz DC (1982) Protection against the cardiotoxic effect of isoproterenol-HCl by dietary polyunsaturated fatty acids and exercise. Toxicology and Applied Pharmacology 62, 152—157
17. Culp BR, Lands WEM, Lucchesi BR, Pitt R, Romson JC (1980) The effect of dietary supplementation of fish oil on experimental myocardial infarction. Prostaglandins 20, 1021—1031
18. Danse LHJC, Verschuren PM (1978) Fish oil-induced yellow fat disease in rats. I Histological changes. Vet Pathol 15, 114—124
19. Das DK, Engelman RM, Rousou JA, Breyer RH, Otani H, Lemeshow SC (1986) Pathophysiology of superoxide radical as potential mediator of reperfusion in pig heart. Basic Res Cardiol 81, 155—166
20. De Deckere EAM (1981) Influences of dietary linoleic acid on coronary flow, left ventricular work and prostaglandin synthesis in the isolated rat heart. Thesis, Erasmus University Rotterdam
21. Dewailly P, Nouvelot A, Sezille G, Fruchart JC, Jaillard J (1977) Changes in fatty acid composition of cardiac mitochondrial phospholipids in rats fed rapeseed oil. Lipids 13, 301—304
22. De Wildt DJ, Speijers GJA (1984) Influence of dietary rapeseed oil and erucic acid upon myocardial performance and hemodynamics in rats. Toxicol Appl Pharmacol 74, 99—108
23. Dyerberg J (1986) Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr Rev 44, 125—134
24. Egwin PO, Kummerow FA (1972) Response of docosapentaenoic acids of rat heart phospholipids to dietary fat. Lipids 7, 567—571
25. Farias RN, Bloj B, Morero RD, Sineriz F, Trucco RE (1975) Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. Biochim Biophys Acta 415, 231—251
26. Fragiskos B, Chan AC, Choi PC (1986) Competition of n-3 and n-6 polyunsaturated fatty acids in the isolated perfused rat heart. Ann Nutr Metabol 30, 331—334
27. Goodnight SH, Harris WS, Connor WE, Illingworth DR (1982) Polyunsaturated fatty acids, hyperlipedemia, and thrombosis. Arteriosclerosis 2, 87—113
28. Gross RW (1984) High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: A fast atom bombardment mass spectroscopic and gaschromatography — mass spectroscopic characterization. Biochemistry 23, 158—165
29. Gudjarnason S, Hallgrimsson J (1976) Prostaglandis and polyunsaturated fatty acids in heart muscle. Acta Biol Med Germ 35, 1969—1978
30. Gudbjarnason S, Oskerdòttir G, Doell B, Hallgrimsson J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 25, 130—144
31. Hammer CT, Wills ED (1978) The role of lipid components of the diet in the regulation of the fatty acid composition of the rat liver endoplasmic reticulum and lipidperoxidation. Biochem J 174, 585—593

32. Hartog JM, Lamers JMJ, Montfoort A, Becker AE, Klompe M, Morse H, Ten Cate FJ, van der Werf L, Hülsmann WC, Hugenholtz PG, Verdouw PD (1987) The effects of a mackerel-oil and a lard-fat enriched diet on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am J Clin Nutr (in press)
33. Hartog JM, Lamers JMJ, Verdouw PD (1986) The effects of dietary mackerel oil on plasma and cell membrane lipids, on hemodynamics and cardiac arrhythmias during reccurrent acute ischemia in the pig. Basic Res Cardiol 81: 567—580
34. Hartog JM, Lamers JMJ, Achterberg PW, van Heuven-Nolsen D, Nijkamp FP, Verdouw PD (1987) The effects of dietary mackerel oil on the recovery of cardiac function after acute ischaemic events in the pig. Basic Res Cardiol (this volume)
35. Herold PM, Kinsella JE (1986) Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am J Clin Nutr 43, 566—598
36. Hoffmann P (1986) Cardiovascular actions of dietary polyunsaturated and related mechanisms. A state-of-the art review. Prostaglandins 21, 113—147
37. Holmes RP, Mahfouz M, Travis BD, Yoss NL, Keenan MJ (1983) The effect of membrane lipid composition on the permeability of membranes to Ca^{2+}. NY Acad Sci 414, 44—56
38. Hsu CML, Kummerow FA (1977) Influence of elaidate and erucate on heart mitochondria. Lipids 12, 486—508
39. Innis SM, Clandinin MT (1981) Dynamic modulation of mitochondrial inner-membrane lipids in rat heart by dietary fat. Biochem J 193, 155—167
40. Iritani N, Fujikawa S (1982) Competitive incorporation of dietary n-3 and n-6 polyunsaturated fatty acids into tissue phospholipids in rats. J Nutr Sci Vitaminol 28, 621—629
41. Karmazyn M and Dhalla NS (1983) Physiological and pathophysiological aspects of cardiac prostaglandins. Can J Physiol Pharmacol 61, 1207—1225
42. Koster JF, Biemond P, Stam H (1987) Lipid peroxidation and myocardial ischaemic changes: Cause or consequence. Basic Res Cardiol (this volume)
43. Kramer JH, Mak IT, Weglicki WB (1984) Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipidperoxidation. Circ Res 55, 120—124
44. Kramer JKG (1980) Comparative studies on composition of cardiac phospholipids in rats fed different vegetable oils, Lipids 15, 651—660
45. Kramer JKG, Farnworth ER, Thompson BK, Corner AH (1982) The effect of dietary fatty acids on the incidence of cardiac lesions and changes in the cardiac phospholipids in male rats. Prog Lipid Res 20, 491—499
46. Kummerow FA (1983) Modification of cell membrane composition by dietary lipids and its implication for atherosclerosis NY Acad Sci 414, 29—43
47. Lamers JMJ, van der Werf L, Montfoort A, Hartog JM, Verdouw PD, Hülsmann WC (1986) Alterations in fatty acid profile of heart sarcolemma phospholipids by dietary fish oil and effects on functional activities of the membrane. J Mol Cell Cardiol 18, 88 (abstract)
48. Lands WEM (1986) Renewed questions about polyunsaturated fatty acids. Nutr Rev 44, 189—195
49. Lepran I, Nemecz GY, Kottai M, Szekeres L (1981) Effect of a linoleic acid-rich diet on the acute phase of coronary occlusion in conscious rats: influence of indomethacin and aspirin. J Cardiovasc Pharmacol 3, 847—853
50. Logan RL, Larking P, Nye ER (1977) Linoleic acid and susceptibility to fatal ventricular fibrillation in rats. Atherosclerosis 27, 265—269
51. McCutcheon JS, Kmermura T, Bhatnagar MK, Walker BL (1976) Cardiopathogenity of rapeseed oils and oil blends differing in erucic, linoleic and linolenic acid content. Lipids 11, 545—552
52. McLennan PL, Abeywardena MY, Charnock JS (1985) Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. Can J Physiol Pharmacol 63, 1411—1417
53. McMurchie EJ, Abeywardena MY, Charnock JS, Gibson RA (1983) Differential modulation of rat heart mitochondrial membrane-associated enzymes by dietary lipid. Biochim Biophys Acta 760, 13—24
54. Menon NK, Dhopeshwarkar GA (1983) Differences in the fatty acid profile and β-oxidation by heart homogenates of rats fed cis and trans octadecenoic acids. Biochim Biophys Acta 751, 14—20
55. Montfoort A, van der Werf L, Hartog JM, Hugenholtz PG, Verdouw PD, Hülsmann WC, Lamers JMJ (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart

phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol 81, 289—302
56. Norum KR, Drevon CA (1986) Dietary n-3 fatty acids and cardiovascular diseases. Arteriosclerosis 6, 352—355
57. Okumura K, Ogawa K, Satake T (1983) Phospholipid methylation in canine cardiac membranes. Relation to β-adrenergic receptors and digitalis receptors. Jpn Heart J 24, 215—235
58. Opstvedt J, Svaar H, Hansen P, Pettersen J, Langmark FT, Barlow SM (1978) Comparison of lipid status in the heart of piglets and rats on short term feeding of marine lipids and rapeseed oils. Lipids, 14, 356—371
59. Ortega A, Mas-Oliva J (1984) Cholesterol effect on enzyme activity of the sarcolemmal (Ca^{2+} + Mg^{2+}) ATPase from cardiac muscle. Biochim Biophys Acta 773, 231—236
60. Robblee NM, Clandinin MT (1984) Effect of dietary fat level and polyunsaturated fatty acid content on the phospholipid composition of rat cardiac mitochondrial membranes and mitochondrial ATPhase activity. J Nutr 114, 263—269
61. Roine PE, Uksila H, Teir H, Rapola J (1960) Histopathological changes in rats and pigs fed rapeseed oil. Zeitschrift Ernährungswissenschaften 1, 118—124
62. Rona G (1985) Editorial review: Catecholamine toxicity. J Mol Cell Cardiol 17, 291—306
63. Rothman JE, Lenard J (1977) Membrane asymmetry. The nature of membrane asymmetry provides clues to the puzzle of how membranes are assembled. Science 195, 743—753
64. Ruiter A, Jongbloed AW, Van Gent CM, Danse LHJC, Metz SHM (1978) The influence of dietary mackerel oil on the condition of organ and on blood lipid composition in the young growing pig. Am J Clin Nutr 31, 2159—2166
65. Scarpace PJ, O'Connor SW, Abrass IB (1985) Cholesterol modulation of β-adrenergic receptor characteristics. Biochim Biophys Acta 845, 520—525
66. Schrör K (1985) Prostaglandins and other eicosanoids in the cardiovascular system. Karger, Basel 1985, 1—6 (editorial)
67. Singer P, Jaeger W, Witth M, et al (1983) Lipid and blood pressure lowering effect of mackerel diet in man. Atherosclerosis 49, 99—108
68. Stam H, Koster JF (1985). Fatty acid peroxidation in ischemia. In: Schrör K (ed) Prostaglandins and other eicosanoids in the cardiovascular system. Karger, Basel, pp 131—148
69. Stubbs CD, Smith AD (1984) The modification of mammalian polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 779, 89—137
70. Swanson JE, Kinsella JE (1986) Dietary n-3 polyunsaturated fatty acids: modification of rat cardiac lipids and fatty acid composition. J Nutr 116, 514—523
71. Szuha BF, McCarl RL (1973) Fatty acid composition of rat hearts as influenced by age and dietary fatty acids. Lipids 8, 241—245
72. Tahin QS, Blum M, Carafoli E (1981) The fatty acid composition of subcellular membranes of rat liver, heart and brain: diet-induced modifications. Eur J Biochem 121: 5—13
73. Takamura H, Narita H, Urade R, Kito M (1986) Quantitative analysis of polyenoic phospholipid molecular species by high performance liquid chromatography. Lipids 21, 356—361
74. Ten Hoor F (1980) Cardiovascular effects of dietary linoleic acid. Nutrition and metabolism 24, 162—180
75. Van Vleet JF, Ferrans VJ (1977) Ultrastructure of hyaline microthrombi in myocardial capillaries of pigs with spontaneous "mulberry heart disease". Am J Vet Res 38, 2077—2080
76. Vles RO (1975) Nutritional aspects of rapeseed oil. In: Vergroesen AJ (ed) The role of fats in human nutrition Acad Press, London, New York, San Francisco, pp 433—477
77. Werns SW, Shea MJ, Lucchesi BR (1986) Free radicals and myocardial injury: pharmacologic implications. Circulation 74, 1—5
78. Yasuda S, Kitagawa Y, Sugimoto E, Kito M (1980) Effect of erucic acid on the phospholipid molecular species compositions of rat heart and liver. J Biochem, Tokyo, 87, 1511—1517
79. Yeagle PL (1985) Cholesterol and the cell membrane. Biochim Biophys Acta 822, 267—287

Authors' address:

J.M.J. Lamers, PhD, Department of Biochemistry I, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

CHAPTER 4

Comparison of mackerel-oil and lard-fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance, and morphology in young pigs[1–3]

Johannes M Hartog, MD; Jos MJ Lamers, PhD; Ad Montfoort, PhD; Anton E Becker, MD, PhD; Marjolein Klompe, PhD; Howard Morse, BS; Folkert J ten Cate, MD, PhD; Leonore van der Werf, BS; Willem C Hülsmann, MD, PhD; Paul G Hugenholtz, MD; and Pieter D Verdouw, PhD

ABSTRACT Purified mackerel-oil extract or lard fat (9.1% wt/wt) was added to a basal diet of young pigs for 8 wk. Effects on plasma lipids, glucose and insulin, cardiac membrane phospholipids, cardiovascular performance, and morphology were studied. A time-dependent reduction of plasma triglyceride (62%), total cholesterol (41%), and HDL cholesterol (47%) was found in the mackerel-oil-fed pigs. The postprandial glucose and insulin response may indicate a mackerel-oil-induced resistance of insulin receptors. Although the polyunsaturated fatty acid composition of cardiac sarcolemma widely differed between the two groups, all determined indices of heart function were equal. It is concluded that consumption of a fish-oil diet with a low content of monoenes and supplemented with antioxidants reduces plasma lipid levels without producing pathological side effects. *Am J Clin Nutr* 1987;46:258–66.

KEY WORDS Fish oil, lipids, phospholipids, cardiac function, morphology, pig, selenium, vitamin E, insulin, glucose

Introduction

Epidemiological studies have revealed direct correlations between heart-attack rate (and mortality) and the level of serum cholesterol. For almost 30 y it has been appreciated that polyunsaturated fatty acids can lower serum cholesterol. The hypocholesterolemic effect of vegetable oils, which contain predominantly ω-6 polyunsaturated fatty acids, has been attributed to the polyunsaturated:saturated fatty acid (P:S) ratio and to the cholesterol content of the oil (1).

Bang and Dyerberg (2, 3) showed that ingestion of fatty seafish, containing more ω-3 than ω-6 fatty acids, is also negatively related to morbidity and mortality of coronary heart disease. In some dietary studies in rats and pigs, the hypocholesterolemic effects of fish-oil-enriched food was equivalent to that of the vegetable-oil-enriched food despite the higher degree of unsaturation of the ω-3 fatty acids in fish oil (4–6). In chicken, fish oil was shown to be more hypocholesterolemic than a diet containing linolenic or linoleic acids (7).

In men the hypolipidemic effects of fish-oil and vegetable-oil diets have been reported to be similar (8, 9), but in these studies the relative amount of fish oil present in the food was lower than that of vegetable oil. Moreover, the fish-oil diet contained higher amounts of cholesterol than the vegetable-oil diet. This suggests that dietary ω-3 fatty acids are more potent than ω-6 fatty acids in reducing serum cholesterol. Indeed, a more marked hypocholesterolemic effect of fish oil has been observed in hyperlipidemic patients following diets with equal amounts of cholesterol and fat (10).

Some scepticism exists about the use of dietary fish oil to prevent atherosclerosis because rather high levels of cetoleic acid (C22:1 ω-11), an isomer of erucic acid (C22:1 ω-9) and gadoleic acid (C20:1 ω-9), are present. Erucic

[1] From the Laboratory for Experimental Cardiology (JMH, FJtC, LvdW, PGH, PDV), Thoraxcenter, Department of Biochemistry I (JMJL, WCH), and Department of Pathology I (AM), Erasmus University Rotterdam, Rotterdam; Department of Pathology (AEB), Academic Medical Center, Amsterdam; Oogziekenhuis (MK), Rotterdam; and Hope Farms (HM), Woerden, The Netherlands.

[2] Supported by a grant from the Dutch Heart Foundation.

[3] Address reprint requests to Pieter D Verdouw, PhD, Laboratory for Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands.

Received May 21, 1986.

Accepted for publication October 14, 1986.

acid has been shown to cause transient myocardial lipidosis and fibrosis in experimental animals (11, 12). However, in men these fatty acids could not be detected in the serum after intake of high levels of fish oil probably due to a poorer resorption or a more rapid metabolism (GP Walsh, unpublished observations).

Because of the high-unsaturation degree of the fish-oil fatty acids, an increased need for exogenous antioxidants is expected. Indeed, vitamin E deficiency causing *yellow-fat disease* (6) or even *mulberry-heart disease* (13) occurs in animals fed with fish oil. In one study yellow-fat disease could not be prevented by dietary supplement of vitamin E, but because the dietary selenium appears to be low in this report, the supplemented vitamin E may still be suboptimal (6). Fish oil, depending on origin of species and manner of preparation, may also contain high levels of vitamin A and D. Toxic effects of these vitamins can be expected after consumption of large amounts of fish oil. Recently, purified fish-oil extracts have been produced that are low in vitamins A and D, cetoleic acid, and gadoleic acid and to which optimal amounts of mixed tocopherols are added. The use of these extracts could circumvent most of the above-mentioned problems (14).

In this study we describe the effects of addition of a purified mackerel-oil extract, enriched in vitamin E and selenium, to the diet of young pigs. A control group of animals received a diet enriched with equal weight of lard fat. Plasma triglyceride and cholesterol were followed during the dietary period. Because it is known that dietary lipids affect fatty acid composition of cardiac membranes and thereby may cause changes in cardiac performance (15–17), sarcolemmal membranes, isolated from myocardial biopsies at the end of the feeding period, were characterized. Cardiac performance was recorded during and at the end of the nutritional period. At the end of the nutritional period, several organs were examined morphologically to see whether lipidosis or fibrosis of heart or liver, mulberry-heart disease, and yellow-fat disease had occurred.

Materials and methods

Experimental animals

Twenty-four newborn Yorkshire piglets (0.8–1.3 kg) of either sex were nourished by sows and supplied with pig starter gruel during the first 4 wk. During the first week the piglets received vitamin and iron injections. In the second week all boars were castrated. After 4 wk the pigs were separated from the sows and housed individually in slat-bottomed cages in temperature-controlled animal quarters. Subsequently they were divided arbitrarily over two groups, each group consisting of six male and six female piglets, and the two diets were followed for 8 wk. The National Research Council's guide for the care and use of laboratory animals was followed.

Experimental diets

The basal diet of both experimental groups was identical. The composition of the basal diet was described by Ruiter et al (6) except for the selenium and vitamin E contents. The present basal diet was prepared by Hope Farms (Woerden, The Netherlands) and low in fat (< 2% wt/wt) although sufficient amounts of linoleic acid remained present. For the control animals 9.1% wt/wt lard fat (Gebro Smilde BV, Heerenveen, The Netherlands) and 0.01% wt/wt mixed tocopherols were added to the basal diet. For the experimental animals 9.1% wt/wt mackerel oil (AS Johan C Martens en Co, Bergen, Norway) and 0.01% wt/wt mixed tocopherols were added to the basal diet. Care was taken to minimize oxidation of mackerel-oil fatty acids before consumption. To this end the mackerel oil, delivered in vacuum-sealed cans, was divided into small batches for daily use and sealed under nitrogen gas. The compositions of the two isocaloric diets are shown in **Tables 1 and 2.** A difference in the cholesterol content was noticed but, because of the rather low amounts, no adjustment was made. The animals were fed in the morning and the portions were consumed within 1 h. To avoid problems during adaptation to the new diet, the animals were fed only 200 g/d during the first week. In the following weeks the daily food intake was gradually increased equally in both groups to 800 g/d in the last week. No scours occurred during the experimental period.

The day before the start of the experimental dietary period the 24-h fasted animals were anesthetized with im injections of 30 mg/kg ketamine (Aescoket®, Aesculaap BV, Boxtel, The Netherlands) and inhalation of a mixture of O_2:N_2O (1:2) to which 1% halothane (Fluothane®, Macclesfield, UK) was added. Echocardiography was carried out using a two-dimensional apparatus (77020A Hewlett-Packard Ultrasound System, Andover, MA) with a 5 MHz transducer of 32 elements giving an examination sector of 90°. The two-dimensional images were recorded

TABLE 1
Composition (g/100 g) of the two experimental diets

	Lard-fat diet	Mackerel-oil diet
Corn (extruded)	32	32
Wheat (extruded)	18	18
Soybean meal	14	14
Wheat middlings	9	9
Dehydrated skimmed-milk powder	14	14
$CaHPO_4 \cdot 2H_2O$	1.3	1.3
CaCo	1.1	1.1
$NaCl^3$, iodized	0.3	0.3
MgO	0.05	0.05
$MgSO_4$	0.05	0.05
$KH_2PO_4 \cdot 2H_2O$	0.36	0.36
Choline chloride 50%	0.18	0.18
Vitamin C coated	0.02	0.02
Vitamin and trace-element mixes*	0.68	0.68
Lard fat	9.1	—
Mackerel oil	—	9.1
Mixed tocopherols	0.01	0.01
Cholesterol	0.01	0.03

* Vitamin and trace element mixes supply the following per 100 g food: vitamin A, 1400 IU; cholecalciferol, 140 IU; vitamin E, 8 mg; menadione, 0.2 mg; thiamin hydrochloride, 1.8 mg; riboflavin, 1.8 mg; pyridoxine hydrochloride, 1.4 mg; niacin, 3.6 mg; *d*-Ca pantothenate, 3.6 mg; folic acid, 0.4 mg; vitamin B-12, 0.004 mg; biotin, 0.1 mg; inositol, 4.5 mg; Iron subcarbonate (57% Fe), 9.1 mg; $FeSO_4 \cdot H_2O$ (30% Fe), 14 mg; Copper carbonate (55% Cu), 2.3 mg; ZnO (78% Zn), 11 mg; MnO (62% Mn), 9.1 mg; sodium selenite (45% Se), 0.08 mg; Ca $(IO_3)_2$ (65% I), 0.2 mg; and $CoCO_3$ (47% Co), 0.09 mg.

TABLE 2
Fatty acid composition (%) of the two experimental diets

Fatty acids	Lard-fat diet	Mackerel-oil diet
14:0	2	7
16:0	24	18
16:1	3	8
18:0	10	1
18:1	42	17
18:2 ω-6	15	8
18:3	1	1
20:1	1	6
20:5 ω-3	—	17
22:1	—	4
22:6 ω-3	—	9
24:1	—	1
others	2	3

on videotape (Panasonic VHS, Matsushita Electric Trading Ltd, Osaka, Japan) with a rate of 30 frames/s. Both parasternal long-axis and short-axis cross sections at several levels of the heart (mitralvalve, papillary muscles, apex) were used. In addition, we recorded M-mode echo tracings of the anterior wall of the left ventricle. Electrocardiographic recordings were made via Einthoven leads I, II, and III. This procedure was repeated every 2 wk during the dietary period to screen for disturbances in left ventricular wall motion and other signs of selenium-vitamin E deficiency (ie, hydropericardium).

Chemical analysis of plasma during the dietary period

At the time of the echocardiografic recordings, a 10 mL bloodsample was taken by puncturing the subclavian vein. Part of the blood sample was used for determination of hemoglobin (OSM 2 Hemoximeter, Radiometer, Copenhagen, Denmark) and the remainder was centrifuged. The plasma was frozen in liquid nitrogen and stored at −80°C.

After 8 wk a catheter was placed in the right internal jugular vein in eight animals of each group. This was done under halothane anesthesia after pretreatment with an im injection of a mixture of 300 000 U procain penicillin-G and 300 000 U bezathin penicillin-G (Duplocilline®, Gist Brocades, Delft, The Netherlands). The catheters were flushed twice a day with 4 mL saline containing 20 IU heparin. Two days after insertion of the catheter the animals were starved for 24 h. After withdrawal of a bloodsample the animals received their daily food, which was consumed by all animals within 1 h. Subsequent bloodsamples were taken at hourly intervals for 8 h postprandially.

Cholesterol, triglyceride, free fatty acids, and glucose concentrations were determined in the plasma. Glucose (18) and cholesterol (19) were assayed spectrophotometrically with hexokinase and cholesterol oxidase-peroxidase reactions, respectively, with test combinations from Boehringer (Mannheim, FRG). Insulin was measured by radioimmunoassay with an Ins-Ria-100 kit (IRE-Medgenix, Fleurus, Belgium) using human insulin standards, which have a 100% cross reactivity with porcine insulin. Triglycerides (20) were estimated using the peroxidase reaction-coupled assay by the Test Combination 17 of Biomed (Oberschleissheim, FRG). The HDL fraction was determined after centrifugal separation (13 000 g, 5 min) of the Mn^{2+}-heparin precipitate (21). The cholesterol content of the HDL-containing supernatant was determined enzymatically (19).

On the next day the procedure of postprandial follow-up was repeated. After withdrawal of a 4-h postprandial blood sample, an intravenous bolus of heparin (50 IU/kg) was administered to induce the release of lipoprotein lipase into the blood. Four minutes after heparin administration a blood sample was collected and the lipoprotein lipase activity was measured with radioactive labeled triolein as a substrate (22). At the end of the dietary period, plasma levels of selenium, α-tocopherol, and vitamin A were determined (23, 24).

Hemodynamic evaluation after the dietary period

After ~8 wk all animals were sedated with 120 mg azaperone (Stresnil®, Janssen Pharmaceutica, Beerse, Belgium) im and 15 min later were anesthetized with 150 mg metomidate (Hypnodil®, Janssen Pharmaceutica, Beerse, Belgium) and intravenous pentobarbital (20 $mg \cdot kg^{-1} \cdot min^{-1}$). Subsequently, the animals were intubated and connected to a respirator for artificial ventilation with a mixture of O_2:N_2O (1:2). Left ventricular and central aortic pressures were obtained from microtipped Millar catheters. After exposure of the heart via a midsternal split, electromagnetic flow probes (Skalar, Delft, The Netherlands) were placed around the ascending aorta and the proximal left anterior descending coronary artery (LAD) and the great cardiac vein was cannulated. Myocardial wall thickness was monitored with an epicardial 5 MHz ultrasonic transducer (Krautkramer-Branson, Lewistown, PA). From the tracings systolic wall thickening (swt) was calculated by

$$swt(\%) = 100 \times (EST - EDT)/EDT \quad (1)$$

in which EST and EDT are the wall thickness at end-systole and at end-diastole, respectively. The mean velocity of systolic wall thickening (vswt) was calculated by

$$vswt(mm/s) = (EST - EDT)/LVET \quad (2)$$

in which LVET is the left ventricular ejection time in seconds. Hemodynamic measurements were obtained after a stabilization period of at least 30 min. Except for anesthetics, heparin, and antibiotics no other drugs have been used in this study.

Chemical analysis of myocardial biopsies taken at the end of the dietary period

After completion of the hemodynamic measurements, the heart was excised and rapidly cooled in ice. The posterior wall of the left ventricle was cut out and homogenized in buffer and heart sarcolemmal membranes were separated (25). The phospholipids were extracted from the sarcolemmal membranes with 19 vol of a mixture of chloroform:methanol (2:1 vol/vol) according to the method of Folch (26). The phospholipids were separated from the other lipids by thin-layer chromatography. The developing system was a mixture of hexane:ether (70:3 vol/vol). The plates were washed with the same mixture and activated at 110°C for 30 min. Phospholipid hydrolysis, formation of fatty acid methylesters, subsequent extraction of these methylesters, and gas-chromatographic separation have all been described. (16).

Morphological examination

Tissue samples of the heart, aorta, liver, spleen, kidneys, adrenal, lymph nodes, skeletal muscle, subcutaneous fat, and perirenal fat tissue were fixed in 10% formalin for morphological examination. The tissues were routinely processed and embedded in paraffin. Sections were cut at 7 μm thickness and stained with hemotoxilline and eosine, alcian blue, periodic acid Schiff (with and without diastase digestion), and different connective tissue stains. Oil red O and sudan III stains were used on formalin-

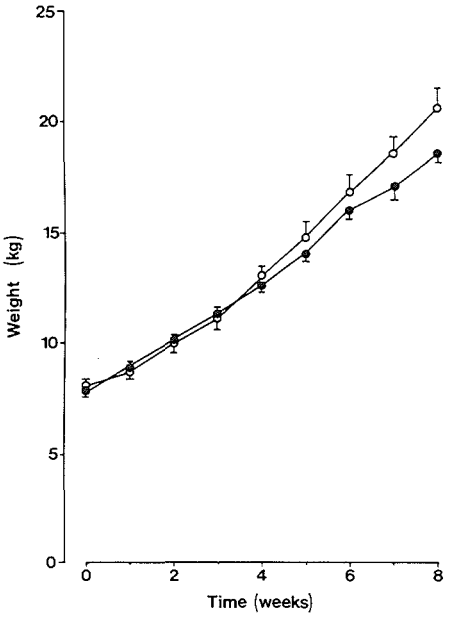

FIG 1. Weight gain of the animals during the dietary period. ● = lard-fat-fed animals (n = 12); ○ = mackerel-oil-fed animals (n = 12).

fixed frozen sections. Additional stains, such as fibrin stains, were used only when considered necessary.

Statistical analysis

In many cases data are expressed as percentage of values obtained at the beginning of the dietary period. For comparison of the values at the different times, the Student's t test was used. For statistical evaluation of the difference between the two dietary groups, analysis of variance was used. Statistical significance was accepted at $p < 0.05$ (two-tailed). All data have been expressed as mean ± SEM.

Results

Performance of the pigs during the dietary period

The growth patterns of the two groups of animals are shown in **Figure 1**. During the first 4 wk, the weight gain was nearly identical in the lard-fat- and mackerel-oil-fed animals and then the mackerel-oil-fed animals gained weight faster than the lard-fat-fed animals. The electrocardiographic recordings and the analysis of the echocardiograms showed no abnormalities in either group during the feeding period. At the end of the dietary period, the hemoglobin content of both groups of animals had stabilized at slightly lower than predietary values (−24 ± 2% vs −23 ± 2% in the lard-fat- and mackerel-oil-fed pigs, respectively, **Table 3**).

Alpha-tocopherol was not detectable in the plasma of both animal groups. Plasma selenium was 136 ± 8 μg/L vs 143 ± 8 μg/L (1.72 ± 0.1 μmol/L vs 1.81 ± 0.1 μmol/L) in the lard-fat- and mackerel-oil-fed animals, respectively. The plasma vitamin A level was significantly higher in the mackerel-oil-fed animals (392 ± 49 μg/L or 13.7 ± 1.7 μmol/L) than in the lard-fat-fed animals (278 ± 20 μg/L or 9.7 ± 0.7 μmol/L).

Plasma levels of lipids and glucose during the dietary period

The plasma levels of triglyceride, cholesterol (total and HDL fraction), and glucose, determined in starved animals, are presented in **Figure 2.** In the lard-fat-fed animals plasma triglyceride levels decreased by 20 ± 4% ($p < 0.05$) after 2 wk but returned later to predietary values. During the first 2 wk of the dietary period, plasma triglycerides in the mackerel-oil-fed animals decreased by 40 ± 8% ($p < 0.05$) and after 8 wk the total decrease was 62 ± 6% ($p < 0.05$ vs 2-wk value). Plasma cholesterol increased slightly in the lard-fat-fed animals (+23 ± 7%, $p < 0.05$) but decreased in the mackerel-oil-fed animals (−28 ± 7%, $p < 0.05$, after 2 wk and −41 ± 4%, $p < 0.05$ vs 2-wk value). HDL cholesterol increased by 29 ± 8% ($p < 0.05$) in the lard-fat-fed pigs but decreased by 47 ± 5% ($p < 0.05$) in the mackerel-oil-fed animals. However, the HDL:total cholesterol ratio remained 0.48 in both groups. The plasma glucose levels of the starved animals remained constant at ~5 mM in both groups during the entire dietary period, and because no differences existed between the two groups, the data were pooled.

Postprandial increase of plasma triglyceride level determined after 8 wk showed a similar pattern for both groups with peak values ~5 h (+183 ± 64% for the lard-fat-fed animals vs +247 ± 86% for the mackerel-oil-fed animals, **Fig 3**). The postprandial curve of free fatty acids of the lard-fat-fed animals peaked sharply again ~5 h postprandially (+283 ± 121%). However, no sharp, defined maximum was observed in the free fatty acid curve

TABLE 3
Blood hemoglobin content (mM) of animals in the two experimental groups during the dietary period

	Week				
	0	2	4	6	8
Lard-fat diet (n = 12)	8.6 ± 0.2	8.1 ± 0.2*	7.7 ± 0.1*	7.7 ± 0.1*	6.4 ± 0.1*
Mackerel-oil diet (n = 12)	8.9 ± 0.1	7.5 ± 0.2*	7.5 ± 0.2*	6.9 ± 0.2*	6.9 ± 0.2*

* = $p < 0.05$ vs predietary value.

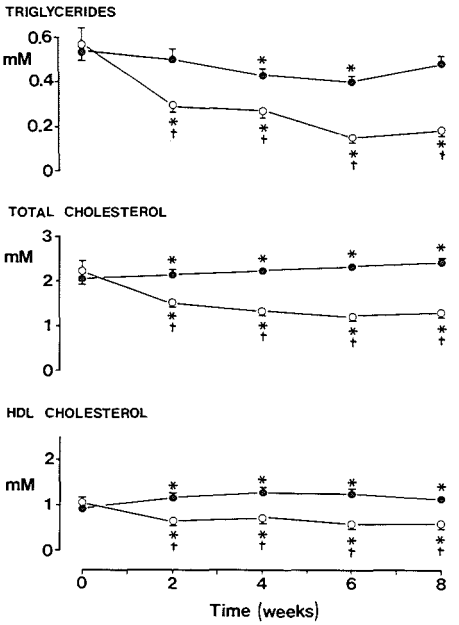

FIG 2. Plasma lipids during the dietary period. ● = lard-fat-fed animals ($n = 12$); ○ = mackerel-oil-fed animals ($n = 12$); * = $p < 0.05$ vs predietary value; † = $p < 0.05$ vs lard-fat-fed animals.

of the mackerel-oil-fed pigs as these animals exhibited a plateau which lasted ~5 h. Plasma cholesterol showed a minor response to the food intake in both dietary groups as can be expected from the low-cholesterol content of either diet. Because of the difference in plasma triglyceride levels between the two groups at the end of the dietary period, it was of interest to know the clearing factor activity when peak plasma triglyceride level was reached. The heparin releasable lipoprotein lipase showed the same activity in both groups: 168 ± 17 mU/mL vs 188 ± 18 mU/mL (2.80 ± 0.28 μkat/L vs 3.13 ± 0.30 μkat/L) plasma for the mackerel-oil- and lard-fat-fed pigs, respectively.

In the lard-fat diet group the plasma glucose level was unchanged after the food intake, but in the mackerel-oil group it had increased by 45 ± 12% ($p < 0.05$ vs lard-fat-fed animals) 2 h after food intake. Insulin levels also were determined in these samples. The preprandial insulin levels were similar in both groups (6.6 ± 1.0 mU/L or 47 ± 7 pmol/L in the lard-fat- and 7.7 ± 1.3 mU/L or 55 ± 9 pmol/L in the mackerel-oil-fed animals), but the insulin level 2 h after the meal was significantly higher in the mackerel-oil-fed animals (34.4 ± 4.3 mU/L vs 23.6 ± 3.6 mU/L or 247 ± 31 vs 169 ± 26 pmol/L in the lard-fat-fed group).

Fatty acid pattern of cardiac membrane phospholipids

The P:S ratio of fatty acids esterified in cardiac sarcolemmal phospholipids did not differ significantly between groups of animals (0.76 ± 0.06 vs 0.81 ± 0.06 in the lard-fat- and mackerel-oil-fed animals, respectively). However, as illustrated in **Figure 4**, the distribution of the polyunsaturated fatty acid molecules became markedly different. In the mackerel-oil-fed animals the ω-6:ω-3 ratio was 0.69 ± 0.3 vs 17.9 ± 2.2 ($p < 0.05$) in the lard-fat-fed animals. This results in a wide variation of double-bond indices: 1.85 ± 0.06 and 1.28 ± 0.06 ($p < 0.05$) for mackerel-oil- and lard-fat-fed animals, respectively.

Global hemodynamics at the end of the dietary period

No significant differences in blood pressure, left ventricular filling pressure, cardiac output, myocardial contractility, and peripheral resistance were found between the two experimental groups (**Table 4**). Regional myocardial performance expressed as coronary blood flow, coronary venous oxygen saturation, and systolic wall thickening were not different between the different groups. The quantitative analysis of regional wall function at the end of the dietary period was in agreement with the qual-

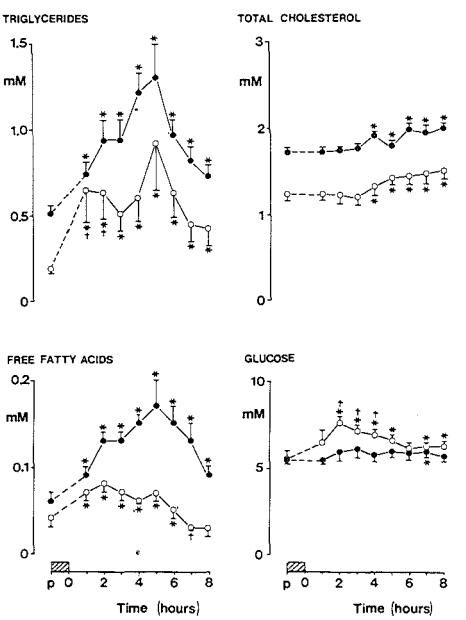

FIG 3. Postprandial responses of plasma triglyceride, free fatty acids, total cholesterol, and glucose. p = preprandial value; ▨ = feeding period; ● = lard-fat-fed animals ($n = 8$); ○ = mackerel-oil-fed animals ($n = 8$); * = $p < 0.05$ vs preprandial value; † = $p < 0.05$ vs lard-fat-fed animals.

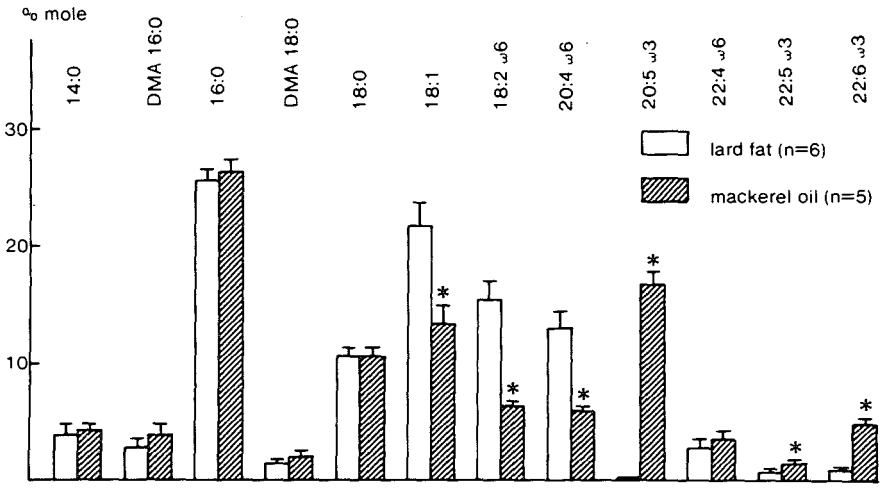

FIG 4. Fatty acid pattern of heart sarcolemma phospholipids in lard-fat- and mackerel-oil-fed animals. * = $p < 0.05$ vs lard-fat-fed animals. DMA = dimethylated acetal.

itative analysis of the two-dimensional and M-mode echorecordings made during the dietary period.

Morphology

Macroscopic evaluation of the different organs revealed no signs of liver enlargement or hypertrophy of the adrenal glands. Microscopic examination did not provide any evidence for yellow-fat disease, cardiac or hepatic lipidosis, and fibrosis.

Discussion

The markedly lower plasma triglyceride levels in the animals on the mackerel-oil diet agree with other reports (4–9). However, at variance with these studies, we found a dramatic reduction of plasma cholesterol content. Phillipson et al (10) observed similar hypocholesterolemic effects in a study with hyperlipidemic patients who consumed about the same daily dose of fish oil, but the effect of a lower dose of fish oil in normolipidemic man was

TABLE 4
Hemodynamic values after the 8-wk dietary period

		Lard-fat diet ($n = 8$)	Mackerel-oil diet ($n = 8$)
Heart rate	(b/min)	101 ± 8	106 ± 15
Systolic arterial blood pressure	(mmHg)	105 ± 5	111 ± 6
Diastolic arterial blood pressure	(mmHg)	69 ± 3	78 ± 6
Mean arterial blood pressure	(mmHg)	89 ± 4	94 ± 6
Left ventricular end-diastolic pressure	(mmHg)	8.3 ± 0.9	10.2 ± 1.5
Stroke volume	(mL)	18 ± 1	19 ± 2
Cardiac output	(L/min)	1.9 ± 0.2	1.8 ± 0.1
Peak rate of rise in left ventricular pressure	(mmHg/s)	2290 ± 340	2080 ± 300
Systemic vascular resistance	(mmHg · min/L)	50 ± 3	53 ± 4
Coronary blood flow	(mL/min)	34 ± 5	36 ± 7
Coronary venous blood oxygen saturation	(%)	21 ± 2	23 ± 3
End-diastolic wall thickness	(mm)	11.4 ± 0.5	11.0 ± 0.9
End-systolic wall thickness	(mm)	15.4 ± 0.6	14.5 ± 0.9
Systolic wall thickening	(%)	36 ± 3	33 ± 3
Mean velocity of systolic wall thickening	(mm/s)	6.3 ± 0.6	6.0 ± 0.5

less (9). The decrease in cholesterol took place predominantly in the first 2 wk (−28 ± 7%), but at the end of the dietary period the total decrease of plasma cholesterol was 41 ± 4% ($p < 0.05$ vs 2-wk value). The HDL fraction of plasma cholesterol fell similarly (−47 ± 5%) but the HDL: total-cholesterol ratio was unchanged (0.48 ± 0.04). The values for HDL:total-cholesterol ratio in the pig are rather high as compared with those found in men (0.25). In a parallel study of animals receiving only 4.6% wt/wt mackerel oil in their diet, the reduction in total cholesterol was less, but the HDL cholesterol was unchanged, resulting in a HDL:total-cholesterol ratio of 0.49 ± 0.02. The large decrease in total cholesterol in animals receiving 9.1% wt/wt mackerel oil in their diet probably leads to a reduction in HDL cholesterol. The lard-fat diet, containing mainly saturated fatty acids, only slightly influenced the plasma cholesterol and triglyceride levels during the nutritional period.

It is unlikely that a difference in the rate of fat resorption explains the hypolipidemia after mackerel-oil nutrition because postprandial responses of plasma lipids after a 24-h period of starving were similar in both diet groups. The changes in plasma lipids in mackerel-oil-fed animals are probably also not due to a different rate of plasma lipid breakdown because the activity of the postheparin lipoprotein lipase, measured 4 h after a morning meal, was similar in both nutritional groups. Changes in the composition of the lipoprotein particles may account for enhanced clearance (8) but a more likely cause is a decreased rate of VLDL synthesis after fish-oil nutrition (27–30). The hypolipidemic effect of mackerel oil probably is dose-dependent because in animals receiving 4.5% wt/wt mackerel oil mixed with 4.5% wt/wt lard fat in their diet plasma cholesterol was lowered by 19 ± 8% compared with 41 ± 4% for the 9.1% wt/wt mackerel-oil-fed animals, and plasma triglycerides fell by 49 ± 8% vs 67 ± 6% in the 9.1% wt/wt mackerel-oil fed group (compare Fig 2). Therefore it is not surprising that in human studies in which lower doses of fish oil are used, no effect on plasma cholesterol level is observed (28–30).

The postprandial response of plasma glucose was significantly higher in the mackerel-oil-fed pigs. The insulin levels in plasma, however, were significantly higher in the mackerel-oil-fed animals. It has been suggested that insulin receptor mobility in a more fluid membrane bilayer of fatty acids with a high double bond index is increased (31–33). As long as data on membrane cholesterol:phospholipid ratio and spinprobe mobilities are not available, fluidities of membranes with a different polyunsaturated fatty acid composition are difficult to predict (34). Others have reported increased insulin receptor sensitivity after fish-oil supplementation although membrane fluidity was unchanged (35). An alternative factor producing changes in plasma glucose could be an increased intestinal resorption of glucose due to variations in membrane fluidity. A relatively slow fatty acid resorption after a saturated fatty acid load has been reported previously (36). This may result in a prolonged increase of gastric inhibitory polypeptide release, which promotes insulin release and stimulates the lipoprotein lipase reaction thereby increasing free fatty acid levels in plasma. The postprandial plasma lipid response of the mackerel-oil-fed animals, however, did not indicate a slower rate of fatty acid absorption and plasma free fatty acids increased to less extent (Fig 3).

Different effects of polyunsaturated fatty acids on arterial blood pressure have been reported. A hypotensive effect of linoleic acid has been found in rat (37–40) and human (41–48). More recently, a similar effect has been shown for fish oils in animal experiments (49) and in human studies (50–52). The fall in blood pressure could be explained by changes in the synthesis of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the kidneys (39, 53). Scherhag (54) on the other hand claimed that diets providing fish oil induce hypertension. We found no differences in blood pressure between the lard-fat- and mackerel-oil-fed animals during anesthesia. This is in agreement with the results reported by others for studies in man using diets with high P:S ratios (55, 56).

Dietary linoleic acid in rats augments coronary flow and contractility of the Langendorff perfused heart (15). In this in vivo study no differences between myocardial contractility, systolic wall thickening, and coronary blood flow of both dietary groups were detectable despite marked changes in polyunsaturated fatty acid composition of cardiac membranes.

The decrease in blood hemoglobin content is probably age dependent and not diet induced as in the experiments by Ruiter et al (6). In a parallel series with a longer dietary period, a similar decrease was found after 8 wk but the hemoglobin content returned to baseline value after 16 wk. Although the plasma levels of vitamin A were significantly higher in animals after the mackerel-oil diet (392 ± 49 μg/L or 13.7 ± 1.7 μmol/L), the levels are still within the normal range for men (200–500 μg/L or 3.5–17.5 μmol/L).

No signs of vitamin E deficiency nor of any other pathology (11, 12) were found after morphological examination of several organs of the mackerel-oil-fed pigs. The low content of monoenes in the mackerel-oil preparation used is one favorable factor. Furthermore, the present diets were not only enriched in vitamin E but also in selenium (Table 1). This was not the case in the diets used by Ruiter et al (6), who observed that increased supply only of vitamin E did not prevent yellow-fat disease.

In conclusion, this study shows that a mackerel-oil diet markedly lowered plasma lipids, in particular total cholesterol. In cardiac membrane phospholipids ω-6 fatty acids were partially replaced by ω-3 fatty acids. These changes in fatty acid composition of sarcolemma did not lead to any variation in cardiac function. Moreover, pathological side effects of mackerel-oil nutrition were not found. The favorable effects of consumption of purified mackerel-oil extracts by the pig, an animal which is very sensitive for vitamin E deficiency, indicate that high dosage of fish oil can be applied, provided that selenium and vitamin E delivery is controlled.

The authors wish to thank Prof Dr AJ Vergroesen (Department of Biochemistry I) for encouragement and advice. The staff of the Laboratory for Surgery is thanked for their assistance.

References

1. Illingworth DR, Connor WE. Present status of polyunsaturated fats in the prevention of cardiovascular disease. In: Santos WJ, Lopes N, Barbosa JJ, Chavez D, Valente JC, eds. Nutrition and food science. Vol 3. New York, NY: Plenum Publ Corp, 1980:365–78.
2. Bang HO, Dyerberg J. Lipid metabolism in Greenland Eskimos In: Draper HH, ed. Advanced nutrition research. Vol 3. New York, NY: Plenum Press, 1980:1–22.
3. Bang HO, Dyerberg J, Nielsen AB. Plasma lipid and lipoprotein pattern in Greenlandic west-coast Eskimos. Lancet 1971;1:1143–5.
4. Hauge JG, Nicolaysen R. The serum cholesterol depressive effect of linoleic, linolenic acids and of cod liver oil in experimental hypercholesterolaemic rats. Acta Physiol Scand 1959;45:326–30.
5. Peifer JJ, Lundberg WO, Ishio S, Warmanen E. Studies of the distribution of lipids in hypercholesterolemic rats. 3 Changes in hypercholesteremia and tissue fatty acids induced by dietary fats and marine oil fractions. Arch Biochem Biophys 1965;110:270–83.
6. Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM. The influence of dietary mackerel oil on the condition of organs and on blood lipid composition in the young growing pig. Am J Clin Nutr 1978;31:2159–66.
7. Kahn SG, van de Putte J, Wind S, Yacowiz H. A study of the hypercholesterolemic activity of the ethyl esters of the polyunsaturated fatty acids of cod liver oil in the chicken. 1 Effect on total serum cholesterol. J Nutr 1963;80:403–13.
8. Goodnight SH, Harris WS, Connor WE, Illingworth DR. Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. Arteriosclerosis 1982;2:87–113.
9. Harris WS, Connor WE, McMurry MP. The comparative reduction of plasmalipids and lipoproteins by dietary polysaturated fats: salmon-oil versus vegetable oils. Metabolism 1983;32:179–84.
10. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR. Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. N Engl J Med 1985;312:1210–6.
11. Food and Agriculture Organization of the United Nations. Dietary fats and oils in human nutrition, a joint FAO WHO report. Rome, Italy: FAO, 1977. (FAO Food and Nutrition Paper No 3.)
12. Beare-Rogers JL. Docosenoic acids in dietary fats. Prog Chem Fats Other Lipids 1977;15:29–56.
13. Van Vleet JF, Ferrans VJ. Ultrastructure of hyaline microthrombi in myocardial capillaries of pigs with spontaneous "mulberry heart disease." Am J Vet Res 1977;38:2077–80.
14. Sanders TAB, Roshanai F. The influence of different types of w-3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. Clin Sci 1983;64:91–9.
15. De Deckere EAM, Ten Hoor F. Influences of dietary fats on coronary flow rate and left ventricular work of the isolated rat heart: sunflower seed oil versus lard. Nutr Metab 1980;24:396–408.
16. Montfoort A, van der Werf L, Hartog JM, et al. The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol, 1986;81:289–302.
17. Charnock JS, McLennan PL, Abeywardena MY, Dryden WF. Diet and cardiac arrhythmia: effects of lipids on age-related changes in myocardial function in the rat. Ann Nutr Metab 1985;29:306–18.
18. Schmidt FH. Die enzymatische Bestimmung von Glucose und Fructose nebeneinander. Klin Wochenschr 1961;23:1244–7.
19. Siedel J, Schlumberger H, Klose S, Ziegenhorn J, Wahlefeld AW. Improved reagent for the enzymatic determination of serum cholesterol. J Clin Chem Clin Biochem 1981;19:838–9.
20. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 1982;280:2077–80.
21. Warnick GR, Albers JJ. A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. J Lipid Res 1978;19:65–76.
22. Nilsson-Ehle P, Schotz MC. A stable, radioactive substrate emulsion for assay of lipoprotein lipase. J Lipid Res 1976;17:536–41.
23. de Bruin M, Korthoven PJM, Bode P. Evaluation of a system for routine instrumental neutron activation analysis. J Radioanalyt Chem 1982;70:497–512.
24. Bieri JG, Tolliver TJ, Catignani GL. Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. Am J Clin Nutr 1979;32:2143–9.
25. Lamers JMJ, de Jonge-Stinis JT, Hülsmann WC, Verdouw PD. Reduced in vitro ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial ischaemia in anaesthetized pigs. J Mol Cell Cardiol 1986;18:115–25.
26. Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.
27. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL. The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochim Biophys Acta 1984;792:103–9.
28. Nestel PJ, Connor WE, Reardon MF. Suppression by diets rich in fish-oil of very low density lipoprotein production in man. J Clin Invest 1984;74:82–9.
29. Sanders TAB, Sullivan DR, Reeve J, Thompson GR. Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. Arteriosclerosis 1985;5:459–65.
30. Simons LA, Hickie JB, Balasubramaniam S. On the effects of dietary n-3 fatty acids (Maxepa) on plasma lipids and lipoproteins in patients with hyperlipidaemia. Atherosclerosis 1985;54:75–88.
31. Ginsberg BH, Brown TJ, Simon I, Spector AA. Effect of the membrane lipid environment on the properties of insulin receptors. Diabetes 1981;30:773–80.
32. Gould RJ, Ginsberg BH, Spector AA. Lipid effects on the binding properties of a reconstituted insulin receptor. J Biol Chem 1982;257: 477–84.
33. Ginsberg BH, Jabour J, Spector AA. Effect of alterations in membrane lipid unsaturation on the properties of the insulin receptor of Ehrlich ascites cells. Biochim Biophys Acta 1982;690:157–64.
34. Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984;779:89–137.
35. Popp-Snijders C, Schouten JA, Heine RJ, van der Veen EA. Dietary supplementation of omega-3 fatty acids improves insulin sensitivity in non-insulin dependent diabetes. Diabetes Res Clin Practice 1985;1: S451.
36. Hülsmann WC, Kort WJ. Saturated fat feeding, hyperlipidemia and hyperinsulinemia. Biochim Biophys Acta 1983;754:231–7.
37. Triebe G, Block HU, Förster W. On the blood pressure response of salt-loaded rats under different content of linoleic acid in the food. Acta Biol Med Ger 1976;35:1223–4.
38. Ten Hoor F, Van de Graaf HM. The influence of a linoleic acid-rich diet and of acetyl salicylic acid on NaCl-induced hypertension, Na^+- and H_2O-balance and urinary prostaglandin excretion in rats. Acta Biol Med Ger 1978;37:875–7.
39. Weber PC, Siess W, Lorenz R, Scherer B. The role of prostaglandins in essential hypertension. Int J Obes 1981;5(suppl I):125–30.
40. Düsing R, Scherhag R, Glänzer K, Budde U, Kramer HJ. Dietary linoleic acid deprivation: effects on blood pressure and PGI_2 synthesis. Am J Physiol 1983;244:H228–33.
41. Iacono JM, Marshall MW, Dougherty RM, Wheeler MA, Mackin JF, Canary JJ. Reduction of blood pressure associated with high polyunsaturated fat diets that reduce blood cholesterol in man. Prev Med 1975;4:426–43.
42. Comberg HU, Heyden S, Hames CG, Vergroesen AJ, Fleischman

AI. Hypotensive effect of dietary prostaglandin precursor in hypertensive men. Prostaglandins 1978;15:193–7.
43. Vergroesen AJ, Fleischman AI, Comberg HU, Heyden S, Hames CG. The influence of increased dietary linoleate on essential hypertension in man. Acta Biol Med Ger 1978;37:879–83.
44. Oster P, Arab L, Schellenberg B, Heuck C, Mordasini R, Schlierf G. Linolsäure in der Diätbehandlung der Hypertonie. Ernährungs-Umschau 1980;27:143–4.
45. Stern B, Heyden S, Miller D, Latham G, Klimas A, Pilkington K. Intervention study in high school students with elevated blood pressures. Dietary experiment with polyunsaturated fatty acids. Nutr Metab 1980;24:137–47.
46. Rao RH, Rao U, Srikantia SG. Effect of polyunsaturate-rich vegetable oils on blood pressure in essential hypertension. Clin Exp Hypertens 1981;3:27–38.
47. Puska P, Iacono JM, Nissinen A, et al. Controlled, randomised trial of the effect of dietary fat on blood pressure. Lancet 1983;i:1–5.
48. Iacono JM, Puska P, Dougherty RM. Dietary fat and blood pressure. In: Horan MJ, Blaustein M, Kahadorian W, Dunbar JB, Kaplan NM, Simopoulos AP, eds. Proceedings, NIH Workshop, Nutrition and Hypertension. New York, NY: Biomedical Information Corp, 1985:305–23.
49. Lockette WE, Webb RC, Culp BR, Pitt B. Vascular reactivity and high dietary eicosapentaenoic acid. Prostaglandins 1982;24:631–9.
50. Sanders TAB, Vickers M, Haines AP. Effect on blood lipids and haemostasis of a supplement of cod-liver oil, rich in eicosapentaenoic and docosahexaenoic acids, in healthy young men. Clin Sci 1981;61: 317–24.
51. Singer P, Voigt S, Gödicke W. Inverse relationship between linoleic acid in serum and in adipose tissue of patients with essential hypertension. Prostaglandins Leukotrienes Med 1982;9:603–13.
52. Lorenz R, Spengler U, Fischer S, et al. Platelet function, thromboxane formation and blood pressure control during supplementation of the western diet with cod-liver oil. Circulation 1983;67:504–11.
53. Tobian L, O'Donnell M. Renal prostaglandin in relation to sodium regulation and hypertension. Fed Proc 1976;35:2388–92.
54. Scherhag R, Kramer HJ, Düsing R. Dietary administration of eicosapentaenoic and linolenic acid increases arterial blood pressure and suppresses vascular prostacyclin synthesis in the rat. Prostaglandins 1982;23:369–82.
55. Brussaard JH, van Raaij JMA, Stasse-Wolthuis M, Katan MB, Hautvast JGAJ. Blood pressure and diet in normotensive volunteers: absence of an effect of dietary fiber, protein, or fat. Am J Clin Nutr 1981;34:2023–9.
56. Iacono JM, Dougherty RM, Puska P. Reduction of blood pressure associated with dietary polyunsaturated fat. Hypertension 1982;4: 34–42.

CHAPTER 5

Dietary Mackerel Oil in Pigs: Effect on Plasma Lipids, Cardiac Sarcolemmal Phospholipids and Cardiovascular Parameters[1]

JOHANNES M. HARTOG, PIETER D. VERDOUW,[2] MARJOLEIN KLOMPE AND JOS M.J. LAMERS†*

*Laboratory for Experimental Cardiology, Thoraxcenter, *Oogziekenhuis, †Department of Biochemistry I, Erasmus University Rotterdam, 3000 DR Rotterdam, The Netherlands*

ABSTRACT The influence of substitution of eicosapentanoic acid to the diet has been investigated in juvenile domestic pigs (7–8 kg) fed either a mixture of 4.5% (wt/wt) mackerel oil and 4.5% (wt/wt) lard fat (n = 12, ML group) or a 9% (wt/wt) lard fat (n = 12, L group) diet for 16 wk. Plasma triglyceride and total cholesterol did not change in L, but had decreased in ML to 51 ± 8 and 81 ± 8% of the initial values, respectively, at the end of this period; the largest decreases already occurred during the first 8 wk. HDL cholesterol of both L and ML were not affected. After 16 wk, postprandial responses of plasma triglyceride, total cholesterol and glucose and insulin, determined at hourly intervals during the first 8 h postfeeding, did not show any differences for the two dietary groups. In spite of a marked replacement of n-6 fatty acids by n-3 fatty acids in cardiac sarcolemmal membranes in ML, there were no major differences in cardiovascular performance (myocardial contractility, pre- and afterload, cardiac output and myocardial work) between L and ML, when measured under baseline conditions and after the heart was stressed by atrial pacing (heart rate: 160 beats/min). In conclusion, feeding moderate amounts of mackerel oil [0.3 g 20:5 n-3/(kg body weight·d)] to pigs during 16 wk produces decreases of the plasma levels of total cholesterol and triglyceride, but does not affect plasma HDL cholesterol and routine cardiovascular parameters. *J. Nutr.* 117: 1371–1378, 1987.

INDEXING KEY WORDS:

- *fish oil*
- *plasma lipids*
- *cardiac function*
- *pigs*

Consumption of fish oil has a beneficial effect on the development of cardiac disease, probably by modification of risk factors such as high levels of plasma triglyceride and total cholesterol, low levels of plasma HDL cholesterol, high aggregability of platelets, high viscosity of blood and possibly hypertension (1–7). The effects are believed to be caused by the n-3 polyunsaturated fatty acids (PUFAs) present in fish oil. However, the time course of changes in the risk factors after daily intake of fish oil is largely unknown. In many studies, the quantity of consumed fish and, therefore, the resulting n-3 PUFA intake have been in excess of what could reasonably be consumed by the public at large (1). The Greenland Eskimos, for instance, consume approximately 0.1 g 20:5n-3 fatty acid/(kg body weight·d), which they obtain predominantly from seafood (1).

In a recent study we investigated the effect on plasma lipids of a purified mackerel oil extract that we added to a basal diet for young growing pigs (8). The intake of 0.6 g 20:5n-3/(kg body weight·d) over 8 wk decreased not only plasma triglyceride and total cholesterol but also plasma high density lipoprotein (HDL) cholesterol levels (8). In the present study pigs were fed only half the mackerel oil content [0.3 g 20:5n-3/(kg body weight·d)], but for twice the duration to allow a more accurate extrapolation from the results obtained in animals to humans.

It is possible that dietary fish oil also directly influences cardiac function, independent of changes in blood lipids, coronary vasculature and hemostasis because consumption of n-3 PUFAs also leads to changes in fatty acid composition of cardiac membrane phospholipids (9). The exchange of heart sarcolemma n-6 PUFA by dietary n-3 PUFAs alters the double bond index of cardiac membranes and may thereby affect membrane fluidity, receptor, intrinsic enzyme or ion transport properties (9–13). It is also feasible that prostanoid synthesis is affected (1, 9). Moreover, it has also been shown that mortality of rats on a fish oil diet was increased during catecholamine stress (9, 12, 13). Effects on infarct size, recovery of cardiac function and ventricular arrhythmias during coronary artery occlusion and reperfusion in dogs have also been described (14).

The goal of this study was to investigate the effects

[1]This study was supported by a grant from the Dutch Heart Foundation.

[2]Author to whom reprint requests should be addressed.

0022-3166/87 $3.00 Received 26 November 1986. Accepted 13 April 1987.

of the substitution of fish oil to a diet comparable to that of humans (30 E% fat and polyunsaturated-saturated fatty acid ratio of 0.78) on plasma lipid levels, cardiac membrane fatty acid composition and routine cardiovascular parameters. We performed a pacing stress test to see whether this led to cardiovascular dysfunction. The results were compared to pigs fed a lard fat diet (30 E% fat and polyunsaturated-saturated fatty acid ratio of 0.50). The pig was chosen because its lipoprotein metabolism and many aspects of its cardiovascular system resemble those of humans (15, 16).

MATERIALS AND METHODS

Experimental animals. Twenty-four weanling pigs (5 wk old and 7.7 ± 0.2 kg) were separated from their sows and housed individually in slat-bottomed cages in temperature-controlled animal quarters and divided into two groups, each group consisting of six castrated males and six females. Each group followed a different diet for 16 wk.

Experimental diets. The diets were prepared by adding either 9% (wt/wt) lard fat (L group) or a mixture of 4.5% (wt/wt) mackerel oil and 4.5% (wt/wt) lard fat (ML group) to a low fat (< 2%) nonpurified basal diet (Hope Farms BV, Woerden, The Netherlands). Lard fat was obtained from GEBRO Smilde BV (Heerenveen, The Netherlands) and the mackerel oil was purchased from A/S Johan C. Martens and Co. (Bergen, Norway). The mackerel oil was sealed under N_2 gas and added to the basal diet immediately before consumption to minimize oxidation. A detailed description of the two experimental diets is given in **Tables 1 and 2**. Besides the difference in fatty acid composition, there was a difference in cholesterol content of the diets that originated mainly from the addition of mackerel oil extract (Table 1). It should, however, be noted that cholesterol contents of both diets were extremely low. The animals were fed in the morning and the portions were consumed within 1 h. To facilitate adaptation to the new diet, the animals were fed only 200 g/d during the first week. In the following weeks the daily food intake was gradually increased to 1500 g/d in the last week. No scour occurred during the experimental feeding period.

Chemical analysis of plasma during the dietary period. Before the start of the dietary period and thereafter at 4-wk intervals plasma triglyceride, total and HDL cholesterol and hemoglobin levels were determined in blood samples with the methods described earlier (17–19). Blood was collected from animals deprived of food for 24 h by puncturing the subclavian vein. After 16 wk, the postprandial response in plasma triglyceride, glucose, insulin and total and HDL cholesterol (8, 17, 18, 20) was followed for 8 h. Three days before the postprandial follow-up a catheter was placed in the external jugular vein. Because of technical failure, postprandial blood samples could be obtained in only 10 of 12 animals in each group.

Hemodynamic measurements. The day after the postprandial follow-up experiment seven animals (three male and four female), randomly selected from each

TABLE 1

Composition of the two experimental diets fed to the Yorkshire piglets

Ingredient	9% lard fat diet	4.5% mackerel oil + 4.5% lard fat diet
Corn (extruded)	32	32
Wheat (extruded)	18	18
Soybean meal (solvent extracted, toasted)	14	14
Wheat middlings	9	9
Dehydrated skimmed milk powder	14	14
$CaHPO_4 \cdot H_2O$	1.3	1.3
$CaCO_3$	1.1	1.1
NaCl, iodinized	0.3	0.3
MgO	0.05	0.05
$MgSO_4$	0.05	0.05
$KH_2PO_4 \cdot 2H_2O$	0.36	0.36
Choline chloride 50% (wt/wt)	0.18	0.18
Vitamin and trace element mixes[1]	0.7	0.7
Lard fat	9.10	4.55
Mackerel oil	—	4.55
Mixed tocopherols	0.01	0.01
Cholesterol	0.01	0.02

[1]Vitamin and trace element mixes supply the following per 100 g/diet: retinol, 1400 IU; cholecalciferol, 140 IU; α-tocopherol, 8 mg; menadione, 0.2 mg; thiamin hydrochloride, 1.8 mg; riboflavin, 1.8 mg; pyridoxine HCl, 1.4 mg; niacin, 3.6 mg; vitamin C coated, 20 mg; D-calcium pantothenate, 3.6 mg; folic acid, 0.4 mg; cyanocobalamin, 0.004 mg; biotin, 0.1 mg; inositol, 4.5 mg; iron subcarbonate (57% Fe), 9.1 mg; $FeSO_4 \cdot H_2O$ (30% Fe), 14 mg; $Cu_2(OH)_2CO_3$ (55% Cu), 2.3 mg; ZnO (78% Zn), 11 mg; MnO (62% Mn), 9.1 mg; $Na_2Se_3 \cdot 5H_2O$ (45% Se), 0.08 mg; $Ca(IO_3)_2$ (65% I), 0.2 mg; $CoCO_3$ (47% Co), 0.09 mg. The composition (g/100 g) is on an as-fed basis.

TABLE 2

Fatty acid composition (%) of the two experimental diets fed to the Yorkshire piglets

Fatty acids	9% lard fat diet	4.5% mackerel oil + 4.5% lard fat diet
14:0	2	5
16:0	24	21
16:1	3	6
18:0	10	6
18:1	42	29
18:2n-6	15	11
18:3	1	1
20:1	1	4
20:5n-3	—	8
22:1	—	2
22:6n-3	—	5
24:1	—	1
Others	2	1

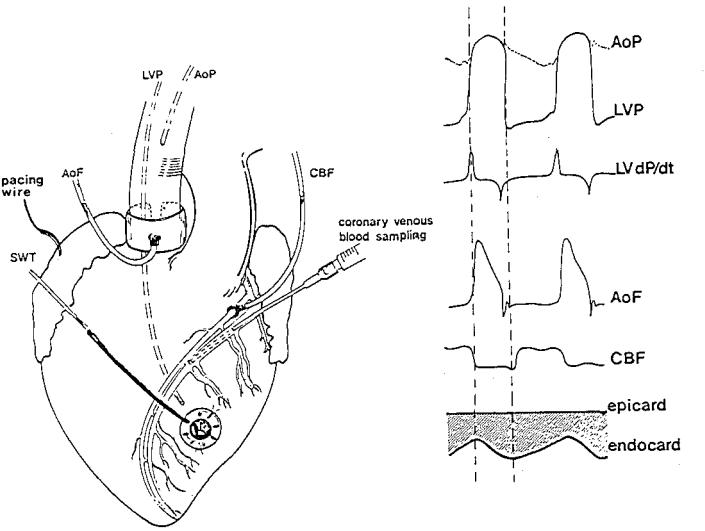

FIGURE 1 Preparation of the heart for hemodynamic measurements, after midsternal thoracotomy. The signals of the transducers are shown on the right: AoP = aortic pressure, LVP = left ventricular pressure, LVdP/dt = rate of change of left ventricular pressure, AoF = ascending aortic blood flow (cardiac output), CBF = coronary blood flow. The ultrasonic crystal measured the change in myocardial wall thickness. SWT = systolic wall thickening, which is calculated as described in the text.

group, were anesthetized and catheterized according to standard procedures (21). The five remaining animals in each group were used for a different protocol in a model of partial coronary artery constriction. After exposure of the heart through a midsternal split, electromagnetic flow probes (Skalar, Delft, The Netherlands) were placed around the ascending aorta and the proximal left anterior descending coronary artery. An electrode was sutured to the right atrium to pace the hearts. Myocardial wall function was monitored with a miniature 5-MHz ultrasound transducer (Krautkramer Branson, Lewiston, PA) sutured onto the epicardium for continuous measurement of regional myocardial wall thickness (21). From the registrations, systolic wall thickening (SWT) was calculated as

$$SWT(\%) = 100(\%) \times (EST - EDT)/EDT,$$

in which EST and EDT are the local wall thicknesses at end-systole and end-diastole, respectively. Finally, the great cardiac vein was cannulated with polyethylene tubing (**Fig. 1**).

Baseline measurements were obtained after stabilization for at least 30 min. Subsequently, the heart rate was raised by atrial stimulation with increments of 20 beats/min at 5-min intervals up to 160 beats/min and hemodynamic measurements were repeated at the end of each 5-min interval.

Chemical analysis of myocardial biopsies. After the last set of hemodynamic measurements were obtained at 160 beats/min, atrial pacing was discontinued and the hearts were excised and immediately cooled on ice. The posterior wall of the left ventricle was excised and a homogenate was prepared in buffer from which heart sarcolemma membranes were isolated from four animals (two male, two female) of each group (8, 22). The procedures for phospholipid hydrolysis, production of fatty acid methyl esters, extraction of methyl esters and their gas chromatographic separation have been described (8, 23, 24).

Morphological examination. Aliquots of heart and liver were fixed in 10% Formalin. The tissues were routinely processed, embedded in paraffin, cut at 7 μm thickness and stained with hematoxylin and eosin, alcian blue, periodic acid Schiff with and without diastase digestion and with different connective tissue stains (8, 25).

Medication. Drugs other than those described in the Materials and Methods section were not administered during the 16-wk dietary period.

Statistical analysis. The values at different time points were compared with analysis of variance. The differences between the two dietary regimens groups were evaluated by Student's *t*-test. Statistical significance was accepted at $P < 0.05$ (two-tailed). All data have been expressed as mean ± SEM.

RESULTS

Performance of the pigs during the 16-wk dietary period. After 16 wk the weight was 50 ± 2 kg in L (initial weight 7.7 ± 0.2 kg) and 46 ± 2 kg in ML (initial weight 8.1 ± 0.2 kg). Anemia, one of the signs of vitamin E deficiency, was not observed. At the end of the dietary period hemoglobin was 10.2 ± 0.3 mmol in L (initial value 10.6 ± 0.3 mmol) and 9.5 ± 0.4 mmol in ML (initial value 10.6 ± 0.2 mmol).

Plasma lipid levels during the dietary period. Plasma triglyceride and total and HDL cholesterol levels did not change in L during the 16-wk dietary period (**Fig. 2**). In ML plasma triglycerides decreased to 51 ± 8% of the initial value after 16 wk; most of the change (to 69 ± 9% of initial value) occurred, however, in the first 4 wk. Total cholesterol decreased to 79 ± 5% of the initial value during the first 8 wk, but did not decrease further during the following 8 wk (81 ± 8% after 16 wk). Although the animals were divided randomly, the predietary HDL cholesterol levels in ML were lower than those in L, but significant changes did not occur in either group during the dietary period. Thus, in spite of the higher cholesterol content in the mackerel oil diet (which is still very low, Table 1), a marked reduction in plasma cholesterol occurred in ML. There were no noticeable sex-related differences in plasma cholesterol, triglyceride and HDL cholesterol. In addition, the plasma lipid responses to the diets were equal when the animals were grouped according to their sex (not shown).

Postprandial plasma lipid and glucose levels at the end of the dietary period. The postprandial responses of plasma triglyceride, total cholesterol and glucose were determined at the end of the dietary period (**Fig. 3**). Because the response of the triglyceride levels in L was highly variable in time, no conclusion about a difference in response compared with ML can be drawn. The data demonstrate that the rate of fat resorption of the two dietary groups was about the same. Postprandial plasma total cholesterol did not change in L but started to increase in ML 4 h after the feeding period. This late rise in total cholesterol could be due to the higher cholesterol content in the diets of ML [0.02 and 0.01% (wt/wt) for ML and L, respectively; see Table 1]. Plasma glucose levels of L were already increased 1 h after ingestion of the meal, whereas it took 2 h in ML (Fig. 3). This small difference cannot be explained by changes in insulin because L insulin rose from preprandial values of 4.0 ± 0.7 to 31.7 ± 3.4 mU/L and ML insulin rose from 6.5 ± 1.2 to 39.6 ± 9.7 mU/L h postprandially. These data do not support a decreased insulin receptor sensitivity caused by mackerel oil feeding as postulated in a previous study (8).

Fatty acid composition of cardiac membrane phospholipids after the 16-wk dietary period. Although the polyunsaturated-saturated fatty acids ratio (P-S ratio) differed between the two diets (0.44 in L and 0.78 in ML), the P-S ratio of cardiac sarcolemma phospholipids was similar for both groups of animals: 1.15 ± 0.07 for L and 1.06 ± 0.02 for ML. However, the distribution of the n-6 and n-3 PUFAs was markedly different (**Fig. 4**). The n-6:n-3 fatty acid ratio was 11.8 ± 1.3 for L and 1.02 ± 0.08 for ML ($P < 0.05$). As can be seen from Fig. 4, 18:2n-6 and 20:4n-6 were partially exchanged for 20:5n-3 and 22:6n-3. This implies that fish oil nutrition caused a large increase in the double bond index (mean number of double bonds per mole fatty acid): 1.65 ± 0.03 for L and 1.94 ± 0.04 for ML ($P < 0.05$).

The 20:3n-9 was not found in the cardiac phospholipids, which indicates that the 18:2n-6 content of the

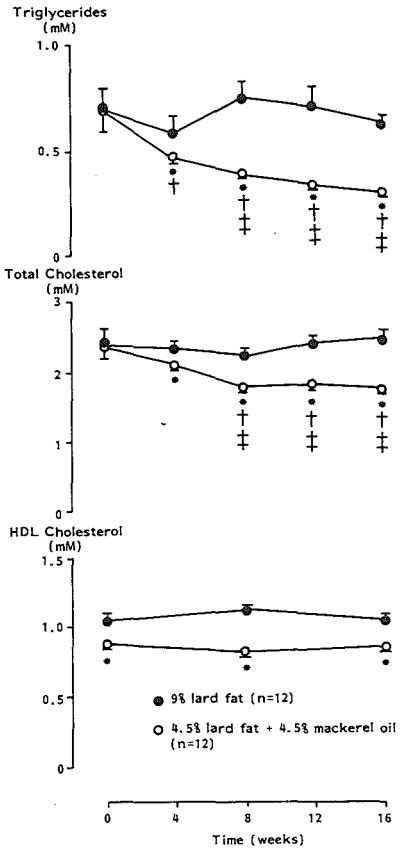

FIGURE 2 Plasma lipid levels of the Yorkshire pigs during the 16-wk experimental period. *$P < 0.05$ vs. initial value; †$P < 0.05$ vs. 9% lard fat diet group; ‡$P < 0.05$ vs. 4 wk value. Data are presented as mean ± SEM.

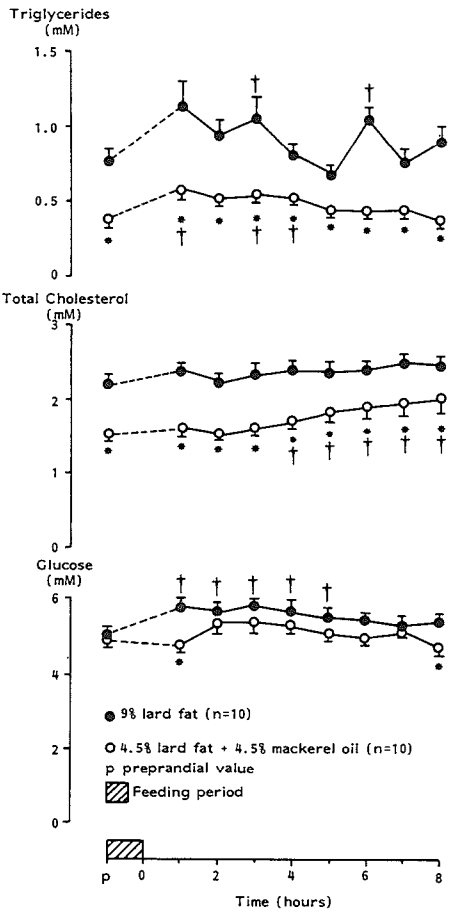

FIGURE 3 Postprandial responses of plasma levels of triglyceride, total cholesterol and glucose at the end of the 16-wk experimental period. p = preprandial value; $^*P < 0.05$ vs. preprandial value; $\dagger P < 0.05$ vs. 9% lard fat diet group. Data are presented as mean ± SEM.

diets (Table 2) was sufficient to prevent essential fatty acid deficiency (26).

Cardiovascular performance after the 16-wk dietary period. Under baseline conditions the heart rate was significantly higher (29%) in ML than in L (**Table 3**), but no differences were found in mean arterial blood pressure, left ventricular filling pressure, cardiac output, max LVdP/dt, coronary blood flow, myocardial work, myocardial O_2 consumption, systolic wall thickening and total systemic vascular resistance (Table 3). There were also no metabolic differences as the arterial-coronary venous pH differences were 0.06 ± 0.02 in L and 0.06 ± 0.02 in ML. The material-coronary venous P_{CO_2} differences were 13.3 ± 1.5 mmHg and 11.1 ± 1.3 mmHg, respectively, in L and ML.

Stress on the heart created by raising its rate gradually to 160 beats/min caused similar responses in corresponding cardiovascular variables in the two groups and, therefore, only the data obtained at 160 beats/min are presented (Table 3). Despite the higher heart rate, coronary blood flow was not increased during pacing at a rate of 160 beats/min. However, it is noteworthy that coronary blood flow increased by 18 ± 9% in L and 20 ± 9%in ML over its baseline value when the hearts were stimulated at 140 beats/min, but started to decrease when the heart rate was further raised to 160 beats/min. The data do not point toward any insufficiency of cardiac function. The arterial-coronary venous differences in pH and P_{CO_2} were not affected by the pacing stress test (not shown).

Morphological examination of heart and liver biopsies. Macroscopic evaluation revealed no cardiac hypertrophy or liver enlargement after mackerel oil feeding. Microscopic examination did not provide any evidence for cardiac or hepatic lipidosis and fibrosis.

DISCUSSION

Anemia, cardiac and hepatic lipidosis or fibrosis have been reported in pigs fed for 4 wk with a diet containing 0.2 g 20:5n-3/(kg body weight·d) (27). However, no such effects were observed with higher doses [0.6 g 20:5n-3/(kg body weight·d)] for 8 wk (8) or 0.3 g 20:5n-3/(kg body weight·d) for 16 wk (present study). Our animals also showed normal and identical growth patterns. The presence of adequate doses of natural antioxidants (vitamin E and selenium) in the diet used in our experiments explains the above difference.

After a rapid decrease during the first 4 wk, plasma triglyceride levels in ML decreased linearly at a slower rate during the next 12 wk. Plasma cholesterol also initially decreased but remained constant after 8 wk. Therefore, we conclude that ML feeding decreased both plasma triglyceride and cholesterol levels during the first 8 wk of the dietary period. It is noteworthy that with this diet, which contained less eicosapentanoic acid [20:5n-3 intake of 0.3 g/(kg body weight·d)] than in our previous study [(20:5n-3 intake of 0.6 g/(kg body weight·d), see ref. 8], the decrease in plasma triglyceride (−35 ± 9 vs. −62 ± 6%) and total cholesterol (−21 ± 5 vs. −41 ± 4%) were also less after 8 wk. Feeding pigs 0.2 g 20:5n-3/(kg body weight·d) during a 4-wk period lowered plasma triglyceride levels by 44%, whereas plasma cholesterol was not affected (27). These

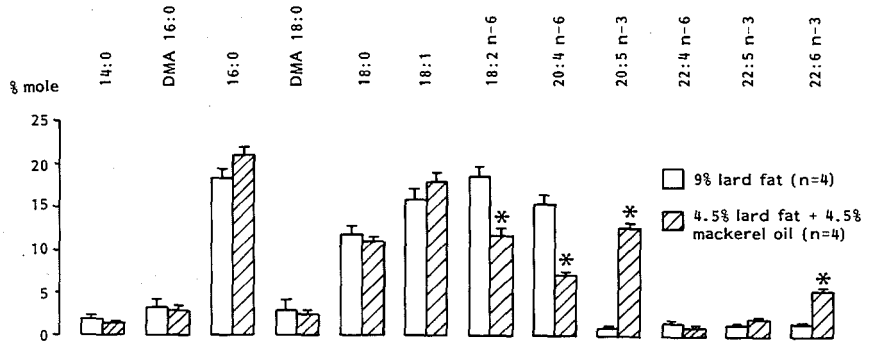

FIGURE 4 Fatty acid pattern of heart sarcolemma phospholipids. *$P < 0.05$ vs. 9% lard fat diet group. DMA = dimethylated acetal. Data are presented as mean ± SEM.

studies confirm other reports that dietary n-3 PUFAs affect plasma triglyceride levels to a larger extent than plasma cholesterol (1, 28–32). Another finding is that plasma HDL cholesterol in ML was not affected during the entire period (Fig. 2). Previously, we observed that plasma HDL cholesterol decreased from 1.06 ± 0.08 to 0.55 ± 0.04 mM in pigs fed with 0.6 g 20:5n-3 g/(kg body weight·d) for 8 wk (8). In the present study the initial HDL cholesterol was 0.88 ± 0.07 mM. It is, therefore, highly unlikely that HDL cholesterol did not decrease in ML because of an initial low value. Some trials in humans and rats showed rises in HDL cholesterol levels during fish oil consumption, but the levels may be more sensitive to the intake of 22:6n-3 than of 20:5n-3 (28, 29). In most other studies using lower doses of fish oil no effect on HDL cholesterol was found, which is in agreement with our present findings (1).

The change in membrane P-S ratio from 0.76 ± 0.06 (see ref. 8) to 1.15 ± 0.07 between 8 and 16 wk of the 9% (wt/wt) lard fat diet compares with our previous study and is primarily due to the increase of the 18:2n-6 content of cardiac membranes. This is in agreement with studies of Gudbjarnason et al. in rats (13). In view of the changes in membrane phospholipid composition (9–14), it is remarkable that 16 wk of 4.5% (wt/wt) mackerel oil consumption (ML) did not cause any dif-

TABLE 3

***Cardiovascular parameter in anesthetized Yorkshire pigs fed 9% lard fat or 4.5% mackerel oil and 4.5% lard fat diets for 16 wk, at baseline and during atrial pacing at a rate of 160 beats/min*[1]**

	9% lard fat (n = 7)		4.5% mackerel oil + 4.5% lard fat (n = 7)	
Parameters	Baseline	Pacing	Baseline	Pacing
HR, *beats/min*	94 ± 6	161 ± 1[a]	121 ± 10[b]	163 ± 1[a]
MAP, *mmHg*	86 ± 3	80 ± 5	84 ± 6	81 ± 8
LVEDP, *mmHg*	9.0 ± 1.0	8.8 ± 1.3	11.6 ± 0.4[b]	10.8 ± 0.6
SV, *mL*	47 ± 4	30 ± 2[a]	33 ± 2[b]	23 ± 3[ab]
CO, *L/min*	4.4 ± 0.5	4.8 ± 0.3	3.9 ± 0.4	3.7 ± 0.5
maxLVdP/dt, *mmHg/s*	1870 ± 310	1930 ± 250	2510 ± 570	2620 ± 560
SVR, (*mmHg·min*)/*L*	21 ± 3	19 ± 1	22 ± 1	23 ± 2
MW, (*mmHg·L*)/*min*	381 ± 41	397 ± 31	339 ± 56	310 ± 61
CBF, *mL/min*	71 ± 6	70 ± 8	66 ± 9	63 ± 8
CVR, (*mmHg·min*)/*mL*	1.27 ± 0.09	1.15 ± 0.09	1.44 ± 0.22	1.44 ± 0.26
cvO_2-sat, %	18 ± 3	18 ± 2	22 ± 3	22 ± 3
$M\dot{V}O_2$, *mmol/min*	0.56 ± 0.06	0.56 ± 0.07	0.50 ± 0.08	0.50 ± 0.04
SWT, %	34 ± 3	26 ± 4	29 ± 3	18 ± 5

[1]Abbreviations: HR = heart rate, MAP = mean arterial blood pressure; LVEDP = left ventricular end-diastolic pressure; SV = stroke volume; CO = cardiac output; maxLVdP/dt = maximum rate of rise in left ventricular pressure; SVR = systemic vascular resistance (MAP/CO); MW = myocardial work (MAP × CO); CBF = coronary blood flow; CVR = coronary vascular resistance (MAP/CBF); cvO_2-sat = coronary venous O_2 saturation; $M\dot{V}O_2$ = myocardial oxygen consumption [hemoglobin × (arterial − coronary venous) O_2-sat × CBF]; SWT = systolic wall thickening; [a]$P < 0.05$ vs. baseline; [b]$P < 0.05$ vs. 9% lard fat diet group.

ference in the cardiovascular parameters when compared with L, except for a higher heart rate and subsequent lower stroke volume in ML. When the heart was paced at a rate of 160 beats/min, no significant differences in cardiovascular performance due to diet were observed. It is premature to suggest that the differences in baseline heart rate of the two groups were due to the differences in diets because the use of anesthesia may also be a contributing factor. Despite the differences in baseline heart rate between L and ML, which is an important determinant of the myocardial O_2 demand, no differences in coronary blood flow and myocardial O_2 consumption were observed. At heart rates of 160 beats/min no increases in coronary blood flow and myocardial O_2 consumption were found. Similar observations during atrial pacing in dogs and pigs have been described (33–35). In earlier studies carried out in pigs, atrial pacing at 160 beats/min and higher led to pulsus alternans and contracture of the left ventricle, resulting in severe hemodynamic deterioration (34, 35). In the present study cardiovascular dysfunction was not observed in either group during atrial pacing.

In summary, in conjunction with our previous study (8) the results of our present investigation show that the addition of mackerel oil [4.5 and 9% (wt/wt)] to basal diets of pigs reduces both plasma triglyceride and cholesterol. HDL cholesterol, which decreases with a 9% (wt/wt) mackerel oil diet, is not affected after intake of 4.5% (wt/wt) mackerel oil. Despite marked changes in fatty acid composition of cardiac membranes, cardiovascular parameters, as well as their changes after atrial pacing, are not significantly different in animals fed with fish oil. This study in pigs indicates that the addition of eicosapentanoic acid to a typical Western diet lowers plasma lipid levels. Moderate amounts of dietary eicosapentanoic acid are required to lower plasma cholesterol, which is one of the major risk factors for coronary heart disease. Cardiovascular parameters were only minimally affected. Changes in cardiac parameters induced by right atrial pacing were independent of the diets.

ACKNOWLEDGMENTS

We thank H. Morse and M. C. Blok (Hope Farms, Woerden, The Netherlands) for their advice and for preparation of the diets, A. J. Vergroesen and W. C. Hülsmann for their encouragement and critical advice and the members of the Laboratory for Surgery for their assistance.

LITERATURE CITED

1. HEROLD, P. M. & KINSELLA, J. E. (1986) Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. *Am. J. Clin. Nutr.* 43: 566–598.
2. GOODNIGHT, S. H., HARRIS, W. S., CONNOR, W. E. & ILLINGWORTH, D. R. (1982) Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arteriosclerosis* 2: 87–113.
3. KROMHOUT, D., BOSSCHIETER, E. B. & DE LEZENNE COULANDER, C. (1985) The inverse relation between fish consumption and 20 year mortality from coronary heart disease. *N. Engl. J. Med.* 312: 1205–1209.
4. DYERBERG, J. (1986) Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. *Nutr. Rev.* 44: 125–134.
5. NORUM, K. R. & DREVON, C. A. (1986) Dietary n-3 fatty acids and cardiovascular diseases. *Arteriosclerosis* 6: 352–355.
6. DYERBERG, J. (1986) Platelet-vessel wall interaction in influence of diet. *Philos. Trans. R. Soc. London* B294: 373–387.
7. SINGER, P., JAEGER, W., WIRTH, M., VOIGHT, S., NAUMANN, E., ZIMONTKOWSKI, S., HAJDU, I. & GOEDICKE, W. (1983) Lipid and blood pressure–lowering effect of a mackerel diet in man. *Atherosclerosis* 49: 99–108.
8. HARTOG, J. M., LAMERS, J. M. J., MONTFOORT, A., BECKER, A. E., KLOMPE, M., MORSE, H., TEN CATE, F. J., VAN DER WERF, L., HÜLSMANN, W. C., HUGENHOLTZ, P. G. & VERDOUW, P. D. (1987) Comparison of mackerel-oil and a lard-fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. *Am. J. Clin. Nutr.* 46: 258–266.
9. LAMERS, J. M. J., HARTOG, J. M., VERDOUW, P. D. & HÜLSMANN, W. C. (1987) Dietary fatty acids and myocardial function. *Basic Res. Cardiol.* In press.
10. BERRY, E. M. & HIRSCH, J. (1986) Does dietary linoleic acid influence blood pressure? *Am. J. Clin. Nutr.* 44: 336–340.
11. STUBBS, C. D. & SMITH, A. D. (1984) The modification of mammalian polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta* 779: 89–137.
12. HOFFMAN, P. (1986) Cardiovascular action of dietary polyunsaturated fatty acids and related mechanisms. A state-of-the-art review. *Prostaglandins. Leukotrienes Med.* 21: 113–147.
13. GUDBJARNASON, S., OSKARSDOTTIR, G., DOELL, B. & HALLGRIMSSON, J. (1978) Myocardial membrane lipids in relation to cardiovascular disease. *Adv. Cardiol.* 25: 130–144.
14. CULP, B. R., LANDS, W. M., LUCCHESI, B. R., PITT, R. & ROMSON, J. (1980) The effect of dietary supplementation of fish oil on experimental myocardial infarction. *Prostaglandins* 20: 1021–1031.
15. CHAPMAN, M. J. (1980) Animal lipoproteins: chemistry, structure and comparative aspects. *J. Lipid Res.* 21: 789–853.
16. VERDOUW, P. D., WOLFFENBUTTEL, B. H. R. & VAN DER GIESSEN, W. J. (1983) Domestic pigs in the study of myocardial ischemia. *Eur. Heart J.* 4: 61–67.
17. FOSSATI, R. & PRENCIPE, L. (1982) Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* 280: 2077–2080.
18. SIEDEL, J., SCHLUMBERGER, H., KLOSE, S., ZIEGENHORN, J. & WAHLEFELD, A. W. (1981) Improved reagent for the enzymatic determination of serum cholesterol. *J. Clin. Chem. Clin. Biochem.* 19: 838–839.
19. WARNICK, G. R. & ALBERS, J. J. (1978) A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J. Lipid Res.* 19: 65–76.
20. SCHMIDT, F. H. (1961) Die enzymatische Bestimmung von Glucose und Fructose nebeneinander. *Klin. Wochenschr.* 23: 1244–1247.
21. HARTOG, J. M. & VERDOUW, P. D. (1986) Alleviation of myocardial ischemia after administration of the cardioselective beta-adrenoceptor antagonist bevantolol. *Cardiovasc. Res.* 20: 264–268.
22. LAMERS, J. M. J., DE JONGE-STINIS, J. T., HÜLSMANN, W. C. & VERDOUW, P. D. (1986) Reduced in vitro ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial

ischaemia in anaesthetized pigs. *J. Mol. Cell. Cardiol.* 18: 115–125.

23. FOLCH, J., LEES, M. & SLOANE-STANLEY, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497–509.
24. MONTFOORT, A., VAN DER WERF, L., HARTOG, J. M., HUGENHOLTZ, P. G., VERDOUW, P. D., HÜLSMANN, W. C. & LAMERS, J. M. J. (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. *Basic Res. Cardiol.* 81: 289–302.
25. ROMEINS, B. (1968) *Mikroskopische Technik* (Oldenbourg, R., ed.), Verlag, Munich.
26. HOUTSMULLER, U. M. T. (1975) Specific biological effects of polyunsaturated fatty acids. In: *The Role of Fats in Human Nutrition* (Vergroesen, A. J., ed.), pp. 331–351, Academic, London.
27. RUITER, A., JONGBLOED, A. W., VAN GENT, C. M., DANSE, L. H. J. C. & METZ, S. H. M. (1978) The influence of dietary mackerel oil on the condition of organs and on blood lipid composition in the young growing pig. *Am. J. Clin. Nutr.* 31: 2159–2166.
28. MORISAKI, N., SHINOMIYA, M., MATSUOKA, N., SAITO, Y. & KUMAGAI, A. (1983) In vivo effect of cis-5,8,11,14,17-20:5(n-3) and cis-4,7,10,13,16,19-22:6(n-3) on serum lipoproteins, platelet aggregation and lipid metabolism in the aorta of rats. *Tohoku J. Exp. Med.* 141: 397–405.
29. SANDERS, T. A. B. & ROSHIMAI, F. (1983) The influence of different types of ω3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. *Clin. Sci.* 6: 491–499.
30. NESTEL, P. J., CONNOR, W. E. & REARDON, M. F. (1984) Suppression by diets rich in fish-oil of very low density lipoprotein production in man. *J. Clin. Invest.* 74: 82–89.
31. SANDERS, T. A. B., SULLIVAN, D. R., REEVE, J. & THOMPSON, G. R. (1985) Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. *Arteriosclerosis* 5: 459–465.
32. SIMONS, L. A., HICKIE, J. B. & BALASUBRAMANIAM, S. (1985) On the effects of dietary n-3 fatty acids (MAXEPA) on plasma lipids and lipoproteins in patients with hyperlipidaemia. *Atherosclerosis* 54: 75–88.
33. VATNER, S. F., HIGGINS, C. B., FRANKLIN, D. & BRAUNWALD, E. (1972) Role of tachycardia in mediating the coronary hemodynamic response to severe exercise. *J. Appl. Physiol.* 32: 380–385.
34. VERDOUW, P. D., TEN CATE, F. J., SCHAMHART, H. C., VAN DER HOEK, T. M. & BASTIAANS, O. L. (1980) Segmental myocardial function during progressive coronary flow reduction and its modification by pharmacologic intervention. In: *Advances in Clinical Cardiology* (Weiss, H. W., ed.), pp. 270–283, Gerhard Witzstrock, New York.
35. SCHEFFER, M. G. & VERDOUW, P. D. (1983) Decreased incidence of ventricular fibrillation after an acute coronary artery ligation in exercised pigs. *Basic Res. Cardiol.* 78: 298–309.

CHAPTER 6

THE EFFECTS OF DIETS SUPPLEMENTED WITH LARD FAT OR MACKEREL OIL ON PLASMA LIPOPROTEIN LIPID CONCENTRATIONS IN DOMESTIC SWINE

Pieter H.E. Groot [1)*], Johannes M. Hartog [2)], Marie-Louise Dubelaar [1)], Leo M. Scheek[1)], Pieter D. Verdouw [2)] and Jos M.J. Lamers [1)].

[1)]Department of Biochemistry I, [2)]Laboratory for Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, Rotterdam, The Netherlands. * Presently affiliated with Smith Kline and French Research Ltd, The Frynthe Welwyn AL6 GAR U.K.

Summary

Levels of plasma lipoprotein were measured in overnight fasted pigs which were fed a diet containing either 21 energy % mackerel oil or 21 energy % lard fat for 8 weeks. Lipoprotein fractionation was performed by density gradient ultracentrifugation or agarose gel chromatography. After 8 weeks levels of plasma triglyceride (-62%) and cholesterol (-55%) were lower in the mackerel oil than in the lard fat fed animals. In triglyceride the fall was exclusively in the VLDL fraction, while cholesterol was reduced in all lipoprotein fractions (VLDL, IDL, LDL and HDL). These results support the hypothesis that regular intake of fish oil reduces VLDL secretion.

Introduction

Studies in man and animal have revealed diverse effects of n-3 polyunsaturated fatty acids on plasma lipid composition [1-9]. Considerable evidence has been presented that these fatty acids reduce levels of plasma cholesterol and triacylglycerol, but the effect on levels of plasma cholesterol is not always evident (see refs. 6 and 7). HDL-

cholesterol has been shown to decrease, not to change as well as to increase. The discrepancies can probably be explained by differences in species, the kind of dietary treatment and the quantitity of n-3 fatty acids consumed [6, 7]. Moreover, some of the used fish oil extracts contain relatively high amounts of cholesterol.

In the present study the pigs received a basal fat-poor diet to which either a purified mackerel oil extract, containing 30% n-3 fatty acids, or lard fat was added. The fish oil extract contained only very low amounts of vitamins A and D and cholesterol. We used pigs because in these animals, similar to man but in contrast to rats, LDL is a prominent cholesterol carrying lipoprotein [10]. Furthermore, we have shown that the changes in the levels of triglyceride, total cholesterol and HDL cholesterol are minimal with 8 weeks of lard feeding while the decreases in total cholesterol and HDL cholesterol by 21 energy% mackerel oil are evident [11]. We therefore determined the lipoprotein profiles only in samples collected after 8 weeks of the dietary period.

Methods

Twenty four young Yorkshire piglets (7.9 ± 0.2 kg) of either sex were divided arbitrarily over two groups and housed individually in slat-bottomed cages in temperature-controlled animal quarters. The pigs were fed isocaloric diets (3830 kCal/kg), consisting of 56 energy % (E%) carbohydrates, 18 E% proteins and 26 E% fat. The latter was composed of a 5 E% basis to which either 21 E% lard fat (Gebro Smilde BV, Heerenveen, The Netherlands) or 21 E% mackerel oil (AS Johan Martens and Co, Bergen, Norway) was added. The composition of the two isocaloric diets are shown in Table 1. A small difference in cholesterol was noticed but because of the rather low amounts, no adjustment was made. The main difference was in the fatty acid composition (Table 2). The animals were fed initially 200 g of the diet daily. This was gradually increased gradually to 800 g daily over a period of 8 weeks. After these 8 weeks blood samples were drawn by puncturing the subclavian vein from the 24 hours fasted animals. Full details of the diets have been described earlier [11].

The lipoprotein fractions were separated by isopycnic density gradient ultracentrifugation. The plasma lipoproteins were also fractionated according to size by 2% agarose gel chromatography using Biogel A-50, 100-200 mesh Bio-Rad (Richmond Calif, USA), and a column of 90 x 1.6 cm, operated at 4°C. Cholesterol and triglyceride were measured by enzymatic procedures using commercially available test kits (CHOD-PAP and GPO-PAP, Boehringer, Mannheim, FRG).

For statistical evaluation of the differences between the 2 dietary groups analysis of variance was used. Statistical significance was accepted at $p<0.05$ (two-tailed). All data have been expressed as mean ± standard error of the mean (X ± SEM).

Table 1
Composition (g/100g) of the diets

	Lard fat	Mackerel oil
corn (extruded)	32	32
wheat (extruded)	18	18
soybean meal	14	14
wheat middlings	9	9
dehydrated skimmed milk powder	14	14
Dicalcium-phosphate	1.3	1.3
$CaCo_3$	1.1	1.1
NaCl, iodinized	0.3	0.3
MgO	0.05	0.05
$MgSO_4$	0.05	0.05
$KH_2PO_4.2H_2O$	0.36	0.36
Choline-chloride 50%	0.18	0.18
Vitamin and trace element mixes$_{a)}$	0.7	0.7
lard fat	9.1	-
mackerel oil	-	9.1
mixed tocopherols	0.01	0.01
cholesterol	0.01	0.03

a) Vitamin and trace element mixes supply per 100 g food: Vitamin A 1400 IU; Vitamin D3 140 IU; Vitamin E 8 mg; Vitamin K3 0.2 mg; Vitamin B1, Thiamin hydrochlorid 1.8 mg; Vitamin B2, Riboflavin 1.8 mg; Vitamin B6, Pyridoxin HCl 1.4 mg; Niacin 3.6 mg; Vitamin C coated 20 mg; d-Ca Pantothenate 3.6 mg; Folic acid 0.4 mg; Vitamin B12 0.004 mg; Biotin 0.1 mg; Inositol 4.5 mg; Iron subcarbonate (57% Fe) 9.1 mg; $FeSO_4.H_2O$ (30% Fe) 14 mg; Coppercarbonate (55% Cu) 2.3 mg; ZnO (78% Zn) 11 mg; MnO (62% Mn) 9.1 mg; Na-selenite (45% Se) 0.08 mg; Ca(IO3)2 (65% I) 0.2 mg; $CoCo_3$ (47% Co) 0.09 mg.

Table 2
Fatty acid composition (g/100g) of the diets

		lard fat	mackerel oil
14:0		2	7
16:0		24	18
16:1		3	8
18:0		10	1
18:1		42	17
18:2	n-6	15	8
18:3		1	1
20:1		1	6
20:5	n-3	-	17
22:1		-	4
22:6	n-3	-	9
24:1		-	1
others		2	3

Results

In the mackerel oil fed animals (M) the levels of serum VLDL, LDL and HDL cholesterol were all approximately 50% and of IDL cholesterol only 25% of those in the lard fat fed animals (L) (Table 3). Levels of serum triglyceride were also only 50% of those in L. The lower plasma triglyceride value in M was entirely due to the lower VLDL levels (40% of L) as IDL, LDL and HDL triglyceride did not differ in both groups.

Table 3
The effect of dietary n-3 fatty acids on serum lipoprotein lipid concentrations (determined by ultracentrifugation) of pigs after an 8 week dietary period

Fraction	Lard fat (n=12)		Mackerel oil (n=12)	
	triglyceride	cholesterol	triglyceride	cholesterol
	μg/ml	μg/ml	μg/ml	μg/ml
Serum	139 ± 14	949 ± 24	67 ± 7*	429 ± 27*
VLDL	98 ± 17	23 ± 3	36 ± 7*	11 ± 2*
IDL	2.9 ± 1.2	16.3 ± 4.0	1.6 ± 0.8	3.8 ± 0.7*
LDL	21 ± 4	436 ± 42	26 ± 4	210 ± 14*
HDL	3.2 ± 1.4	399 ± 10	6.8 ± 2.7	181 ± 13*

Values are expressed as x ± S.E.M.
* $p<0.05$ versus Lard fat group.

Independently, in pooled plasma samples from four animals, the lipoproteins were fractionated in agarose columns. Elution profiles obtained by 2% agarose gel chromatography from 3 sets of experiments are shown in Fig. 1. Plasma cholesterol elutes in two major peaks representing LDL (left peak) and HDL (right peak) while VLDL is eluted just ahead of the LDL peak. The fish oil induced changes in cholesterol and triacylglycerol contents that were calculated from the peak areas agree with those measured after ultracentrifugal separation of VLDL, LDL and HDL (results not shown).

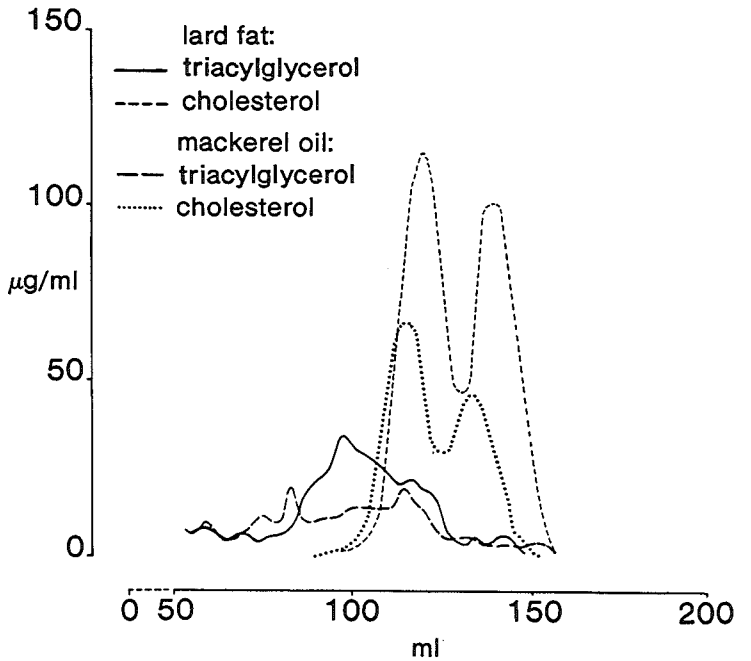

Figure 1. Lipoprotein triglyceride and cholesterol profiles in overnight fasted pigs after an 8 week dietary period,obtained by 2% agarose gel chromatography. The data shown are average values from three independent fractionation studies in pooled samples from four animals each.

Discussion

The increasing interest in the effects of n-3 fatty acids on the suppression and regression of atherosclerosis [6, 12, 14] and the use of pigs in atherosclerotic research [12, 14-18] led us to investigate the effect of n-3 rich fatty diets on serum lipoprotein lipids in domestic pigs. Pigs and man have in common that in both species LDL and HDL are important cholesterol carrying lipoproteins although the total mass of the lipoproteins in particular that of VLDL is somewhat lower in the pig than in man [10, 19].

In the present study we showed that after 8 weeks serum VLDL triglyceride as well as cholesterol present in VLDL, LDL and HDL were all more than 50% lower in the mackerel oil than in the lard fat fed pigs. These lower values are caused by a profound hypotriglyceridemic and hypocholesterolemic action of mackerel oil, rather than by a hyperlipidemic effect of the lard feeding. This conclusion is based on the earlier findings that under the same experimental conditions, dietary lard fat has only a minimal effect on plasma lipids of domestic pigs [11].

A comparison with rats, in which LDL is only a minor fraction of the plasma lipoproteins, is of interest. Balasubramanian et al [4] fed a fat-rich diets (30 E%) containing either coconut oil, sunflower oil or fish oil to rats. In that study fish oil induced a larger decrease (40%) in plasma cholesterol than coconut oil, but had similar to the present study no effect on the ratio of LDL and HDL cholesterol. As in rats, HDL is the prominent cholesterol-carrier in porcine plasma [10, 19], the decrease in cholesterol is to a large extent caused by the decrease in HDL. It should be noted, however, that feeding pigs a diet with a lower mackerel oil content (4.5% wt/wt) reduces total cholesterol but does not affect HDL cholesterol, thereby decreasing the ratio of LDL and 2HDL cholesterol [20].

Fish oil exerts in man a larger effect on plasma VLDL triglyceride than on LDL triglyceride [3, 5-7, 21-24]. The same pattern was seen in the present study. Levels of HDL triglyceride in man have been reported not to change [3], but most reports describe a decrease after a fish oil diet [22-29]. In the present study HDL triglyceride was not significantly affected but it tended to increase rather than decrease.

N-3 polyunsaturated fatty acids decrease the synthesis of VLDL probably due to decreased lipogenesis and increased oxidation of fatty acids [19, 25-28]. Less LDL was secreted from the perfused livers of EPA-fed rats [26]. The suppression of triacylglycerol synthesis of rat hepatocytes in culture by EPA may be caused partially by the inhibition of diacylglycerol acyltransferase [27]. This is, however, not supported by the results of another study in which the effect of fish oil-enriched diet on changes rat liver enzyme activities was investigated [28]. The hypotriglyceridemic action of fish oil may also be related to a stimulation of the peroxisomal beta-oxidation activity in the liver [29]. The reduced levels of plasma VLDL triglyceride are not caused by a higher lipolytic breakdown by lipoprotein lipase and hepatic lipase [30]. As LDL is a metabolic product of VLDL catabolism and surface fragments of VLDL may be used to assemble plasma HDL [24], secondary effects of fish oil-rich diets on LDL and HDL synthesis and plasma concentration can be expected. Our data in pigs are in line with these findings.

In conclusion, the results of the present study performed in domestic pigs support the hypothesis of a primary reduction of VLDL secretion by dietary fish oil. This is probably followed by secondary changes in the cholesterol content of IDL, LDL and HDL fractions.

Acknowledgement

The authors wish to thank Profs. A.J. Vergroesen and W.C. Hülsmann for encouragement and advice and Miss P.H. Vegter for her assistance in the preparation of this manuscript.

REFERENCES

1. Kahn SG, van de Putte J, Wind S, Yacowiz H: A study of the hypercholesterolemic activity of the ethyl esters of the polyunsaturated fatty acids of cod liver oil in the chicken. 1. Effect on total serum cholesterol. J Nutr 80: 403-413, 1963
2. Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM: The influence of dietary mackerel oil on the condition of organs and on blood lipid composition in the young growing pig. Am J Clin Nutr 31: 2159-2166, 1978
3. Harris WS, Connor WE, McMurry MP: The comparative reduction of plasma-lipids and lipoproteins by dietary polysaturated fats: salmon-oil versus vegetable oils. Metabolism 32: 179-184, 1983
4. Balasubramaniam S, Simons LA, Chang S, Hickie JB: Reduction in plasma cholesterol and increase in biliary cholesterol by a diet rich in n-3 fatty acids in the rat. J Lip Res 26: 684-689, 1985
5. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR: Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. New Engl J Med 312: 1210-1216, 1985
6. Herold PM, Kinsella JE: Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am J Clin Nutr 43: 566-598, 1986
7. Goodnight SH, Harris WS, Connor WE, Illingworth DR: Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. Arteriosclerosis 2: 87-113, 1982
8. Rogers S, James KS, Butland BK, Etherington MD, O'Brien JR, Jones JG: Effect of fish oil supplement on serum lipids, blood pressure, bleeding time, haemostatic and rheological variables. Atherosclerosis 63: 137-143, 1987
9. Parks JS, Martin JA, Soubert BL, Bullock BC: Alterations of high density lipoprotein subfractions of nonhuman primates fed fish oil diets. Selective lowering of HDL subfractions of intermediate size and density. Arteriosclerosis 7: 71-79, 1987
10. Chapman MJ: Comparative analysis of mammalian plasma lipoproteins. In JP Segrest and JJ Alberts (eds). Methods in Enzymology vol. 128. Academic Press Incorporation, Orlando Florida, USA, 1986, pp 70-143
11. Hartog JM, Lamers JMJ, Montfoort A, Becker AE, Klompe M, Morse H, Ten Cate FJ, Werf L van der, Hülsmann WC, Hugenholtz PG, Verdouw PD: Comparison of mackerel-oil and lard-fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance, and morphology in young pigs. Am J Clin Nutr 46: 258-266, 1987
12. Weiner BH, Ockene IS, Levine PH, Cuenoud Fisher M, Johnson BF, Daoud AS, Jarmolych J, Hosmer D, Johnson MH, Natale A, Vaudreuil Chr, Hoogasian JJ: Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. New Engl J Med 315: 841-846, 1986
13. Leaf A, Weber PC: Cardiovascular effects of n-3 fatty acids. New Engl J Med 318· 549-557, 1988
14. Sassen LMA, Koning MMG, Dekkers DHW, Lamers JMJ, Verdouw PD: Differential effects of n-3 fatty acids on the regression of atherosclerosis in coronary arteries and the aorta of the pig. Eur Heart J in press.
15. Kammermann KL, Luginbuhl H, Ratcliffe HL: Intramural coronary arteriosclerosis of normal and dwarfed swine. Vet Pathol 13: 104-109, 1976

16. Fuster V, Lie JT, Badimon L, Rosemark JA, Badimon JJ, Bowie EJW: Spontaneous and diet-induced coronary atherosclerosis in normal swine and swine with von Willebrand disease. Arteriosclerosis 5: 67-73, 1985
17. Kim DN, Lee KT, Schmee J, Thomas WA: Quantification of intimal cell masses and atherosclerotic lesions in coronary arteries of control and hyperlipidemic swine. Atherosclerosis 52: 115-122, 1984
18. Fritz KE, Augustyn JM, Jarmolych J, Daoud AS, Lee KT: Regression of advanced atherosclerosis in swine. Arch Pathol Lab Med 100: 380-385, 1976
19. Mills GL, Taylaur CE: The distribution and compositions of serum lipoproteins in eighteen animals. Comp Biochem Physiol 240b: 489-501, 1971
20. Hartog JM, Verdouw PD, Klompe M, Lamers JMJ: Dietary mackerel oil in pigs: effect on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. J Nutr 117: 1371-1378, 1987
21. Nestel PJ, Connor WE, Reardon MF: Suppression by diets rich in fish-oil of very low density lipoprotein production in man. J Clin Invest 74: 82-89, 1984
22. Sanders TAB, Sullivan DR, Reeve J, Thompson GR: Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. Arteriosclerosis 5: 459-465, 1985
23. Simons LA, Hickie JB, Balasubramaniam S: On the effects of dietary n-3 fatty acid (Maxepa) on plasma lipids and lipoproteins in patients with hyperlipidaemia. Atherosclerosis 54: 75-88, 1985
24. Nestel, P. Dietary effects on lipoprotein metabolism. In NH Fidge and P Nestel (eds). Atherosclerosis VII. Elsevier Science Publishers B.V., Amsterdam, The Netherlands, 1986.
25. Daggy B, Arost C, Benjadoun A: Dietary fish oil decreases VLDL production rats. Biochim Biophys Acta 920: 293-300, 1987
26. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL: The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochim Biophys Acta 792: 103-109, 1984
27. Strum-Odin R, Adkins-Finke B, Blake WL, Phinney SD, Clarke SD: Modification of fatty acid composition of membrane phospholipid in hepatocyte monolayer with n-3, n-6 and n-9 fatty acids and its relationship to triacylglycerol production. Biochim Biophys Acta 921: 378-391, 1987
28. Marsh JB, Topping DL, Nestel PJ: Comparitive effect of dietary fish oil and carbohydrate on plasmalipids and hepatic activities of phosphatidate phosphohydrolase, diacylglycerol acyltransferase and neutral lipase activities in the rat. Biochim Biophys Acta 922: 239-243, 1987
29. Haug A, Hostmark AT: Lipoprotein lipases, lipoproteins and tissue lipids in rats fed fish oil or coconut oil. J Nutrition 117: 1011-1017, 1987
30. Yamazaki RK, Shen T, Schade GB: A diet rich in (n-3) fatty acids increases peroxisomal beta-oxidation activity and lowers plasma triacylglycerols without inhibiting glutathion-dependent detoxication activities in the rat liver. Biochim Biophys Acta 920: 62-67, 1987

CHAPTER 7

The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine

A. Montfoort[a]), L. van der Werf[b]), J. M. Hartog[b]), P. G. Hugenholtz[b]), P. D. Verdouw[b]), W. C. Hülsmann[c]), and J. M. J. Lamers[c])

[a]) Department of Pathology I, [b]) Department of Experimental Cardiology and
[c]) Department of Biochemistry I
Medical Faculty, Erasmus University Rotterdam, Rotterdam (The Netherlands)

Summary: The effect of chronic norepinephrine (NE) administration with increasing dosage from 1–4 mg/kg over a period of 2 weeks was studied on cardiac phospholipids and their fatty acid distribution in rats. Animals were fed a control diet or a 10 % cod liver oil (CLO)-enriched diet. The relative distribution of various polyunsaturated fatty acids esterified to the 1- and 2-position of the phosphatidylethanolamine fraction was estimated. NE stress during control feeding significantly reduced the total phospholipid content in rat heart. No differences in the phospholipid class distribution were found. However, CLO feeding as well as chronic NE administration resulted in a decrease of ω 6 fatty acids, mainly C 18:2 ω 6 and C 20:4 ω 6, which was compensated with an increase in ω 3 fatty acids, mainly C 20:5 ω 3 and C 22:6 ω 3. The changes in fatty acid composition qualitatively agree with those reported by Gudbjarnason et al. (23), except that the mortality in our NE-treated control or CLO-fed groups was considerably lower. It can probably be attributed to a different mode of NE administration. On the other hand, at the end of the CLO feeding period in rats treated with NE or not, comparing with control fed rats without NE treatment, the incidence rate of ST segment elevation in electrocardiogram (ECG) recorded under light diethylether-induced anesthesia was higher. Independent of whether the fatty acid composition of myocardial phospholipids was dietary or pharmacologically manipulated, most of the polyunsaturated fatty acids were found at the 2-position of the phosphatidylethanolamine molecules. The polyunsaturated fatty acids account for 45–50 % of the fatty acyl residues and preferentially occupy the 2-position, where they can exchange for each other.

Key words: phospholipids, fish oil diet, catecholamines, polyunsaturated fatty acids, positional fatty acid distribution

Introduction

Disturbances in phospholipid metabolism and subsequent changes in membrane function have been implicated in the development of irreversible cell injury during both chronic catecholamine treatment and myocardial ischemia (7, 8, 22, 25, 27, 31, 50, 51, 53). Accelerated phospholipid degradation is generally thought to result from activation of endogenous membrane-bound phospholipase A_2. During ischemia but also during beta-adrenergic overstimulation of the heart, increased levels of intracellular Ca^{2+} very likely occur. That phospholipase A_2 activation is dependent on Ca^{2+} ions has repeatedly been demonstrated (4, 5, 49). One of the lysophospholipid products can either be reacylated to form newly synthesized phospholipid molecules or can be further degraded by the activity of

lysophospholipase. In normal heart the concentrations of these phospholipid breakdown products are extremely low because lysophospholipids as well as free polyunsaturated fatty acids are detergent-like compounds which at higher concentration can be harmful for membrane functions (27, 31, 53, 56). Membrane phospholipid products indeed have been implicated in the development of cardiac arrhythmias and the pathogenesis of myocardial ischemia (27, 28, 34, 42, 53). Also changes in the phospholipid fatty acid composition due to dietary or hormonal manipulation can give rise to alterations in properties of cardiac membranes, e.g. the fluidity and ion permeability (1, 9, 33, 34, 43). Furthermore it can have implications for enzyme and transport protein properties (27) and thereby for the regulation of transmembrane Ca^{2+}-, Na^{+}- and K^{+} ion gradients. Therefore, it is important to know to what extent heart membrane phospholipids are modified due to changes in external factors like diet, stress or age (2, 23, 37).

Cardiac phospholipids, particularly the phosphatidylethanolamine component, are characterized by their high content of polyunsaturated fatty acids (13, 29, 30, 38). Bang et al. (3) suggested that the low incidence of ischemic heart disease in Greenlandic Eskimos is linked to the high daily intake of polyunsaturated fatty acids (e.g. eicosapentanoic acid, C 20:5 ω 3 and docosahexaenoic acid C 22:6 ω 3) through feeding with fish and marine oils. Diets rich in ω 3 fatty acids derived from fish oils have been demonstrated to lower the plasma concentrations of low density and very low density lipoproteins (35), cholesterol as well as triglyceride content (35, 46) and to affect platelet reactivity (6, 21, 24, 26, 40, 45, 47). These hypolipidemic and antithrombotic effects of fish oil diet may offer the explanation for their beneficial effects in ischemic heart disease. Diets rich in ω 3 fatty acids also influence the biosynthesis of ω 6 fatty acids and supplementation of diets with menhaden oil (rich in C 20:5 ω 3 and C 22:6 ω 3) have been demonstrated to depress the Δ 6 desaturase activity in rats resulting in a diminished synthesis of C 20:4 ω 6 and C 22:4 ω 6 from C 18:2 ω 6 (44). On the other hand, it has been reported that unsaturation of the fatty acids in heart cell membranes may enhance lipid peroxidation. This unfavorable effect of fish oil diet which could occur especially at low vitamin E content of the diet may result in the accumulation of lipofuscin (39) and a disturbance of the membrane integrity producing metabolic and functional abnormalities in the heart. Stress has been found to stimulate myocardial lipid peroxidation (2, 37) and impair the contractile function of the myocardium. Therefore it is interesting to note that the stress tolerance of rats is decreased under the influence of dietary cod liver oil (CLO) (23). In rats subjected to catecholamine stress for two weeks heart phospholipid fatty acid components undergo replacement of linoleic acid (C 18:2 ω 6) by docosahexaenoic acid (C 22:6 ω 3) and this is similar to the response seen after CLO diet or at increasing age (13, 14). Apparently, a large increase in polyene fatty acid content of membrane phospholipids influences the sensitivity of rats to catecholamine-induced necrosis (13, 14, 23). As noted before, increased phospholipase A_2 activity is a factor in catecholamine-induced myocardial necrosis (27, 42). Thus it is interesting to know the relative 1- and 2-position of polyene fatty acids in phospholipids under influence of a dietary change. We therefore analysed the phospholipid composition and fatty acid distribution of the major phospholipids in rats receiving either a control or CLO diet without and after chronic treatment with norepinephrine (NE). The positional fatty acid distribution of phosphatidylethanolamine was determined by stepwise hydrolysis of the phospholipid component of the heart in either group of rats. In the work of Emilsson and Gudbjarnason (14) no indication was given of the cause of death of the rats under the influence of CLO diet or NE treatment. Another recent study, however, suggested correlation between catecholamine and lipidperoxidation-induced damage in heart cells (41). Therefore in the present work attention was paid to possible abnormalities in the electrocardiogram (ECG) pattern at the end of the nutritional and catecholamine treatment periods.

Materials and methods

Animal treatment

Normal Wistar rats of either sex were used. 33 adult rats (2 months old) were divided into 2 groups, 17 animals received a 10 % CLO diet supplied by Unilever, Vlaardingen, The Netherlands, whereas 16 animals were fed with a control diet (standard laboratory food, from Hope Farms, Woerden, The Netherlands) for 6 weeks. No difference existed between vitamin E content of control and CLO diet. Water and food were supplied ad libitum. The diets had the following mean fatty acid composition:

Control diet: 14^0+, 16^0 21.6 %, 16^1 ω 9 2.2 %, 18^0 3.5 %, 18^1 ω 9 26.5 %, 18^2 ω 6 39.3 %, 18^3 ω 3 4.5 %, 22^0 0.5 %, 20^4 ω 6 0.6 %, others 1.3 %.

CLO diet: 14^0 7.2 %, 16^0 15.7 %, 16^1 ω 9 9.0 %, 18^0 3.1 %, 18^1 ω 9 16.8 %, 18^2 ω 6 1.5 %, 18^3 ω 3 0.8 %, 18^4 1.5 %, 20^1 5.1 %, 20^4 ω 6 4.8 %, 20^5 ω 3 13.7 %, 22^4 ω 6 0.6 %, 22^5 ω 3 2.7 %, 22^6 ω 3 11.0 %, others 6.5 %.

Eight animals with control diet and 9 animals with CLO diet after 6 weeks were daily injected with NE over a period of 2 weeks. NE was suspended by sonication in polyethylene glycol (PEG 400). The other animals received only PEG 400. The NE dosage increased from 1 mg/kg during the first 3 days to 4 mg/kg during the last 3 days, which was achieved by increasing the dosage with 1 mg/kg every 4th day (23). The feeding of rats with the two different diets was continued during the daily control or NE injections.

Phospholipid analyses

The lipids were extracted from the heart according to the method of Folch (17) with 19 volumes of a mixture of chloroform-methanol (2:1, by volume). Two dimensional separation of the phospholipid classes and phosphorus analysis was carried out as described before (38). The total phospholipids were separated from the other lipids by thin layer chromatography. The developing system was a mixture of hexane-ether (70:30 v/v). The plates were first washed with the same mixture and activated at 110 °C during 30 min. Phosphatidylcholine and phosphatidylethanolamine were separated from other phospholipids by thin layer chromatography using a solvent mixture of chloroform-methanol-4N NH_4OH (65:35:4, by volume) after the same washing procedure as described before (38). Phospholipase A_2 degradation with snake venom from Crotalis Adamanteus was used to determine the fatty acid composition at the 1- and 2-position of the phosphatidylethanolamine fraction by gaschromatography as described before (38). After separation of the phospholipids (total, phosphatidylcholine or phosphatidylethanolamine fraction) and the breakdown products of phospholipase A_2 degradation (free fatty acids and lysophosphatidylethanolamine) 15 ml of methanolic HCL containing 2.6 % HCL (w/v) were added and the mixture was heated for 2 h at 70 °C. A nitrogen atmosphere was used to prevent oxidation. The methylesters of the fatty acids were extracted with three portions of pentane, after adding 15 ml of water. The combined extracts were dried over anhydrous Na_2SO_4 and after concentration applied on a Carlo Erba gaschromatograph (4160 series) equipped with a 50 m capillary column (0.32 mm diameter, 0.2 μm thick stationary phase CPS1L88) and temperature programming (70–200 °C).

Determination of ECG pattern

At the end of the experimental periods (after 8 weeks, including the 2 final weeks of daily control or NE injections in which the control or CLO diets were continued) light anesthesia was induced by diethylether and peripheral ECG was recorded, after which the animals were sacrificed. Heart rates were calculated and special attention was paid to changes in the ST segment. ST elevation was defined as an elevation higher than 0.1 mV.

Statistical analysis

Where possible, the results were expressed as mean ± S.E.M. Different groups of rats were compared with each other using the t-test.

Results

NE administration

Initial experiments demonstrated that catecholamines dissolved in 0.9 % NaCl solution and given subcutaneously already at the lowest concentration (1 mg/kg) resulted in a 100 % mortality which is higher than the 50 % observed by Emilsson and Gudbjarnason (14) after the same mode of administration. The differences in the sensitivity for stress hormones between various strains of rats could represent one explanation. Nevertheless in subsequent experiments we administered subcutaneously NE suspended in PEG 400 to decrease the rate of plasma uptake. Mortalities: 10 % in the control fed plus NE injected group (1 out of 10) and 22 % in the CLO fed plus NE injected group (2 out of 9). The low incidence of death in the NE injected animals does not allow to draw conclusions as to whether CLO nutrition increased the mortality. It should also be noted that when the animals were sacrificed (after 14 days NE treatment), the extreme changes in polyene fatty acid content of phospholipid as described by Gudbjarnason et al. (13, 14, 23) were already obvious (see later).

Phospholipid content and composition

Differences in the total amount of cardiac phospholipids were found between the respective groups of rats (Table 1). The total phospholipid content was lower, although not

Table 1. The phospholipid content (mean ± SEM) of rat heart after dietary and/or beta-adrenergic treatment.

Diet	Injected	Number of animals	Phospholipid content (μmoles/g)
Control	Solvent	8	40.6 ± 1.6
Control	NE	8	34.9 ± 2.1*)
CLO	Solvent	7	33.6 ± 3.3
CLO	NE	7	30.7 ± 2.2*)

*) $p < 0.05$ with control fed, solvent injected animals

Table 2. Percentual distribution of the major phospholipid classes in rat hearts*).

	Control fed rats		CLO fed rats	
	Solvent injected	NE injected	Solvent injected	NE injected
Lysolecithin	1.3	1.4	1.1	1.2
Sphingomyelin	4.4	5.2	3.4	4.8
Phosphatidylinositol	4.3	3.8	3.7	4.7
Phosphatidylserine	1.5	1.8	1.5	2.4
Phosphatidylcholine	42.4	41.5	39.8	41.9
Phosphatidylethanolamine	26.7	31.1	31.8	28.8
Cardiolipin	14.4	10.4	11.3	13.0
$(Mag)_2P$	1.9	1.2	0.6	0.5
Others	3.1	3.5	6.9	2.7

*) results are the means of 4 determinations of combined phospholipid fractions extracted from hearts of the animal groups mentioned in Table 1. $(Mag)_2P$ is an abbreviation for: bis-(monoacylglyceryl)phosphate

significantly, in the CLO fed animals. A significant decrease ($p < 0.05$) in the total phospholipids per gram wet weight was found upon beta-adrenergic treatment of control rats. A similar but non-significant decrease was found upon NE treatment of CLO rats. It is known that a high dose of catecholamines causes an increase of the influx of Ca^{2+} into and deposition in myocardial cells, the release of FFA by enhanced lipolysis (27, 36), stimulation of phospholipase A_2 activity causing breakdown of the phospholipids (48) and preferentially those which are rich in polyunsaturated fatty acids. It is possible that in addition to changes in total phospholipid content a specific class of phospholipids is affected by catecholamine treatment. Therefore we analysed the phospholipid class distribution to see whether the decrease in the total phospholipids was preferent for a particular phospholipid fraction. Combination of the extracted lipid fractions from hearts of animal groups indicated in Table 1 was required to obtain sufficient material for thin layer chromatography and phosphorus determination. The mean values for the different phospholipid classes in the different groups are presented in Table 2.

The phospholipid class composition agrees well with earlier data obtained with rat hearts by us and by others (29, 38, 52). No dramatic changes were seen after NE treatment which suggests that the distribution of specific classes is not affected by NE treatment.

Percentual distribution of fatty acids in cardiac phospholipids

Changes in the percentual distribution of the fatty acids in cardiac phospholipids induced by diet and stress are presented in Table 3. Chronic administration of beta-adrenergic

Table 3. Percentual distribution of the fatty acids in the total phospholipids in rat heart after dietary and/or beta-adrenergic treatment*).

Fatty acids	Control fed rats		CLO fed rats	
	Solvent injected (n = 8)	NE injected (n = 8)	Solvent injected (n = 7)	NE injected (n = 7)
DMA 16:0	+	+	+	+
16:0	13.2 ± 0.4	13.8 ± 0.6	15.0 ± 0.9	13.6 ± 1.0
DMA 18:0	+	+	+	+
18:0	14.0 ± 2.0	14.4 ± 1.9	15.0 ± 1.7	12.7 ± 2.0
18:1 ω 9	5.9 ± 0.8	7.0 ± 0.5	7.3 ± 0.7	7.9 ± 0.6
18:2 ω 6	25.0 ± 1.3	18.9 ± 1.0[a])	11.2 ± 0.9[c])	9.4 ± 0.4[d])
22:0	0.8 ± 0.3	0.9 ± 0.5	0.4 ± 0.2	1.4 ± 1.1
20:4 ω 6	25.0 ± 1.1	25.7 ± 1.6	19.0 ± 0.9[c])	21.2 ± 0.8
20:5 ω 3	0.0 ± 0.0	0.8 ± 0.5	4.8 ± 0.2[c])	3.7 ± 0.5[d])
22:4 ω 6	+	+	+	+
24:1	1.5 ± 0.6	1.1 ± 0.4	1.4 ± 0.4	1.5 ± 0.5
22:5 ω 3	2.6 ± 0.4	2.3 ± 0.2	2.8 ± 0.1	3.3 ± 0.4
22:6 ω 3	11.9 ± 0.9	15.0 ± 0.6[b])	23.2 ± 0.6[c])	25.4 ± 0.6[d])

*) mean ± SEM

[a]), [b]) refer to significance of differences between column "control + solvent" vs. "control + NE" (a, $p < 0.01$; b, $p < 0.05$)

[c]) refers to significance of differences between column "control + solvent" vs. "CLO + solvent" (c, $p < 0.001$)

[d]) refers to significance of differences between column "CLO + solvent" vs. "CLO + NE" (d, $p < 0.05$)

DMA = dimethylated acetals

amines probably leads to less extreme changes in the fatty acid patterns of CLO fed rats with respect to changes in the C 22:6 ω 3 content. A 10 % CLO diet for 8 weeks resulted in a substantial replacement of ω 6 fatty acids, e.g. C 18:2 ω 6 and C 20:4 ω 6 by ω 3 fatty acids, particularly C 20:5 ω 3, C 22:5 ω 3 and C 22:6 ω 3 as was also described by other investigators (13, 22, 23, 26, 44). These changes are very similar to those induced by catecholamine stress except for C 20:4 ω 6, which is reduced in the case of CLO feeding, but hardly under the influence of stress hormones. The percentual distribution of the saturated fatty acids and the monounsaturated fatty acids was rather constant in all experiments.

The percentual distribution of the polyunsaturated fatty acids has the tendency to change more after CLO feeding than after NE treatment, although the most extreme values are reached when both treatments are combined (Table 3). In view of previously reported plateau levels of docosahexaenoic acid of heart phospholipids in aging rats (23), it is not surprising to find that the C 22:6 ω 3 content of the phospholipids in CLO hearts was hardly increased further by NE treatment. A maximum percentage of docosahexaenoic acid (25.4 % in the total phospholipids of the CLO + NE group compared to 11.9 % in the control group and 37.5 % versus 21.0 % in phosphatidylethanolamine) is probably reached. Norepinephrine stress hovever, gave a significant further reduction in linoleic acid in CLO hearts when the animals were subjected to catecholamine stress (Table 3). Despite a very low mortality rate, the extreme changes in polyene fatty acid content in total heart phospholipid as previously described by Gudbjarnason et al. (23), were found.

Fatty acid composition in phosphatidylcholine and phosphatidylethanolamine

As can be seen from Table 4, a CLO diet induces in phosphatidylcholine a significant decrease in linoleic acid and arachidonic acid, both ω 6 fatty acids. This is compensated by an increase in ω 3 fatty acids, e.g. eicosapentaenoic acid and docosahexaenoic acid. Linoleic acid was reduced by 63 % and arachidonic acid by 17 % respectively. The relative increase of docosapentaenoic acid was 28 % and docosahexaenoic acid even increased to 129 %. In phosphatidylcholine from rat heart on a CLO diet eicosapentaenoic acid was found to represent a quite substantial part of the bound fatty acids (5.0 %). Consequently it can be calculated from these results that the relative sum of ω 3 + ω 6 fatty acids in the phosphatidyl choline molecule is rather constant and so is the saturated/polyunsaturated ratio of the component phospholipid. It is also remarkable that oleic acid (C 18:1 ω 9) did not change very much in its relative amount. The polyunsaturated fatty acids account for about 45 % of the fatty acids independent of the diet or beta-adrenergic treatment. Therefore it is reasonable to assume that the ω 6 fatty acids in phosphatidylcholine are replaced by ω 3 fatty acids. NE stress had no effect on the arachidonic acid content of phosphatidylcholine in the hearts of the animals receiving a control diet (Table 4). Emilsson and Gudbjarnason (14) reported an increase in their experiments. Linoleic acid decreased significantly after daily NE administration.

Docosahexaenoic acid in the total phospholipid as well as the phosphatidylethanolamine fraction was increased significantly by NE administration in the control fed rats (Tables 3 and 5). The differences in effect of NE stress on the arachidonic acid content of phosphatidylcholine in the hearts of rats receiving a control diet as found by us and the data by Gudbjarnason et al. (14) is probably related to the fact that these authors found lower arachidonic acid contents in the control fed rats (15 % versus 29.4 % in our experiments). The relative amount of 30 % of this fatty acid is probably already maximal prior to the dietary or the NE treatment of the rats. In the phosphatidylcholine fraction of hearts from rats receiving a CLO diet the C 18:2 ω 6 content is already low (4.0 %). Still norepinephrine stress reduces this fatty acid with 35 %. NE stress had no significant effect on the arachidonic acid content and the docosahexaenoic acid content in the CLO fed rats.

The changes in the fatty acid composition of phosphatidylethanolamine due to NE stress and a CLO diet are presented in Table 5. Linoleic acid is reduced with 53 % and arachidonic acid with 51 %. Docosahexaenoic acid is increased from 21.0 % to 34.5 %. Like the shifts observed in the phosphatidylcholine fraction, there is a diet-induced increase in C 20:5 ω 3 in the phosphatidylethanolamine fraction. The saturated/unsaturated fatty acid ratio remains constant. The total amount of ω 6 fatty acis in phosphatidylethanolamine decreases from 27.4 % to 12.6 % while there is a simultaneous increase in ω 3 fatty acids from 25.4 % to 40.9 %. NE stress similarly affected the fatty acid distribution in phosphatidylethanolamine as caused by CLO nutrition, although to a less extent (Table 5). Hardly an effect is seen on the saturated and monounsaturated fatty acids. For the linoleic acid content the effect is less pronounced after NE treatment than after CLO nutrition. This holds especially for arachidonic acid which is only slightly reduced by norepinephrine. Also the percentage of docosahexaenoic acid is not raised to the same extent by stress as compared to the CLO diet. As a result of the CLO feeding linoleic acid is already low and docosahexaenoic acid very high in phosphatidylethanolamine and can hardly be influenced by NE treatment. Perhaps this represents the reason that hardly no change is observed in these fatty acids. Compared with the data reported by Gudbjarnason et al. (23) the changes in the fatty acid pattern due to CLO feeding are more pronounced and a relative concentration of 22:6 ω 3 in phosphatidylethanolamine of 34.5 % is reached (Table 5). Linoleic acid was even found to be as low as 2.1 %. From the data can also be concluded that the cardiac

Table 4. Changes in the fatty acid composition of phosphatidylocholine in heart tissue of rats after dietary and/or beta-adrenergic treatment*).

Fatty acids	Control fed rats		CLO fed rats	
	Solvent injected (n = 8)	NE injected (n = 8)	Solvent injected (n = 7)	NE injected (n = 7)
DMA 16:0	+	+	+	+
16:0	17.5 ± 0.4	19.3 ± 0.3[a])	19.5 ± 0.6[c])	17.7 ± 0.8
DMA 18:0	+	+	+	+
18:0	26.7 ± 0.9	25.9 ± 0.4	25.6 ± 0.6	28.0 ± 0.7[e])
18:1 ω 9	5.6 ± 0.8	6.7 ± 0.6	5.9 ± 0.6	5.4 ± 0.5
18:2 ω 6	10.8 ± 0.4	6.6 ± 0.6[b])	4.0 ± 0.4[d])	2.6 ± 0.3[f])
22:0	0.5 ± 0.3	0.7 ± 0.3	0.2 ± 0.1	1.0 ± 0.6
20:4 ω 6	29.4 ± 0.8	29.5 ± 0.9	24.2 ± 0.7[d])	26.4 ± 0.9
20:5 ω 3	0.2 ± 0.1	0.2 ± 0.1	5.0 ± 0.4[d])	3.3 ± 0.3[f])
22:4 ω 6	0.4 ± 0.2	+	+	+
24:1	2.0 ± 0.3	2.3 ± 0.7	1.7 ± 0.4	1.4 ± 0.3
22:5 ω 3	2.1 ± 0.5	2.3 ± 0.3	2.7 ± 0.1	2.6 ± 0.1
22:6 ω 3	4.9 ± 0.6	6.6 ± 0.7	11.2 ± 0.5[d])	11.6 ± 0.4

[a]), [b]) refer to significance of differences between column "control + solvent" vs. "control + NE" (a, $p < 0.01$; b, $p < 0.001$)

[c]), [d]) refer to significance of differences between column "control + solvent" vs. "CLO + solvent" (c, $p < 0.02$; d, $p < 0.001$)

[e]), [f]) refer to significance of differences between column "control + solvent" vs. "CLO + NE" (e, $p < 0.05$; f, $p < 0.01$)

*) mean ± SEM, DMA = dimethylated acetals

Table 5. Changes in the fatty acid composition fo phosphatidylethanolamine in heart tissue of rats after dietary and/or beta-adrenergic treatment*).

Fatty acids	Conrol fed rats		CLO fed rats	
	Solvent injected (n = 8)	NE injected (n = 8)	Solvent injected (n = 7)	NE injected (n = 7)
DMA 16:0	4.5 ± 0.2	4.9 ± 0.2	4.5 ± 0.3	4.8 ± 0.2
16:0	8.2 ± 0.2	9.9 ± 0.6[a])	9.4 ± 0.2[c])	10.0 ± 0.2[e])
DMA 18:0	3.9 ± 0.2	4.1 ± 0.2	2.6 ± 0.2[d])	2.7 ± 0.2
18:0	23.8 ± 0.4	21.9 ± 0.8	22.6 ± 0.5	22.1 ± 0.2
18:1 ω 9	4.5 ± 0.3	3.7 ± 0.2[a])	3.9 ± 0.5	4.4 ± 0.7
18:2 ω 6	6.0 ± 0.5	2.8 ± 0.3[b])	2.1 ± 0.2[d])	1.4 ± 0.4[f])
22:0	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.3	0.8 ± 0.1
20:4 ω 6	21.4 ± 1.2	19.0 ± 1.9	10.5 ± 0.3[d])	8.9 ± 0.3[f])
20:5 ω 3	0.7 ± 0.4	0.3 ± 0.1	3.1 ± 0.2[d])	2.5 ± 0.2
22:4 ω 6	+	+	+	+
24:1	2.2 ± 0.7	1.9 ± 0.3	3.1 ± 1.0	2.1 ± 0.7
22:5 ω 3	3.7 ± 0.4	3.2 ± 0.2	3.3 ± 0.1	2.8 ± 0.1[g])
22:6 ω 3	21.0 ± 1.8	28.0 ± 1.7[b])	34.5 ± 0.9[d])	37.5 ± 0.9[e])

[a]), [b]) refer to significance of differences between column "control + solvent" vs. "control + NE" (a, $p < 0.02$; b, $p < 0.001$)

[c]), [d]) refers to significance of differences between column "control + solvent" vs. "CLO + solvent" (c, $p < 0.01$; d, $p < 0.001$)

[e]), [f]), [g]) refers to significance of differences between column "CLO + solvent" vs. "CLO + NE" (c, $p < 0.05$; f, $p < 0.01$; g, $p < 0.001$)

DMA = dimethylated acetals

*) mean ± SEM

phospholipids from CLO fed rats are less sensitive to changes in their fatty acid patterns due to NE treatment than the cardiac phospholipids of control fed animals.

Positional fatty acid distribution in phosphatidylethanolamine

The fatty acid distribution on the 1- and 2-position of the phosphatidylethanolamine fraction was analyzed. As we had to deal with the determination of the phosphorus content of the different phospholipid components (see above), a combination of the extracted phosphatidylethanolamine fractions of hearts from a whole group of rats was required to obtain sufficient material for determination of the positional distribution of fatty acids. The results are presented in Table 6. As can be seen from this table the polyunsaturated fatty acids are almost exclusively located at the 2-position of the phospholipid molecule. Linoleic acid and docosahexaenoic acid are also found in small amounts at the 1-position. These results agree very well with former analyses described by us on the positional distribution of the fatty acids in phosphatidylethanolamine from rat heart (38). If the results are compared with similar analysis on heart phosphatidylethanolamine from control fed mice (38) which is especially rich in docosahexaenoic acid (39.5 %), no polyunsaturated fatty acids were found at the 1-position except for small amounts of C 18:2 ω 6. In the present work it is demonstrated that an increase or decrease in ω 3 and ω 6 fatty acids either by dietary or pharmacological treatment does not lead to great alterations of the fatty acid distribution over the 1- and 2-position.

Table 6. Percentual distribution fo the fatty acids at the 1- and 2-position of phosphatidylethanolamine in rat heart after dietary and/or beta-adrenergic treatment*).

Fatty acids	Control fed rats				CLO fed rats			
	Solvent injected		NE injected		Solvent injected		NE injected	
	1-pos	2-pos	1-pos	2-pos	1-pos	2-pos	1-pos	2-pos
DMA 16:0	10.6	0.4	12.5	0.6	13.4	0.4	12.4	1.0
16:0	18.9	4.6	18.9	4.9	21.5	5.4	22.3	5.4
DMA 18:0	8.4	–	10.1	–	5.0	–	7.0	0.4
18:0	49.1	5.7	40.2	7.9	46.4	7.6	40.9	6.6
18:1 ω 9	7.2	3.4	5.3	3.8	5.8	2.9	4.3	3.8
18:2 ω 6	2.7	9.7	0.5	5.3	0.9	2.1	1.9	1.6
22:0	+	–	–	–	–	–	–	–
20:4 ω 6	0.9	41.1	4.2	32.0	0.8	14.6	1.0	16.3
20:5 ω 3	–	0.4	1.2	1.2	0.4	5.2	3.1	5.5
22:5 ω 3	0.5	5.1	2.7	6.3	–	5.2	–	5.0
22:6 ω 3	1.7	29.6	4.4	37.0	3.2	55.8	4.6	52.4
others	–	–	–	1.0	2.6	0.8	2.5	2.0

DMA is an abbreviation for: dimethylated acetals; *) results are the means of 2 determinations of combined phospholipid fractions extracted from hearts of the animal groups mentioned in Table 1.

ECG patterns

In Table 7 the experimental groups are shown with the observed mean heart rates and incidence of ST segment elevation in the ECG pattern. The heart rates were not different in either group of rats. In the CLO diet group 86 % of the animals had ST elevation versus 25 % in the control diet group. It is possible that CLO feeding of rats makes their hearts more susceptible to ether anesthesia (without ventilatory assistance-induced ischemia). Measurements of ECG have not been carried out on conscious rats so that no definitive conclusions on this point can be made. The incidence of ST changes was also significantly higher in the CLO fed group treated with NE. This is not surprising if one considers previously described necrotic effects induced by excessive NE administration on rat heart (16, 42). However, it is important to note that NE treatment of CLO fed rats did not further aggravate electrocardiographic abnormalities.

Table 7. Heart rates and occurrence of ST segment elevation in the ECG patterns of rats after dietary and/or beta-adrenergic treatment.

Diet	Injected	Number of animals	Heart rate*) (min^{-1})	Incidence of ST-elevation (%)
Control	Solvent	8	373 ± 10	25
Control	NE	8	353 ± 11	50
CLO	Solvent	7	386 ± 15	86**)
CLO	NE	7	351 ± 11	86**)

*) mean ± SEM

**) $p < 0.05$ with control fed, solvent injected animals. Statistical evaluation of these incidence rate percentages have been carried out with comparative analysis of rate differences and rate ratio.

Discussion

From the results it is clear that both CLO feeding and chronic administration of NE will change the fatty acid distribution in the major phospholipids in the rat heart. A substantial replacement of ω 6 fatty acids by ω 3 fatty acids takes place. At the end of the experimental period phosphatidylethanolamine contains the highest amount of ω 3 fatty acids, but the relative changes are more pronounced in phosphatidylcholine. The data obtained are in qualitative agreement with those reported by Gudbjarnason et al. (13, 14, 23). For rats receiving a control diet a mortality of 50 % within 15 days was reported by Emilsson and Gudbjarnason (14) upon daily administration of NE with increasing dose. Our results show a much lower mortality (10 %) when NE was suspended in PEG 400 despite the same changes in the fatty acid distribution of the phospholipids. Further studies carried out in our laboratory showed that the same amount of catecholamines given in a similar time period but now as a constant flow infusion resulted in no mortality at all, and also much less pronounced changes in the fatty acid composition of the phospholipids were found (unpublished data). This indicates that not only the rat strain but also the mode of catecholamine administration may influence mortality. It also demonstrates that high concentrations of catecholamines are needed for substantial changes in the fatty acyl composition of the phospholipids occur. Gudbjarnason et al. (23) reported an increased sensitivity in rats to catecholamines upon CLO feeding, especially when there is a vitamin E deficiency. In the present study no differences existed between vitamin E content of control and CLO diet. Moreover Ruiter et al. (46) demonstrated that the extra addition of vitamin E to the diet of fish oil fed pigs did not protect against development of yellow fat disease, which is a symptom of vitamin E deficiency. Although in the present work the CLO fed plus NE-treated group had an increased mortality rate (22 %), this value is still far below the previously reported mortality rate (14). Because there is a marked difference between the findings of Emilsson and Gudbjarnason (14) and those of our studies with respect to the mortality rate while changes in fatty acid composition of the phospholipids were similar, we decided to evaluate the occurrence of abnormalities in electrical properties of the heart. Interestingly it was observed that CLO feeding in either control or NE injected rats resulted in increased rate of incidence of ST-elevations, 86 % versus 25 % in the control-fed plus control-injected rats.

Presently there is no information available as to whether enzymes involved in the biosynthesis or breakdown of cardiac phospholipids are affected by CLO feeding and catecholamine stress. Cellular phospholipids are confined to membrane structures and the continuous repair and maintenance of these membranes is the primary function of phospholipid turnover. Above that the biomembranes have to keep their physico-chemical properties within certain limits (11, 19) for proper functioning, which is primarily determined by the fatty acid composition of their phospholipids. The main processes which are known to disrupt the phospholipid molecules in biomembranes are those induced by action of phospholipases and autooxidation. Phospholipid molecules with a high content of polyunsaturated fatty acids, as reported in this study, are favorite substrates for both biochemical processes.

It has been repeatedly demonstrated that polyene fatty acids preferentially occupy the 2-position and saturated fatty acids as well as oleic acid settle at the 1-position (11, 38). Independent of whether changes in the fatty acid composition are induced by CLO diet and/or catecholamine stress, the ratio saturated/polyunsaturated fatty acids is hardly influenced and changes are only observed within the ω 6 and ω 3 fatty acid distribution (Tables 3, 4 and 5). The shifts of ω 6/ω 3 distribution in phosphatidylethanolamine were most pronounced (Table 5). That catecholamines activate phospholipase A_2 thereby directly causing break-

down of cellular phospholipids has been reported (54). In the present study a significant decrease ($p < 0.05$) in the total phospholipids per gram wet cardiac tissue was indeed demonstrated when NE was administered to control rats. A non-significant reduction in the phospholipids was also found in the CLO group with the same treatment. Apparently, a tendency of total phospholipids to decrease upon CLO feeding may have obscured the NE-induced reduction. In fact, diet-induced phospholipid changes may also occur through change in plasma NE and tissue sensitivity to NE in view of previous findings of suppression of NE-induced thermogenesis in human obesity by diet and weight loss (15). Cellular membranes contain phospholipase A_1 and/or A_2, which may undergo changes under the influence of dietary and catecholamine treatments, although no phospholipase activity measurements have been carried out in the present study.

It is believed that polyunsaturated fatty acids are exchanged in biomembranes by reacylation of lysophospholipids rather than by de novo synthesis from the corresponding diglycerides (20). NE probably also, activates phospholipase A_2 activity in tissues other than heart (e.g. adipose tissue), favouring the remotion of fatty acids, thereby inducing changes in phospholipid fatty acid composition in the heart. However, it is also demonstrated for the heart that an exchange of whole phospholipid molecules takes place between membranes mediated by phospholipid exchange proteins (10). To what extent each pathway contributes to formation of a steady state phospholipid composition of the heart membranes is as yet unclear. Finally, autooxidation of polyunsaturated fatty acids, especially those containing four and six double bonds, leads to a more rigid membrane structure (12, 18) resulting in a disturbance of membrane-localized biochemical processes. Furthermore peroxidized lipids have been shown to become preferred substrates for endogenous phospholipases (57). Therefore, by what factors the positional distribution of the fatty acids in phospholipids are determined is still unclear.

It has been demonstrated that mitochondrial and plasmalemmal membranes do not have the capacity for the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine. So it is most likely that the positional fatty acid distribution in these membranes is determined by acylation-reacylation processes and by phospholipid exchange of presynthesized molecules. Discrimination between plasma membranes, mitochondria and sarcoplasmatic reticulum has not been carried out in the present work and would offer more insight. Vasdev and Kako (56) showed that in rat myocardium, differences in preference exist between long chain fatty acids to be incorporated in phospholipids, even a profound difference in incorporation between phosphatidylcholine and phosphatidylethanolamine. Phosphatidylethanolamine is mainly characterized by a relatively higher content of long chain polyunsaturated fatty acids than phosphatidylcholine (compare Tables 4 and 5). Despite dietary and beta-adrenergic treatment, resulting in a replacement of ω 6 fatty acids for ω 3 fatty acids with increased chain length and unsaturation, this different character of fatty acid composition of phosphatidylcholine and phosphatidylethanolamine does not change. In the present work it appears that the polyunsaturated fatty acids account for 45–50 % of the fatty acyl residues and preferentially occupy the 2-position of phosphatidylethanolamine at which they can exchange for each other due to changes in diet or beta-adrenergic treatment. Thus supports the idea that reacylation represents the major pathway for biosynthesis of these unsaturated molecular species.

Acknowledgements

This work was supported by a grant of the Dutch Heart Foundation. The control rats and CLO fed rats were kindly supplied by Unilever (Vlaardingen, The Netherlands). The authors wish to thank Dr. A. J. Vergroesen (Unilever, Vlaardingen, The Netherlands) for advice and Mrs. M. Hanegraaff and Mrs. P. H. Vegter for their help in preparing the manuscript.

References

1. Andrich MP, Vanderkooi JM (1976) Temperature dependence of 1,6 diphenyl 1,3,5 hexatriene fluorescence in phospholipid artificial membranes. Biochemistry 15:1257–1261
2. Arkhipenko Yu V, Kagan VE, Meerson FZ (1983) Mechanisms of heart sarcoplasmic reticulum damage. In: Jacob R, Gülch RW, Kissling G (eds) Cardiac adaptation to hemodynamic overload, training and stress. Steinkopff, Darmstadt. pp 258–264
3. Bang HO, Dyerberg J, Brondum Nielsen A (1971) Plasma lipid and lipoprotein pattern in Greenlandic west-coast eskimos. Lancet 1:1143–1146
4. Björnstad P (1966) Phospholipase activity in rat liver microsomes studied by the use of endogenous substrates. Biochim Biophys Acta 116:500–510
5. Bonsen PPM, de Haas GH, Pieterson WA, van Deenen LLM (1972) Studies on phospholipase A and its zymogen from porcine pancreas IV. The influence of chemical modification of the lecithin structure on substrate properties. Biochim Biophys Acta 270:364–382
6. Budowski P (1981) Review: nutritional effects of ω 3 polyunsaturated fatty acids. Isr J Med Sci 17:223–231
7. Burton KP, Hagler HK, Templeton GH, Willerson JT, Buja LM (1977) Lanthanum probe studies of cellular pathophysiology by hypoxia in isolated cardiac muscle. J Clin Invest 60:1289–1302
8. Chien KR, Pfau RG, Farber JL (1979) Ischemic myocardial cell injury. Am J Pathol 97(3):505–522
9. Cogan U, Shinitzky M, Weber G, Nishida T (1973) Microviscosity and order in the hydrocarbon region of phospholipid and phospholipid-cholesterol dispersions determined with fluorescent probes. Biochemistry 12:521–528
10. Dawson RMC (1973) The exchange of phospholipids between cell membranes. Sub Cell Biochem 2:69–89
11. van Deenen LLM (1965) Phospholipids and biomembranes. In: Holman RT (ed) Progress in the chemistry of fats and other lipids 8 (1). Pergamon Press, pp 45–46
12. Dobretsov GE, Borschevskaya TA, Petrov VA, Vladimirov Yu A (1977) The increase of phospholipid bilayer rigidity after lipid peroxidation. FEBS Lett 84:125–128
13. Emilsson A, Gudbjarnason S (1983) Reversible alterations in fatty acid profile of glycerophospholipids in rat heart muscle induced by repeated norepinephrine administration. Biochim Biophys Acta 750:1–6
14. Emilsson A, Gudbjarnason S (1981) Changes in fatty acyl chain composition of rat heart phospholipids induced by noradrenalin. Biochim Biophys Acta 664:82–88
15. Finer N, Swan PC, Mitchell FT (1985) Suppression of norepinephrine-induced thermogenesis in human obesity by diet and weight loss. Int J Obesity 9:121–126
16. Fleckenstein A, Janke J, Döring HJ, Pachinger O (1973) Ca-overload as the determinant factor in the production of catecholamine induced myocardial lesions. In: Bajusz E, Rona G (eds) Cardiomyopathies recent advances in cardiac structure and metabolism, Vol 2. Baltimore University Park Press, pp 455–466
17. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from annual tissues. J Biol Chem 226:497–509
18. Fukuzawa K, Chida H, Tokumura A, Tsukatani H (1981) Antioxidative effect of alpha-tocopherol incorporation into lecithin liposomes on ascorbic acid Fe^{++}-induced lipid peroxydation. Arch Biochem Biophys 206:173–180
19. van Golde LMG, van Deenen LLM (1966) The effect of dietary fat on the molecular species of lecithin from rat liver. Biochim Biophys Acta 125:496–509
20. van Golde LMG, Scherphof GL, van Deenen LLM (1969) Biosynthetic pathways in the formation of individual molecular species of rat liver phospholipids. Biochem Biophys Acta 176:635–637
21. Goodnight Jr SH, Harris WS, Connor WE, Illingworth DR (1982) Polyunsaturated fatty acids, hyperlipidemia and thrombosis: a review. Arteriosclerosis 2:87–113.
22. Gudbjarnason S (1983) Biochemical alterations in the ischemic and infarcted heart following coronary artery occlusion. In: Peeters H, Gresham GA, Paoletti R (eds) Arterial Pollution. Plenum Press, pp 125–142
23. Gudbjarnason S, Oskarsdottir G, Doell B, Hallgrimson J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 25:130–144

24. Hamberg M, Svensson J, Samuelsson B (1975) Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci (USA) 72:2994–2998
25. Herdson PB, Sommers NH, Jennings RB (1963) A comparative study of the fine structure of normal and ischemic dog myocardium with special reference to early changes following temporary occlusion of a coronary artery. Am J Pathol 46:367–386
26. Iritani N, Narita R (1984) Changes of arachidonic acid on N-3 polyunsaturated fatty acids of phospholipid classes in liver, plasma and platelets during dietary fat manipulation. Biochim Biophys Acta 793:441–447
27. Katz AM, Messineo FC (1981) Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. Circ Res 48:1–16
28. Kimelberg HK (1977) Dynamic aspects of cell surface organization. In: Poste G, Nicolson GL (eds) Cell Surface Reviews 3, pp 205–293
29. Kramer JKG (1980) Comparative studies on composition of cardiac phospholipids in rat fed different vegetable oils. Lipids 15 (9):651–660
30. Kreisberg RA (1966) Effect of epinephrine on myocardial triglyceride and free acid utilization. Am J Physiol 210:385–389
31. Lamers JMJ, Stinis HT, Montfoort A, Hülsmann WC (1984) The effect of lipid intermediates on Ca^{++} and Na^{+} permeability and $(Na^{+} + K^{+})$ ATPase of cardiac sarcolemma: a possible role in myocardial ischemia. Biochim Biophys Acta 774:127–137
32. Lentz BR, Barenholz Y, Thompson TE (1976) Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers, I. Single component phosphatidylcholine liposomes. Biochemistry 15:4521–4528
33. Lentz BR, Barenholz Y, Thomspon TE (1976) Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers, II. Two component phosphatidylcholine liposomes. Biochemistry 15:4529–4537
34. Liedtke AJ (1981) Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. Prog Cardiovasc Dis 23:321–336
35. von Lossonczy TO, Ruiter A, Bronsgeest-Schoute HC, van Gent CM, Hermus RJJ (1978) The effect of a fish diet on serum lipids in healthy human subjects. Am J Clin Nutr 31:1340–1346
36. Mallow S (1983) Role of calcium and free fatty acids in epinephrine induced myocardial necrosis. Toxicol Appl Pharmacology 71:280–287
37. Meerson FZ (1980) Disturbances of metabolism and cardiac function under the action of emotional painful stress and their prophylaxis. Basic Res Cardiol 75:479–500
38. Montfoort A, Rutten-van Beysterveld CCM, Wortelboer MR (1983) Molecular species of diacylphosphatidylethanolamine in rat and mouse heart given the same diet. Biochem Intern 6 (5):569–580
39. Nafstad I, Tollersrud S (1970) The vitamin E-deficiency syndrome in pigs. Acta Vet Scand 11:452
40. Needleman P, Minkes MS, Raz A (1976) Thromboxanes: selective biosynthesis and distinct biological properties. Science 193:163–165
41. Noronko-Dutra AA, Steen EM, Woolf N (1985) The correlation between catecholamine and lipid peroxidation-induced damage in heart cells. Bas Res Cardiol 80 (Suppl 1):133–136
42. Opie LH (1975) Metabolism of free fatty acids, glucose and catecholamines in acute myocardial infarction. Relation to myocardial ischemia and infarct size. Am J Cardiol 36:938–953
43. Papahadjopoulos D (1973) In: Hawthorn JN, Dawson RMC (eds) Form and functions of phospholipids. BBA Library Vol 3, pp 143–169
44. Popp-Snijders C, Schouten JA, de Jong AP, van der Veen EA (1984) Effect of dietary cod liver oil on the lipid composition of human erythrocyte membranes. Scand J Clin Lab Invest 44:39–46
45. Raz A, Minkes MS, Needleman R (1977) Endoperoxides and thromboxanes. Structural determinants for platelet aggregation and vasoconstriction. Biochim Biophys Acta 488:305–311
46. Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM (1978) The influence of dietary mackerel oil on the conditions of organs and on blood lipid composition in the young growing pig. Am J Clin Nutr 31:2159–2166
47. Sanders TAB, Vickers M, Haines HP (1981) Effect on blood lipids and haemostasis of a supplement of cod liver oil rich in eicosapentaenoic and docosahexaenoic acid in healthy young men. Clin Sci 61:317–324

48. Saxon ME, Filipov AK, Porotikov UI (1984) The possible role of phospholipase A_2 in cardiac membrane destabilization under calcium overload conditions. Basic Res Cardiol 79:668–678
49. Scherphof GL (1967) Metabolic conversions of mitochondrial and microsomal phospholipids. Thesis, Utrecht, The Netherlands, p 28–41
50. Shaikh NA, Downar E (1981) Time course of changes in porcine myocardial phospholipid levels during ischemia – A reassessment of the lysolipid hypothesis. Circ Res 49 (2):316–325
51. Shen AC, Jennings RB (1972) Myocardial calcium and magnesium in acute ischemic injury. Am J Pathol 67:417–440
52. Simon G, Rouser G (1969) Species variations in phospholipid class distribution of organs II: heart and skeletal muscle. Lipids 4 (6):607–614
53. Sobel BE, Corr PB, Robinson AK, Goldstein RA, Witkowski FX, Klein MS (1978) Accumulation of lysophosphoglycerides with arrhythmogenic properties in ischemic myocardium. J Clin Invest 62:546–553
54. Stam H, Hülsmann WC (1981) Release of lipolytic products from rat heart, hormonal stimulation, intracardiac origin and pharmacological modification. Biochem Int 2:477–484
55. Vasdev SC, Kako KJ (1977) Incorporation of fatty acids into rat heart lipids. In vivo and in vitro studies. J Mol Cell Cardiol 9:617–631
56. Weglicki WB (1980) Degradation of phospholipids of myocardial membranes. In: Wildenthal K (ed) Degradative processes in heart muscle. Elsevier North Holland Biomedical Press, pp 377–388
57. Weglicki WB, Dickens BF, Tong Mak I (1984) Enhanced lysosomal phospholipid degradation and lysophospholipid production due to free radicals. Biochem Biophys Res Commun 124:229–235
58. Willerson JT, Scales F, Mukherjee A, Platt MR, Templeton GH, Fink GC, Buja LM (1977) Abnormal myocardial fluid retention as an early manifestation of ischemic injury. Am J Pathol: 87:159–188

Received June 10, 1985

Authors' address:

Dr. A. Montfoort, Department of Pathology I, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

CHAPTER 8

The effects of dietary mackerel oil on plasma and cell membrane lipids, on hemodynamics and cardiac arrhythmias during recurrent acute ischemia in the pig

J. M. Hartog, J. M. J. Lamers*, and P. D. Verdouw

Laboratory for Experimental Cardiology, Thoraxcenter, * Department of Biochemistry I, Medical Faculty, Erasmus University Rotterdam, Rotterdam (The Netherlands)

Summary: Fish-oil nutrition leads to a change in fatty acid composition of cellular membranes. To investigate whether this affects cardiac responses to acute myocardial ischemia, pigs were fed a 9.1 % (w/w) mackerel-oil (n = 8) or 9.1 % (w/w) lard-fat diet (n = 8). Eight weeks mackerel-oil feeding reduced plasmacholesterol (51 %) and triglyceride (48 %), while the n-6 fatty acids of cardiac and platelet membrane phospholipids were partially replaced by n-3 fatty acids. After 8 weeks the animals were anesthetized and the left anterior descending coronary artery was occluded 6 times for a period of 5 min at 15 min intervals. Recovery of cardiac function and the incidence of cardiac arrhythmias were similar for both dietary groups. However, during the last reperfusions, hyperemic responses were less in magnitude and shorter lasting in the lard-fat (81 ± 17 ml/min) than in the mackerel-oil group (129 ± 18 ml/min). This may be caused by a difference in thromboxane synthesis, as coronary venous blood thromboxane B_2 levels were higher in the lard-fat (77 ± 6 pg/ml) than in the mackerel-oil group (19 ± 7 pg/ml, $P < 0.05$) during peak hyperemia. The low baseline levels of both thromboxane B_2 and 6-keto prostaglandin F_{1alpha} in fish-oil fed animals originate from the reduced content of precursor fatty acid in the membrane phospholipids. In conclusion, despite marked changes in membrane fatty acid composition induced by prolonged feeding with fish oil, no modification of recovery of cardiac function and of incidence of cardiac arrhythmias was found during acute recurrent ischemia.

Key words: fish oil, phospholipids, myocardial ischemia, ventricular arrhythmias, cardiac function, prostaglandins.

Introduction

Epidemiological studies have revealed a negative correlation between the consumption of fatty sea-fish and coronary heart disease which has been explained by the plasma lipid lowering and antithrombotic effects of dietary n-3 fatty acids (2, 11). The exchange of cell membrane n-6 fatty acids by dietary n-3 fatty acids and subsequent alterations in prostanoid synthesis may represent independent factors influencing cardiac function, ischemia-reperfusion injury and/or incidence of arrhythmias. Several groups of investigators have reported that dietary polyunsaturated fatty acids affect arterial blood pressure (26, 28, 35, 39, 42) and the susceptibility to arrhythmias (6, 9, 10, 15–17, 29). However, the results are not conclusive as hypotensive (26, 28, 35, 42) as well as hypertensive (39) actions have been reported. Likewise, the effect of dietary fish oil on the incidence of ventricular arrhythmias is controversial as antiarrhythmic (10, 17, 29) as well as arrhythmogenic (16) actions have been reported. The harmful effects of diets using cod liver oil might have resulted from the toxic side effects of the high levels of vitamin A or D and cetoleic acid or the low level of vitamin E in these diets (16, 19). This problem can be overcome by using purified fish oil

extracts (EPA) which are supplemented with tocopherols (35). The increase of n-3 fatty acids in membrane phospholipids after consumption of high doses of fish oil could contribute to a higher susceptibility for cardiac arrhythmias through reduced levels of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) (7, 19, 20). Long-term consumption of a linoleic acid rich diet increased the survival rate and reduced the occurrence of arrhythmias after coronary artery ligation in rats (25, 27). The prevention by indomethacin of this beneficial effect suggested the involvement of increased prostaglandin synthesis due to replacement of membrane bound fatty acids of n-3 to n-6 type. Arachidonic acid, a prostaglandin of the E type, and PGI_2 have been shown to exhibit antiarrhythmic activity and to offer protection in experimental myocardial ischemia (21, 24, 33) whereas TXA_2 has been reported to be arrhythmogenic (7, 8). Dietary supplementation with fish oil, which is rich in n-3 fatty acids eicosapentanoic and docosahexanoic acids, also exerts a protective effect on experimental cerebral infarction in cats (5) and myocardial infarction in dogs (10). Therefore at present no clearcut data have been provided to what extent fish-oil nutrition can influence cardiac function, ischemia-reperfusion injury and/or the incidence of ventricular arrhythmias.

The purpose of the present study was therefore to evaluate the effect of dietary fish-oil (EPA) on the recovery of cardiac function and the susceptibility to arrhythmias during coronary artery occlusion and reperfusion in anesthetized pigs. Experiments were performed in a model (multiple occlusion and reperfusion) which has been shown to result in a partial loss of regional function for a substantial period of time (31). Even so, in pigs the earliest phase of ventricular arrhythmias already occurs within minutes following coronary artery occlusion which frequently ends in ventricular fibrillation (3, 44).

Materials and Methods

Experimental animals

Sixteen Yorkshire piglets of either sex, and 5 weeks of age (7.9 ± 0.2 kg), were housed individually in slat-bottomed cages in temperature-controlled animal quarters and divided arbitrarily into two groups. Each group followed a different diet for a period of 8 weeks.

Experimental diets

The basal diet of both experimental groups was identical (Hope Farms, Woerden, The Netherlands) and low in fat content (< 2 % w/w) but with sufficient levels of linoleic acid. The control diet was prepared by addition of 9.1 % w/w lard fat (Gebro Smilde BV, Heerenveen, The Netherlands) to the basal diet. In order to minimize the oxidation of the mackerel oil in the experimental diet, the 9.1 % w/w mackerel oil (EPA extract obtained from A/S Johan C. Martens and Co., Bergen, Norway) was added

Table 1. Composition (g/100 g) of the diets fed to two groups of young Yorkshire pigs.

	9.1 % lard-fat diet	9.1 % mackerel-oil diet
Carbohydrates	54	54
Crude protein	17	17
Crude fat	2	2
Crude fiber	8	8
Moisture	9	9
Lard fat	9.1	–
Mackerel oil	–	9.1

Table 2. Fatty acid compositions (%) of the lard-fat and mackerel-oil diets fed to young Yorkshire pigs.

Fatty acid		9.1 % lard-fat diet	9.1 % mackerel-oil diet
14:0		2	7
16:0		24	18
16:1		3	8
18:0		10	1
18:1		42	17
18:2	n–6	15	8
18:3	n–3	1	1
20:1		1	6
20:5	n–3	–	17
22:1		–	4
22:6	n–3	–	9
24:1		–	1
others		2	3

just prior to consumption. For this reason the oil was packed and sealed under N_2 gas in small batches for daily use. Composition of the two diets have been presented in Tables 1 and 2.

The animals were fed in the morning and the portions were usually consumed within one hour.

Chemical analysis of plasma, platelets and myocardial biopsies

Before the start and at the end of the dietary period blood plasma triglyceride, total- and HDL-cholesterol were determined in samples collected by puncturing the subclavian vein (13, 40, 47). Thromboxane B_2 (TXB_2) and 6-keto prostaglandin F_{1alpha} (6-keto PGF_{1alpha}), stable products of the prostaglandin synthesis, were determined by radioimmunoassay in coronary venous blood samples collected prior to the first coronary artery occlusion and during peak hyperemia of the last reperfusion period (49).

During catheterization 50 ml blood was collected and separated platelets were washed and homogenized in buffer. The extracted phospholipids were separated from other lipids by thin layer chromatography (12, 30). After completion of the occlusion-reperfusion experiment (see later) the heart was excised and cooled on ice. The non-ischemic posterior wall of the left ventricle was rapidly cut out and homogenized in buffer. Homogenate was prepared as previously described (23). Homogenate phospholipids were separated (12, 30). The procedures for phospholipid hydrolysis, formation of fatty acid methyl esters, extraction of methylesters and gaschromatographic separation have all been described (30).

Hemodynamic measurements

After 8 weeks, the 16 animals (now weighing 20.4 ± 0.7 kg), were anesthetized and catheterized according to standard procedures (18). Following exposure of the heart via a midsternal split, electromagnetic flow probes (Skalar, Delft, The Netherlands) were placed around the ascending aorta and the proximal left anterior descending coronary artery (LADCA). The great cardiac vein was cannulated.

Baseline measurements were obtained after a stabilization period of at least 30 minutes. Subsequently, the LADCA was clamped for 5 minutes and declamped for 10 minutes. This occlusion-reperfusion procedure was repeated five more times. When ventricular fibrillation occurred, the animals were promptly defibrillated. If such attempts were not successful within 1 minute, the animals were excluded from further study. Except for the anesthetics and pancuronium bromide no other drugs were used.

Statistical analysis

The changes in biochemical and hemodynamic variables from baseline and/or post-dietary values were calculated separately in each experiment and the significance of these changes was determined by using the Student's T-test. For the evaluation of the overall difference between the two dietary groups analysis of variance for repeated measures was used. Statistical significance was accepted at $p < 0.05$ (two-tailed). All data have been expressed as mean ± standard error of mean (mean ± SEM).

Results

Plasmalipid levels after the dietary period

Before the start of the dietary period the plasmalipid levels of the two groups were the same (Table 3). After the dietary period of 8 weeks plasma triglyceride, total- and HDL-cholesterol decreased by 52 ± 7 %, 37 ± 4 % and 38 ± 3 %, respectively in the mackerel-oil

Table 3. Levels of plasma lipids of young Yorkshire pigs before and after 8 weeks of feeding 9.1 % lard-fat or 9.1 % mackerel-oil diets.

Plasmalipid	Diet	Pre-dietary (n = 8)	Post-dietary (n = 8)
Triglyceride (mM)	lard fat	0.50 ± 0.06	0.40 ± 0.03
	mackerel oil	0.49 ± 0.08	0.21 ± 0.01[a,b]
Total cholesterol (mM)	lard fat	2.17 ± 0.11	2.45 ± 0.10[a]
	mackerel oil	1.92 ± 0.12	1.21 ± 0.09[a,b]
HDL cholesterol (mM)	lard fat	0.91 ± 0.09	1.13 ± 0.04[a]
	mackerel oil	0.97 ± 0.09	0.60 ± 0.04[a,b]

a = $p < 0.05$ versus pre-dietary; b = $p < 0.05$ versus lard-fat fed animals at comparable time of feeding; all data have been presented as mean ± SEM.

Table 4. Fatty acid composition (%) of the phospholipids of cardiac and platelet homogenates of young Yorkshire pigs fed with 9.1 % lard-fat or 9.1 % mackerel-oil diets for 8 weeks.

Fatty acid	Myocardium		Platelets	
	9.1 % lard fat (n = 6)	9.1 % mackerel oil (n = 5)	9.1 % lard fat (n = 6)	9.1 % mackerel oil (n = 5)
16:0	19 ± 1	19 ± 1	21 ± 2	23 ± 1
18:0	11 ± 1	10 ± 1	16 ± 1	16 ± 4
18:1	19 ± 1	13 ± 3[a]	19 ± 3	17 ± 1
18:2 n–6	24 ± 1	13 ± 1[a]	9 ± 1	7 ± 1[a]
20:4 n–6	19 ± 1	7 ± 1[a]	14 ± 5	6 ± 1[a]
20:5 n–3	0.5 ± 0.2	21 ± 1[a]	0	12 ± 1[a]
22:4 n–6	1.7 ± 0.5	1.5 ± 0.4	3.9 ± 2.4	1.2 ± 0.3
22:5 n–3	1.2 ± 0.2	1.4 ± 0.3	0	2.7 ± 0.3[a]
22:6 n–3	1.6 ± 0.1[a]	6.1 ± 0.1[a]	1.1 ± 0.2	1.8 ± 0.3

[a] = $p < 0.05$ versus lard-fat diet animals. All data have been presented as mean ± SEM. The data on dimethylated acetals of 14:0 and 16:0, which are present in minor amounts, are not included.

fed pigs, whereas no significant change in plasma triglyceride and slightly elevated plasma total- and HDL-cholesterol levels were found in lard-fat fed animals (Table 3).

Fatty acid composition of cardiac and platelet membrane phospholipids

The n-6 fatty acids 18:2 and 20:4 in the cardiac phospholipids of the mackerel-oil fed pigs were exchanged for the n-3 fatty acids 20:5 and 22:6 (Table 4). Although no change occurred in the total polyunsaturated fatty acid content a dramatic increase of double bond indices

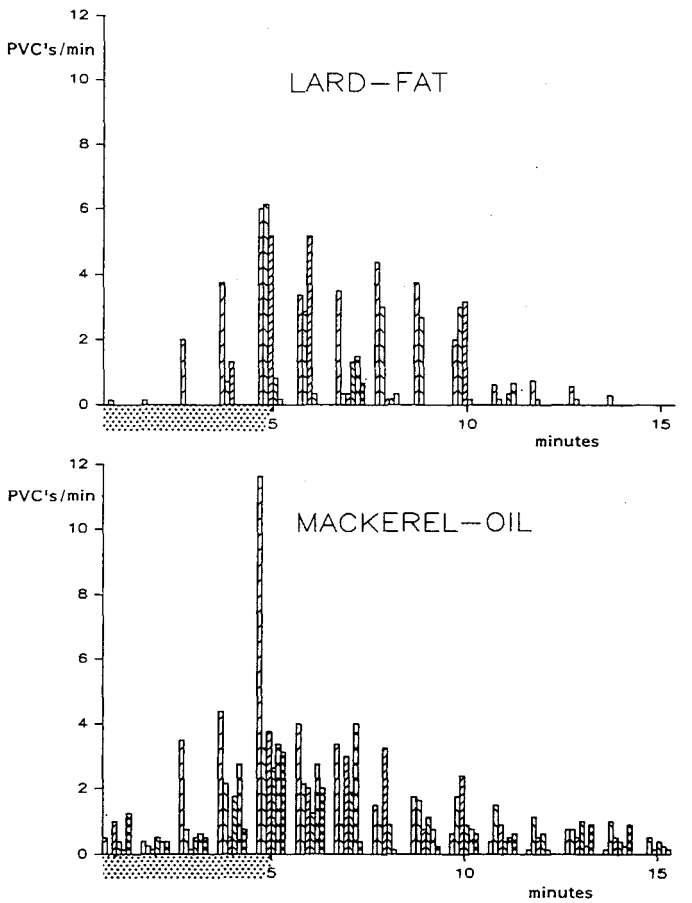

Fig. 1. Premature ventricular contractions during 6 consecutive 5 min occlusions followed by 10 min of reperfusion in anesthetized pigs which had been on a 9.1 % lard-fat (top) or 9.1 % mackerel-oil diet (bottom) for 8 weeks. The mean of the ventricular arrhythmias per min for each occlusion (0–5 min) and reperfusion (5–15 min) have been depicted. Because the differences in the incidence of ventricular arrhythmias between the two groups were not statistically significant, the SEM bars have been omitted for the sake of clarity. Each group consisted of 8 animals, but in the lard-fat dietary group two animals died of ventricular asystole; one during the first reperfusion and one during the second reperfusion period. ▨ = first period; ▧ = second period; ▨ = third period; ▧ = fourth period; ▩ = fifth period; ▩ = sixth period.

(1.85 ± 0.06 versus 1.28 ± 0.06) in the mackerel-oil fed versus the lard-fat fed pigs was observed. Similar changes occurred in platelet membrane phospholipids of the mackerel-oil fed pigs (Table 4).

Ventricular arrhythmias

No ventricular arrhythmias were observed during the periodical ECG recordings in the dietary period and during the stabilization period before the stress-test. On the other hand, severe arrhythmias were observed in both groups during the occlusion and reperfusion periods. In the lard-fat fed group 61 ± 24 premature ventricular beats per animal were observed, whereas in the mackerel-oil fed group 114 ± 23 premature ventricular contractions per animal were seen (Fig. 1). In 6 of the lard-fat fed pigs and 7 of the mackerel-oil fed animals several periods with more than 5 premature ventricular contractions per minute were observed. Bigeminies were only seen in 4 of the mackerel-oil fed animals. Periods of ventricular tachycardia were observed in 3 lard-fat fed pigs, but were also more common in the mackerel-oil fed group, because 7 animals had periods of ventricular tachycardia. Usually the duration of the ventricular tachycardia was not longer than 15 seconds, but if so it passed into ventricular fibrillation. There were 8 periods of ventricular fibrillation (in 3 pigs) in the lard-fat fed animals, and 11 periods of ventricular fibrillation mackerel-oil fed animals (in 4 animals). However, defibrillation was unsuccessful in 2 of 3 lard-fat fed animals as they went in asystole after defibrillation, respectively, in the first and second reperfusion period. The 4 mackerel-oil fed animals, which encountered ventricular fibrillation, could be promptly defibrillated.

Table 5. Cardiovascular performance before the first occlusion and at the end of the last reperfusion period of 6 consecutive 5 minute coronary artery occlusions interrupted by 10 minutes of reperfusion in anesthetized young Yorkshire pigs fed with 9.1 % lard-fat or 9.1 % mackerel-oil diets for 8 weeks.

		9.1 % lard fat		9.1 % mackerel oil	
		baseline (n = 8)	last reperfusion (in % of baseline) (n = 8)	baseline (n = 8)	last reperfusion (in % of baseline) (n = 8)
HR	$b \cdot min^{-1}$	110 ± 9	87 ± 8	107 ± 15	96 ± 9
MAP	mm Hg	86 ± 5	76 ± 10	94 ± 5	75 ± 3
LVEDP	mm Hg	7.7 ± 0.6	114 ± 13	8.7 ± 1.3	128 ± 22
CO	$l \cdot min^{-1}$	1.8 ± 0.2	57 ± 2	1.8 ± 0.2	65 ± 4
max LVdP/dt	$mm Hg \cdot s^{-1}$	2570 ± 590	59 ± 13	2030 ± 280	62 ± 4
SVR	$mm Hg \cdot min \cdot l^{-1}$	48 ± 3	132 ± 16	54 ± 6	118 ± 9
CBF	$ml \cdot min^{-1}$	31 ± 5	112 ± 16	32 ± 5	161 ± 21[a]
CVR	$mm Hg \cdot min \cdot ml^{-1}$	3.9 ± 0.9	81 ± 18	3.4 ± 0.5	53 ± 6[a]
cv O_2-sat	%	25 ± 3	145 ± 12	23 ± 3	231 ± 27[a]
swt	%	37 ± 3	78 ± 12	28 ± 3	77 ± 8

Abbreviations: HR = heart rate; MAP = mean arterial blood pressure; LVEDP = left ventricular enddiastolic pressure; CO = cardiac output; max LVdP/dt = peak rate of rise in left ventricular pressure; SVR = systemic vascular resistance; CBF = left anterior descending coronary artery flow; CVR = coronary vascular resistance; cvO_2-sat = coronary venous O_2-saturation; swt = systolic wall thickening; [a] = $p < 0.05$ versus 9.1 % lard-fat diet group. All data have been presented as mean ± SEM.

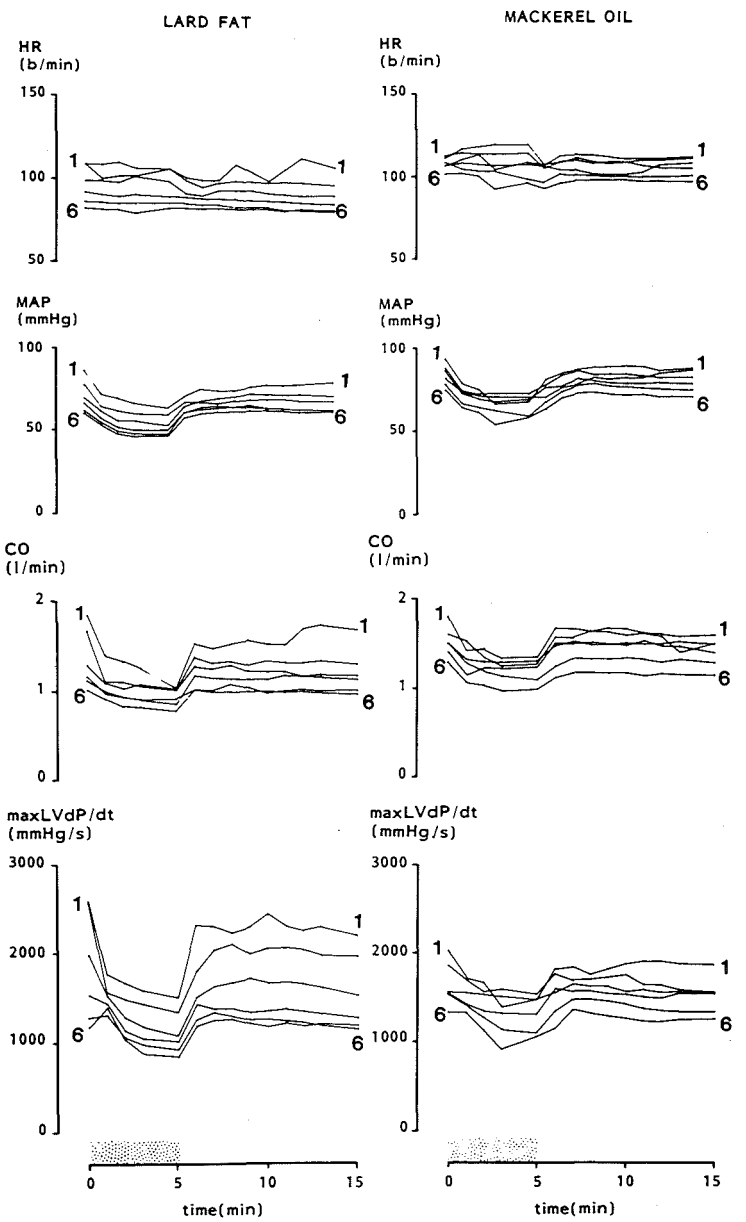

Fig. 2. Systemic hemodynamics during 6 consecutive 5-min occlusions interrupted by 10 min of reperfusion in anesthetized pigs which had been on a 9.1 % lard-fat (left hand panel) or 9.1 % mackerel-oil diet (right hand panel) for 8 weeks. From top to bottom are shown heart rate (HR), mean arterial blood pressure (MAP), cardiac output (CO) and the maximum rate of rise of left ventricular pressure (max LVdP/dt). For the number of observations in each occlusion-reperfusion period see Fig. 1. The SEM bars have been omitted for the sake of clarity. No differences existed between the two groups.

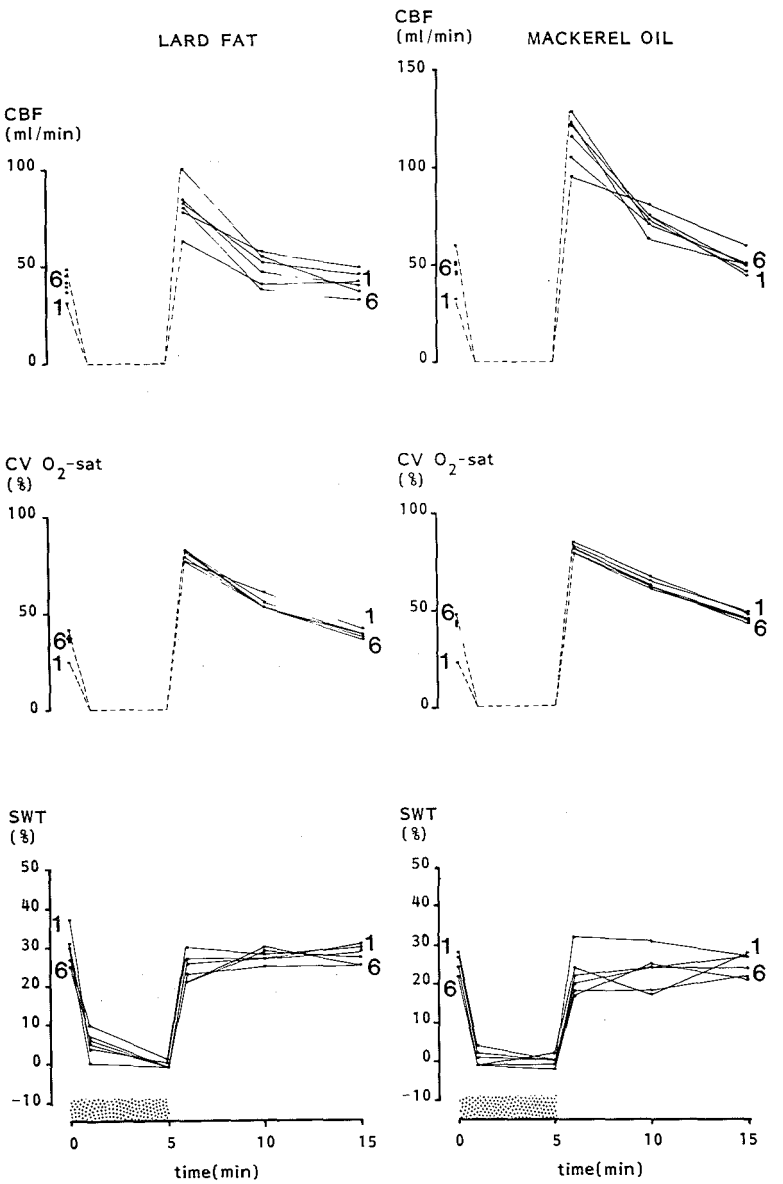

Fig. 3. Myocardial performance of the ischemic segment during 6 consecutive 5-min occlusions interrupted by 10 min of reperfusion in anesthetized pigs which had been on a 9.1 % lard-fat (left hand panel) or 9.1 % mackerel-oil diet (right hand panel) for 8 weeks. From top to bottom are shown: left anterior descending coronary artery blood flow (CBF), O_2-saturation in the great cardiac vein (cvO_2-sat) and systolic wall thickening (swt). For the number of observations in each occlusion-reperfusion period, see Fig. 1. The SEM bars have been omitted for the sake of clarity. Reactive hyperemic flow and O_2-saturation in the great cardiac vein were significantly ($p < 0.05$) larger in the mackerel-oil than in the lard-fat dietary group during the last reperfusion periods.

Hemodynamics

Baseline values at the end of the dietary period

No significant differences in cardiovascular performance of the two experimental groups were found after the 8-week dietary period (Table 5).

Multiple coronary artery occlusion-reperfusion periods

In the lard-fat fed animals mean arterial blood pressure (MAP) fell to 75 ± 2 % of baseline ($p < 0.05$) during the first occlusion, but returned to 95 ± 2 % ($p < 0.05$ vs pre-occlusion) at the end of the first reperfusion period. During the following occlusion and reperfusion periods a similar pattern was observed. Consequently at the end of the last reperfusion period MAP had decreased to 76 ± 10 % of baseline ($p < 0.05$, Fig. 2, left hand panel). Left ventricular end-diastolic pressure (LVEDP, pre-occlusion: 7.7 ± 0.6 mm Hg) was elevated to 11.6 ± 1.2 mm Hg during the first occlusion period but fell to 8.9 ± 0.9 mm Hg during reperfusion. During the next occlusion and reperfusion periods the same pattern was observed resulting in a LVEDP of 9.1 ± 0.9 mm Hg at the end of the last reperfusion period. Similar patterns were also found for cardiac output and max LVdP/dt: a decrease during coronary artery occlusion followed by a partial recovery during reperfusion (Fig. 2, left hand panel). Consequently, at the end of the last reperfusion period these parameters had fallen below the first pre-occlusion measurement (Table 5).

In the mackerel-oil fed animals the changes in MAP, LVEDP (not shown), SV, CO and max LVdP/dt due to ischemia-reperfusion were almost similar to those in the lard-fat fed animals (Fig. 2, right hand panel and Table 5).

In both the lard-fat and mackerel-oil fed animals there was a complete loss of systolic wall thickening immediately following each occlusion, but almost complete recovery occurred upon reperfusion (Fig. 3). At the end of the last reperfusion period systolic wall thickening was still 79 ± 9 % of the pre-occlusion value in lard-fat fed animals and 77 ± 8 % of the pre-occlusion value in the mackerel-oil fed animals, which was not statistically different (Table 5). O_2 saturation in the great cardiac vein was compared to pre-occlusion values more than tripled in the lard-fat fed pigs (77 ± 2 %) and in the mackerel-oil fed pigs (79 ± 2 %) during peak reactive hyperemia in the early reperfusion (Fig. 3). The reactive hyperemic response of the mackerel-oil fed animals exceeded that of the lard-fat fed animals (129 ± 18 ml/min vs 81 ± 17 ml/min) during the last reperfusions ($p < 0.05$), (Fig. 3 and Table 5).

Prostaglandin plasma levels in coronary venous blood

TXB_2 and 6-keto PGF_{1alpha} levels were measured in blood samples collected from the great cardiac vein. Prior to the first LADCA occlusion significant higher levels of TXB_2 were observed in the lard-fat fed animals (39 ± 8 pg/ml) than in the mackerel-oil fed pigs (16 ± 5 pg/ml, Table 6). On the other hand, much higher TXB_2 levels were found in the lard-fat fed animals (77 ± 6 pg/ml, vs 19 ± 7 pg/ml, in mackerel-oil fed pigs, $p < 0.05$) during peak hyperemia of the last reperfusion (Table 6). The same phenomenon was seen with the 6-keto PGF_{1alpha}: Preocclusion plasma levels were 133 ± 35 pg/ml and 82 ± 6 pg/ml, respectively in the lard-fat and mackerel-oil fed animals. During peak hyperemia of the last reperfusion period we found a 3-fold increase in the lard-fat fed animals (371 ± 40 pg/ml) and only a 50 % increase in the mackerel-oil fed pigs ($p < 0.05$, 123 ± 7 pg/ml, Table 6).

Table 6. Coronary venous plasma levels of prostaglandins before the first occlusion and during peak reactive hyperemia of the last reperfusion period of 6 consecutive 5-minute coronary artery occlusions interrupted by 10 minutes of reperfusion in anesthetized young Yorkshire pigs fed with 9.1 % lard-fat or 9.1 % mackerel-oil diets for 8 weeks.

		9.1 % lard fat		9.1 % mackerel oil	
		baseline	peak hyperemia (last reperfusion)	baseline	peak hyperemia (last reperfusion)
		(n = 8)	(n = 6)	(n = 8)	(n = 6)
Thromboxane B_2	pg/ml	39 ± 8	77 ± 6[b]	16 ± 5[a]	19 ± 7[a]
6-keto PGF_{1alpha}	pg/ml	133 ± 35[c]	371 ± 40[b]	82 ± 6[a]	123 ± 7[a]
TXB_2/6-keto PGF_{1alpha}		0.44 ± 0.13[c]	0.22 ± 0.03[b]	0.20 ± 0.06[a]	0.16 ± 0.06

Abbreviations: TXB_2 = Thromboxane B_2; 6-keto PGF_{1alpha} = 6-keto prostaglandin F_{1alpha}; [a] = $p < 0.05$ versus lard-fat diet group; [b] = < 0.05 versus baseline; [c] = $n = 7$. All data have been presented as mean ± SEM.

Discussion

In the present study a dietary dose of fish oil was employed that supplied equal amounts of n-3 fatty acids as consumed by the eskimos reported in the studies by Bang and Dyerberg (2). The hypolipidemic effect of a mackerel-oil diet that we observed in pigs is in agreement with results obtained in a study on hyperlipidemic patients consuming a nearly identical dose of n-3 fatty acids (34). The large decrease in total plasma cholesterol was accompanied by a similar fall in the HDL cholesterol. Consequently the HDL-/total-cholesterol ratio remained constant. An enhanced clearance of VLDL-particles may have contributed in the decrease of plasmalipid levels in the mackerel-oil fed animals, but a decreased rate of VLDL-synthesis in the liver is probably the most important factor (14, 32, 36, 41, 48). At any rate the dramatic plasma lipid changes observed in the present study confirms the efficacy of the fish-oil dose (19). Indeed the cellular membrane phospholipids showed a marked polyunsaturated fatty acid response as well.

Despite the marked fish oil-induced changes in fatty acid composition of platelet and cardiac membranes there were no differences in myocardial performance between the mackerel-oil and lard-fat dietary groups. It cannot be entirely excluded that slight differences in performance were masked by the presence of the anesthetics during the measurements. Pigs are very susceptible to ventricular fibrillation, probably due to the lack of collateral flow (3, 37, 45). In addition, coronary artery occlusions from 10–30 min already lead to a complete loss of function for a substantial period of time (3, 4, 44, 46). In order to evaluate the effect of changes in cardiac membrane phospholipids on ventricular arrhythmias and cardiac function, it is desirable that a substantial number of animals survive the ischemic events and that partial recovery of regional function occurs. Multiple coronary artery occlusions of 5 min interrupted by 10 min of reperfusion provide such a model (31). In the present study recovery was about the same in both the mackerel-oil and lard-fat diet groups. Moreover, the incidence of ventricular arrhythmias was very similar. Hence, the changes in cardiac membrane phospholipids do not affect cardiovascular performance during acute episodes of myocardial ischemia. Because of the high susceptibility to ventricular fibrillation, this porcine model is not very suitable for the study of long periods of

ischemia. Even in pigs with smaller ischemic segments, the incidence of ventricular fibrillation remains very high (18, 38, 43).

The only difference was the more pronounced hyperemic response during the last reperfusion periods in the mackerel-oil fed animals. Since arterial blood pressures were similar for both groups a larger vasodilatory response of the coronary resistance vessels must have occurred in these animals. This difference in coronary resistance can be explained by the lower levels of TXA_2 in the coronary venous blood. However, it appeared that PGI_2 levels were also reduced due to mackerel-oil nutrition. Apparently the TXA_2/PGI_2 ratio, which was significantly lower in mackerel-oil fed animals, has determined the decrease in coronary vascular tone. It is possible that due to the changes in polyunsaturated fatty acid composition of the platelets and probably also of the endothelial membranes, the synthesis of TXA_3 and PGI_3 has become predominant (20). Some contribution of the 3-series of prostacyclin to the hyperemic response may exist.

Except for minor but non-significant differences in the incidence of bigeminies or ventricular tachycardias no differences in the incidence of ventricular arrhythmias were observed between the two dietary groups. Differences in the incidence of ventricular arrhythmias of the two dietary groups could have been expected because: 1) the increase of docosahexanoic acid in the cardiac membrane phospholipids has been associated with a higher susceptibility for cardiac arrhythmias in rats, after consumption of high doses of fish-oil (15, 16) and 2) altered concentrations of TXA_2 and PGI_2 have been reported to influence the susceptibility to ventricular arrhythmias during ischemia (7, 8, 19, 20, 22, 24, 25, 27, 33). In the present study the n-6 fatty acids of platelet and heart membrane phospholipids were partially replaced by the n-3 fatty acids eicosapentanoic acid and to a lesser extent by docosahexanoic acid. This has affected the synthesis of both TXA_2 and PGI_2 as reduced baseline levels of both TXA_3 and 6-keto PGF_{1alpha} were found in mackerel-oil fed pigs in the present study. Although the baseline TXA_2/PGI_2 ratio in the mackerel-oil fed pigs was significantly reduced, no influence on the incidence of ventricular arrhythmias was observed (7, 8). Perhaps the levels of TXA_3 and PGI_3 should also be taken into account, but these data are not available. It should be noted that the TXB_2/6-keto PGF_{1alpha} ratio became similar at peak hyperemic response, when most arrhythmias occurred. Supposing that not the level of TXA_2 and PGI_2 but their ratio is important. The absence of dietary-induced changes in ventricular arrhythmias can be related to the similar TXB_2/6-keto PGF_{1alpha} ratios. The 5 min occlusion periods are too short to provoke serious reperfusion arrhythmias (46) and the dietary effect on this type of ventricular arrhythmias remains to be studied.

We conclude that consumption of high doses of mackerel oil leads to marked decreases in plasma triglyceride- and cholesterol-levels and replacement of linoleic and arachidonic acid by eicosapentanoic and docosahexanoic acid in platelet and cardiac membrane phospholipids. Although the membrane changes also reduced prostaglandin levels in plasma, no differences in heart function, recovery and incidence of ventricular arrhythmias were found in this open-chest pig model during multiple coronary artery occlusion-reperfusion periods.

Acknowledgements

The authors are grateful to Dr. M. Klompe, Mr. J. Endeveld, F. J. Zijlstra, Miss L. van der Werf, and Mrs. M. Groh-Hoogenboom for their help with the biochemical analysis and Dr. A. Montfoort and Prof. Dr. A. J. Vergroesen for their advice. Mr. J. Kasbergen, R. C. Spruyt, E. C. Collij, E. Ridderhof and Miss J. de Kam (Laboratory for Surgery) are thanked for their assistance during the dietary period and Mr. R. H. van Bremen, R. J. Rensen, and Miss A. M. Rutteman (Laboratory for Experimental Cardiology) for their assistance during the hemodynamic measurements. Mr. H. Morse and Dr. M.

Blok (Hope Farms, Woerden, The Netherlands) are thanked for the careful preparation of the special diets used in this experiment. The authors thank Miss P. H. Vegter for her assistance in the preparation of the manuscript. This study was supported by a grant from the Dutch Heart Foundation.

References

1. Balke CW, Kaplinsky E, Michelson EL, Naito M, Dreifus LS (1981) Reperfusion ventricular tachyarrhythmias: Correlation with antecedent coronary artery occlusion tachyarrhythmias and duration of myocardial ischemia. Am Heart J 101:449–456
2. Bang HO, Dyerberg J (1984) Plasmalipids and lipoproteins in Greenlandic west coast Eskimos. Acta Med Scand 251(3):351–364
3. Bergey JL, Nocella K, McCallum JD (1982) Acute coronary artery occlusion-reperfusion-induced arrhythmias in rats, dogs and pigs: antiarrhythmic evaluation of quinidine, procainamide and lidocaine. Eur J Pharmacol 81:205–216
4. Bergey JL, Wendt RL, Nocella K, McCallum JD (1984) Acute coronary artery occlusion-reperfusion arrhythmias in pigs: antiarrhythmic and antifibrillatory evaluation of verapamil, nifedipine, prenylamine and propranolol. Eur J Pharmacol 97:95–103
5. Black KL, Culp B, Madison D, Randall OS, Lands WEM (1979) The protective effect of dietary fish oil on cerebral infarction. Prostaglan Med 5:257–268
6. Charnock JS, McLennan PL, Abeywardena MY, Dryden WF (1985) Diet and cardiac arrhythmia: effect of lipids on age-related changes in myocardial function in the rat. Ann Nutr Metab 29:306–318
7. Coker SJ, Parratt JR, Ledingham McA, Zeitlin IJ (1981) Thromboxane and prostacyclin release from ischaemic myocardium in relation to arrhythmias. Nature 291:323–324
8. Coker SJ, Parratt JR, Ledingham McA, Zeitlin IJ (1982) Evidence that thromboxane contributes to ventricular fibrillation induced by reperfusion of the ischaemic myocardium. J Mol Cell Cardiol 14:483–485
9. Crandall DL, Griffith DR, Beitz DC (1982) Protection against the cardiotoxic effect of isoproterenol – HCL by dietary polyunsaturated fatty acids and exercise. Toxicol Appl Pharmacol 62:152–157
10. Culp BR, Lands WEM, Lucchesi BR, Pitt R, Romson J (1980) The effect of dietary supplementation of fish oil on experimental myocardial infarction. Prostaglandins 20:1021–1031
11. Dyerberg J, Bang HO (1979) Haemostatic function and platelet polyunsaturated fatty acids in eskimos. Lancet ii:433–435
12. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
13. Fossati P, Prencipe L (1982) Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 280:2077–2080
14. Goodnight SH, Harris WS, Connor WE, Illingworth DR (1982) Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. Arteriosclerosis 2:87–113
15. Gudbjarnason S, Hallgrimsson J (1976) Prostaglandins and polyunsaturated fatty acids in heart muscle. Acta Biol Med Ger 35:1069–1080
16. Gudbjarnason S, Oskarsdottir G, Doell B, Hallgrimsson J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 25:130–144
17. Gudbjarnason S, Benediktsdottir E (1985) Role of arachidonic acid metabolism in development of fatal ventricular fibrillation in rats. J Mol Cell Cardiol 17(3):112 (abstract)
18. Hartog JM, Bremen RH van, Verdouw PD (1986) On the cardiovascular and antiarrhythmic actions of the cardioselective beta-adrenoceptor antagonist bevantolol in the pig. Drug Dev Res 7:23–33
19. Herold PM, Kinsella JE (1986) Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am J Clin Nutr 43:566–598
20. Hornstra G, Christ-Hazelhof E, Haddeman E, ten Hoor F, Nugteren DH (1981) Fish oil feeding lowers thromboxane- and prostacyclin production by rat platelets and aorta and does not result in the formation of prostaglandin I_3. Prostaglandins 21:727–738

21. Hutton I, Parratt JR, Laurrie TDV (1973) Cardiovascular effects of prostaglandin E, in experimental myocardial infarction. Cardiovasc Res 7:149–155
22. Karmazyn M, Dhalla NS (1983) Physiological and pathological aspects of cardiac prostaglandins. Can J Physiol Pharmacol 61:1207–1225
23. Lamers JMJ, de Jonge-Stinis JT, Hülsmann WC, Verdouw PD (1986) Reduced in vitro ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial ischaemia in anaesthetized pigs. J Mol Cell Card 18:115–125
24. Lefer AM, Ogletree ML, Smith JB, Silver MJ, Nicolan KC, Bernette WE, Gasic GP (1978) Prostacyclin: a potentially valuable agent for preserving myocardial tissue in acute myocardial ischemia. Science 200:52–54
25. Lepran J, Nemecz GY, Koltai M, Szekeres L (1981) Effect of a linoleic acid-rich diet on the acute phase of coronary occlusion in conscious rats: influence of indomethacin and aspirin. J Cardiovasc Pharmacol 3:847–853
26. Lockette WE, Webb RC, Culp BR, Pitt B (1982) Vascular reactivity and high dietary eicosapentaenoic acid. Prostaglandins 24(5):631–639
27. Logan RL, Larking P, Nye ER (1977) Linoleic acid and susceptibility to fatal ventricular fibrillation in rats. Atherosclerosis 27:265–269
28. Lorenz R, Spengler U, Fischer S et al (1983) Platelet function, thromboxane formation and blood pressure control during supplementation of the western diet with cod-liver oil. Circulation 67:504–511
29. McLennan PL, Abeywardena MY, Charnock JS (1985) Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. Can J Physiol Pharmacol 63:1411–1417
30. Montfoort A, Van der Werf L, Hartog JM, Hugenholtz PG, Verdouw PD, Hülsmann WC, Lamers JMJ (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol 81:289–302
31. Murphy ML, Kane JJ, Peng CF, Straub KD (1982) Wall motion and metabolic changes after coronary occlusion and reperfusion. J Surg Res 32:143–149
32. Nestel PJ, Connor WE, Reardon MF (1984) Suppression by diets rich in fish-oil of very low density lipoprotein production in man. J Clin Invest 74:82–89
33. Ogletree ML, Lefer AM (1978) Prostaglandin-induced preservation of the ischemic myocardium. Circ Res 42:218–224
34. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR (1985) Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. New Engl J Med 312:1210–1216
35. Sanders TAB, Vickers M, Haines AP (1981) Effect on blood lipids and haemostasis of a supplement of cod-liver oil, rich in eicosapentaenoic and docosahexaenoic acids, in healthy young men. Clin Sci 61:317–324
36. Sanders TAB, Sullivan DR, Reeve J, Thompson GR (1985) Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. Arteriosclerosis 5:459–465
37. Schaper W (1979) The collateral circulation of the heart. Vol 1; Black DAK (ed) Elsevier/North-Holland, Amsterdam
38. Scheffer MG, Verdouw PD (1983) Decreased incidence of ventricular fibrillation after an acute coronary artery ligation in exercised pigs. Basic Res Cardiol 78:298–309
39. Sherhag R, Kramer HJ, Düsing R (1982) Dietary administration of eicosapentaenoic and linolenic acid increases arterial blood pressure and suppresses vascular prostacyclin synthesis in the rat. Prostaglandins 23(3):369–382
40. Siedel J, Schlumberger H, Klose S, Ziegenhorn J, Wahlefeld AW (1981) Improved reagent for the enzymatic determination of serum cholesterol. J Clin Chem Clin Biochem 19:838–839
41. Simons LA, Hickie JB, Balasubramaniam S (1985) On the effects of dietary n-3 fatty acids (Maxepa) on plasma lipids and lipoproteins in patients with hyperlipidaemia 54:75–88
42. Singer P, Voigt S, Gödicke W (1982) Inverse relationship between linoleic acid in serum and in adipose tissue of patients with essential hypertension. Prostagl Leukotr Med 9:603–613
43. Verdouw PD, Deckers JW, Conard GJ (1979) Antiarrhythmic and hemodynamic actions of flecainide acetate (R-818) in the ischemic porcine heart. J Cardiovasc Pharmacol 1:473–486

44. Verdouw PD, Wolffenbuttel BHR, Ten Cate FJ (1983) Nifedipine with and without propranolol in the treatment of myocardial ischemia: effect on ventricular arrhythmias and recovery of regional wall function. Eur Heart J 4:101–108 peroxide
45. Verdouw PD, Wolffenbuttel BHR, Giessen WJ van der (1983) Domestic pigs in the study of myocardial ischemia. Eur Heart J 4:61–67
46. Verdouw PD, van Bremen RH, Verkeste CM, van der Giessen WJ (1985) Antiarrhythmic action and protection of ischaemic myocardium after beta-blockade with bevantolol. Eur J Pharmacol 111:377–380
47. Warnick GR, Albers JJ (1978) A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. J Lip Res 19:65–76
48. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL (1984) The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochim Biophys Acta 792:103–109
49. Zijlstra FJ, Van Vliet HHDM, Vincent JE (1983) Thrombotic thrombocytic purpura and thromboxane B_2 levels. Thromb Res 30:535–538

Received September 1, 1986

Authors' address:

J. M. Hartog, M.D., Laboratory for Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

CHAPTER 9

The effects of dietary mackerel oil on the recovery of cardiac function after acute ischaemic events in the pig

J. M. Hartog, J. M. J. Lamers[1], P. W. Achterberg, D. van Heuven-Nolsen[2], F. P. Nijkamp[2] and P. D. Verdouw

Laboratory for Experimental Cardiology, Thoraxcenter, and [1]Department of Biochemistry I, Erasmus University Rotterdam, Rotterdam, The Netherlands and [2]Institute for Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, Utrecht, The Netherlands

Summary

To investigate the effects of fish oil nutrition on cardiac haemodynamics and the biochemical response to ischaemia-reperfusion, young pigs (5 weeks old) were fed a 9% lard fat diet or a mixed diet of 4.5% mackerel oil and 4.5% lard fat for 16 weeks. In the mackerel oil fed pigs plasma cholesterol and triglyceride levels decreased by 22% and 58% (both $p < 0.05$), respectively, while levels in the animals which received only lard fat did not change. The n-6 fatty acids present in cardiac and platelet membrane phospholipids underwent a partial replacement by n-3 fatty acids in the mackerel oil fed pigs. Under anaesthesia, multiple coronary artery occlusions (5 min) were interrupted by 10 min of reperfusion. The extent of recovery of cardiac function and reduction of adenine nucleotide levels were similar for both dietary groups. The incidence of reperfusion arrhythmias was significantly lower and the reactive hyperaemic responses were of longer duration in the mackerel oil fed animals. These effects cannot be explained by diet-induced alterations in thromboxane B_2/6-keto-$PGF_{1\alpha}$ ratio, although a marked reduction in absolute levels of both prostaglandins was seen in the mackerel oil fed pigs ($p < 0.05$). In conclusion, dietary fish oil caused changes in membrane fatty acid composition and plasma prostaglandin levels, although these did not affect alterations of cardiac performance during and after short periods of ischaemia.

Key words: fish oil, ischaemia-reperfusion, cardiac function, arrhythmias, prostaglandins, phospholipids, pigs.

Introduction

The plasma lipid lowering and antithrombotic effects of dietary fish oils, rich in n-3 fatty acids, have been well established (1, 5, 11, 14, 20). Effects of dietary n-3 fatty acids have been reported on blood pressure (19, 20, 25, 26) cardiac performance (2, 17, 18), infarct size (4), ischaemic insult (13), susceptibility to catecholamine-induced heart necrosis (6, 17, 21) and arrhythmias (4, 17). Because fish oil nutrition induces replacement of n-6 by n-3 fatty acids, the precursor fatty acid 20:4 n-6 for prostaglandin synthesis is less available (14). Since prostaglandins have been shown to play a role in the recovery of regional myocardial function and arrhythmias following release of coronary artery occlusion (3, 27), an effect of prolonged feeding with fish oil through alteration of prostaglandin synthesis could be present.

The purpose of the present study was to evaluate the effect of a mackerel oil diet on arterial and coronary venous prostaglandin levels, recovery of cardiac function and the incidence of ventricular arrhythmias during multiple coronary artery occlusions and reperfusions in pigs. Tissue levels of ATP, which are generally believed to be critically involved in the recovery of the myocardial cell from an ischaemic event, were also measured.

Materials and Methods

Thirteen Yorkshire piglets (5 weeks of age and 7.7 ± 0.2 kg), were fed either 9% w/w lard fat (L, n = 6) or 4.5% w/w mackerel oil plus 4.5% w/w lard fat (ML, n = 7) for a period of 16 weeks as previously described (9, 10). Table 1 shows the fatty acid composition of the two diets. Cardiac sarcolemma was isolated from heart biopsies by differential centrifugation (16). Plasma cholesterol and triglyceride (9, 10), phospholipid composition of platelet and cardiac sarcolemmal membranes (10, 21) and arterial and coronary venous plasma levels of TXB_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were measured by radioimmunoassay as previously described (24, 26). Prostaglandin synthesis of the coronary vascular bed was calculated as the product of coronary blood flow and the difference in arterial and coronary venous prostaglandin plasma levels.

Table 1. Fatty acid compositions (% mole) of the 9% lard fat (L) and 4.5% mackerel oil + 4.5% lard fat (ML) diets fed to the Yorkshire pigs

Fatty acids	L	ML
14:0	2	5
16:0	24	21
16:1	3	6
18:0	10	6
18:1	42	29
18:2 n−6	15	11
18:3 n−3	1	1
20:1	1	4
20:5 n−3	–	8
22:1	–	2
22:6 n−3	–	5
24:1	–	1
others	2	1

After 16 weeks, the animals (now weighing 48.5 ± 1.5 kg) were anaesthetized and prepared according to standard procedures (8, 10). Subsequently, the left anterior descending coronary artery was six times clamped for 5 min and declamped for 10 min. At the end of the last reperfusion period two needle biopsies were taken from the ischaemic as well as from the non-ischaemic segment (posterior wall) of the left ventricle for the determination of adenine nucleotides (7).

Results

Plasmalipid levels and fatty acid composition of cardiac and platelet membranes

In L plasma triglyceride (from 0.76 ± 0.12 mM to 0.64 ± 0.09 mM), total cholesterol (from 2.39 ± 0.41 mM to 2.35 ± 19 mM) and HDL cholesterol (from 1.02 ± 0.16 mM to 1.01 ± 0.11 mM) did not change ($p > 0.05$) during the dietary period. In ML, HDL cholesterol (from 0.87 ± 0.10 mM to 0.83 ± 0.04 mM) was not affected, but triglyceride (from 0.76 ± 0.10 mM to 0.31 ± 0.03 mM) and total cholesterol (from 2.40 ± 0.28 mM to 1.75 ± 0.11 mM) decreased by 58 ± 3% and 22 ± 9%, respectively (both $p < 0.05$ versus pre-dietary value and L).

In ML, the relative content of n-6 fatty acids of heart sarcolemmal phospholipids was lower, but the n-3 fatty acid content was higher than in L (Fig. 1). The total polyunsaturated fatty acid content was similar for both dietary groups. Because of the higher unsaturation degree of the n-3 fatty acids, the double bond index of ML (1.94 ± 0.04) was appreciably higher than that of L (1.65 ± 0.03; $p < 0.05$ versus ML). Analogous shifts of n-6 to n-3 fatty acids were found in the fatty acid composition of the platelet membrane phospholipids of both groups (Fig. 2).

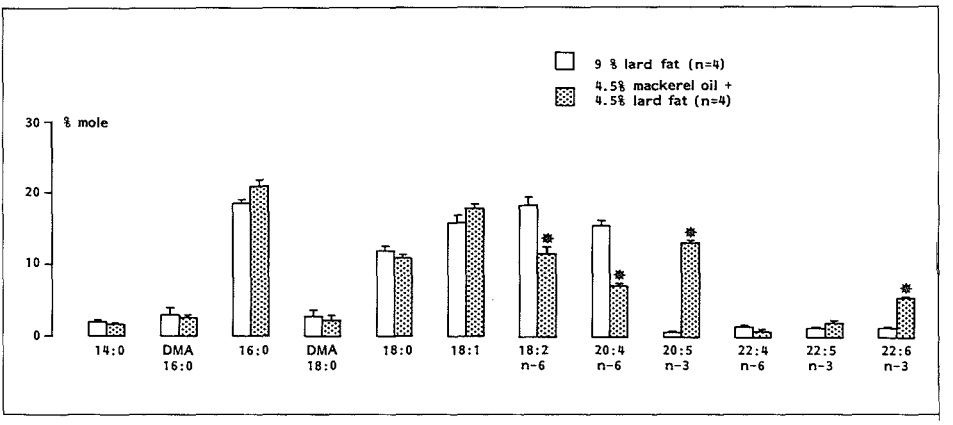

Fig. 1. Fatty acid composition of heart sarcolemmal phospholipids in pigs after a 16 week dietary period. ★ = $p < 0.05$ versus 9% lard fat fed animals. DMA = dimethylated acetals.

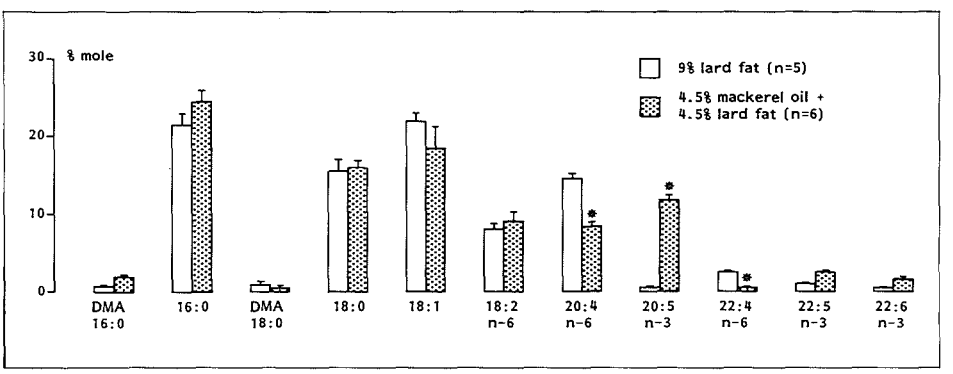

Fig. 2. Fatty acid composition of platelet homogenate phospholipids in pigs after a 16 week dietary period. ★ = $p < 0.05$ versus 9% lard fat fed animals. DMA = dimethylated acetals.

Ventricular arrhythmias during coronary artery occlusion and reperfusion

The incidence of premature ventricular contractions (PVC's) (Fig. 3) was not different during occlusion (15 ± 12 and 23 ± 11 PVC's/animal in L and ML, respectively), but during reperfusion more PVS's were seen in L (26 ± 7 PVC's/animals) than in ML (9 ± 3 PVC's/animal, $p < 0.05$ versus L). Eight animals (four in each group) had several periods with more than 5 PVC's per minute. Bigeminies were seen in one animal of each group. One animal in L and two animals in ML had periods of ventricular tachycardia (all during occlusion). Ventricular fibrillation (VF) was not observed in L, but in ML three animals encountered seven episodes of VF (five of these during occlusion). Defibrillation was unsuccessful in one animal, of the ML group, which died of ventricular asystole during the fifth reperfusion.

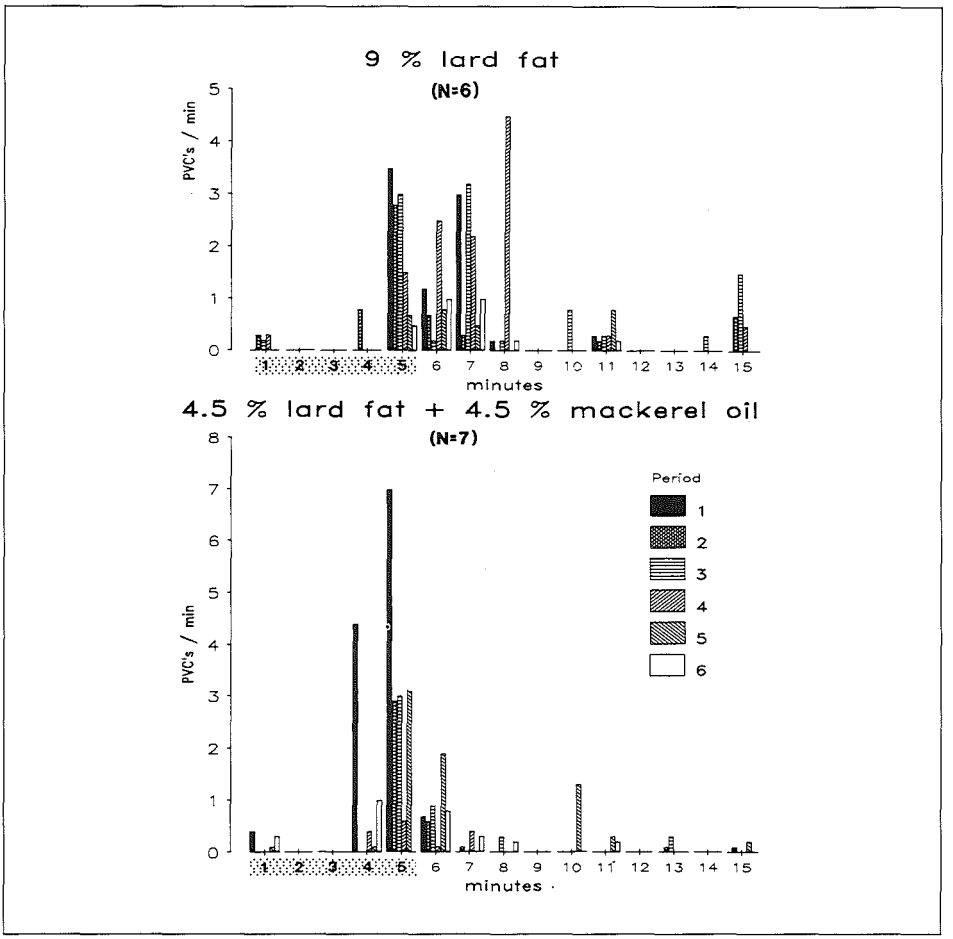

Fig. 3. Premature ventricular contractions (PVC's) during 6 consecutive episodes of 5 min occlusion — 10 min reperfusion in pigs, which had been on a diet for 16 weeks. The mean of the premature ventricular contractions per minute (PVC's/min) has been depicted for each minute during all 6 occlusions (0—5 min) and reperfusions (5—15 min).

Systemic haemodynamics

In neither of the two groups heart rate, which was significantly higher in ML during pre-occlusion, was affected by the multiple occlusions and reperfusions (Fig. 4). Mean arterial blood pressure (MAP), however, decreased to approximately 80% of the pre-occlusion value during the first occlusion in both groups but returned to 95% of the pre-occlusion value at the end of the first reperfusion. With each following occlusion-reperfusion there was a gradual decrease in MAP. Hence, at the end of the last reperfusion MAP had fallen to 81% ±4% in L and to 79 ±4% in ML. Similar patterns were observed for cardiac output (78 ± 6% in L and 75 ± 5% in ML at the end of the last reperfusion), and max LVdP/dt (75 ± 8% in L and 72 ± 5% in ML). As expected, left ventricular end-diastolic blood pressure increased during occlusion, but returned to

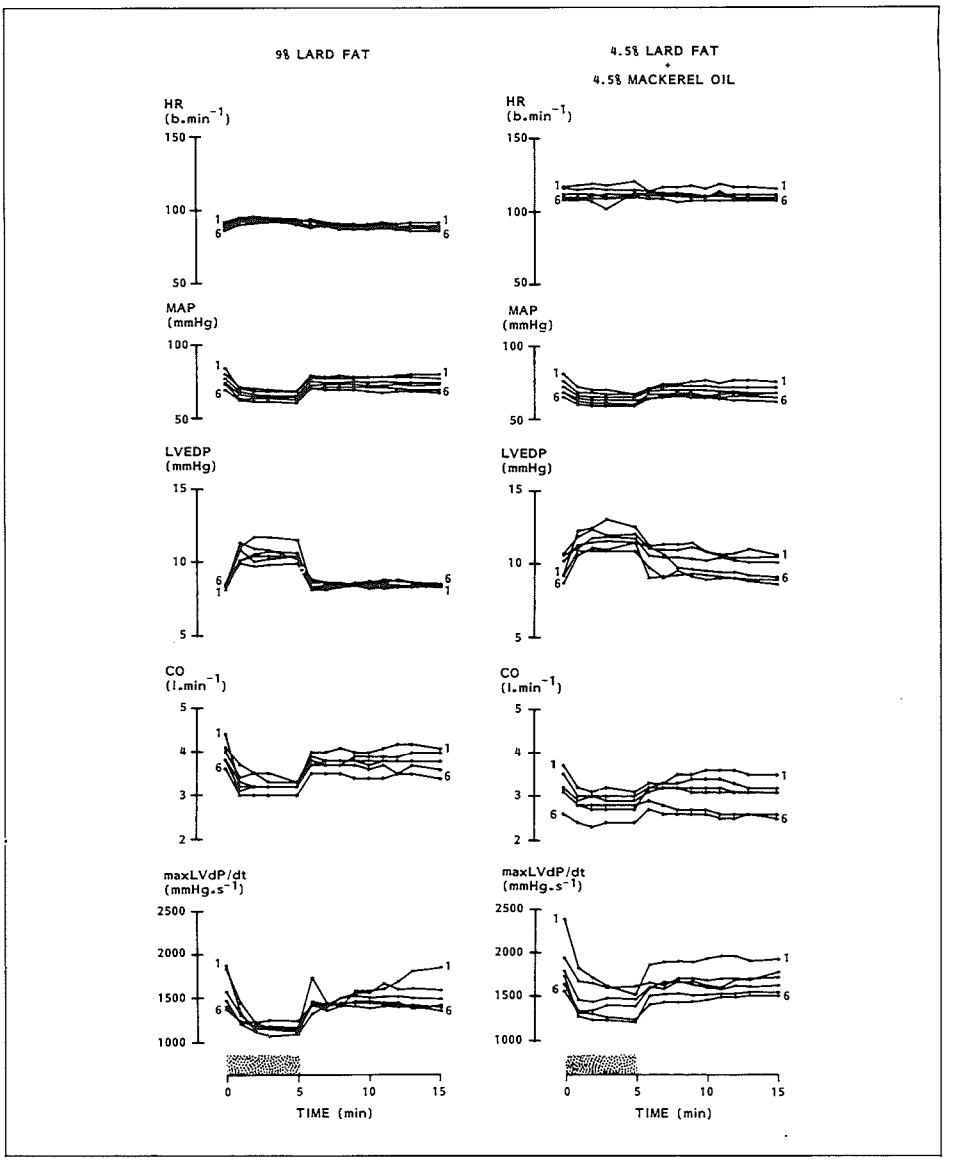

Fig. 4. Systemic haemodynamics during 6 consecutive episodes of 5 min occlusion — 10 min reperfusion in pigs, which had been on the diet for 16 weeks. From top to bottom are shown: heart rate (HR), mean arterial blood pressure (MAP), left ventricular end-diastolic pressure (LVEDP), cardiac output (CO) and maximum rate of rise in left ventricular pressure (max LVdP/dt).

occlusion value with each reperfusion. Systemic vascular resistance (not shown) was not affected by these procedures.

Left ventricular performance

Upon release of the occlusion, reactive hyperaemia occurred in both groups. Peak flows were similar, but the hyperaemic response lasted longer in ML than in L (Fig. 5). Therefore, at the end of the last reperfusion coronary blood flow was in ML above (24 ± 13%) and in L below

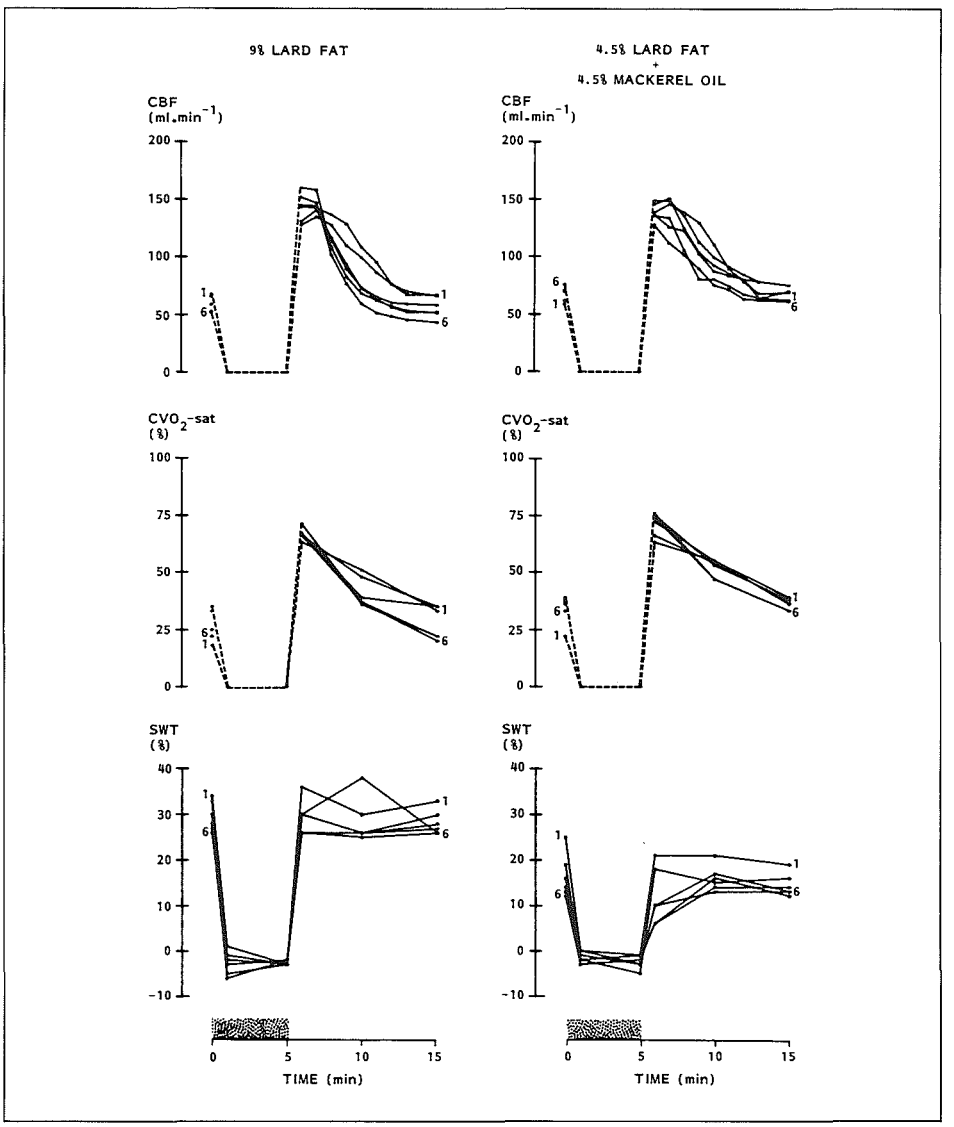

Fig. 5. Myocardial performance of the ischaemic segment of the left ventricle during 6 consecutive episodes of 5 min occlusion — 10 min reperfusion in pigs, which had been on the diet for 16 weeks. From top to bottom are shown: left anterior descending coronary artery blood flow (CBF), O_2 saturation in the great cardiac vein (CVO_2-sat) and systolic wall thickening (SWT).

(—32 ± 5%, $p < 0.05$) the pre-occlusion value. During occlusion, coronary venous O_2 saturation (CVO_2-sat) could not be measured, but immediately upon release of the obstruction the O_2 saturation in the great cardiac vein rose in both dietary groups to approximately 75% (pre-occlusion values 20—25%). At the end of the last reperfusion CVO_2-sat was still elevated in ML, whereas pre-occlusion values were re-established in L.

In both groups a complete loss of regional systolic wall thickening (SWT), which was slightly lower in ML during pre-occlusion ($p < 0.05$ versus L), occurred with each occlusion. Recovery was incomplete at the end of each reperfusion and occurred more slowly in ML during the last reperfusions. Hence SWT decreased to 71 ± 13% of the pre-occlusion value in L and to 56 ± 14% of that in ML at the end of the last reperfusion.

Prostaglandin plasma levels in arterial and coronary venous blood

Before occlusion, arterial as well as coronary venous plasma levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ were appreciably lower in ML than in L. The arterial TXB_2/6-keto-$PGF_{1\alpha}$ ratio was similar for both groups, but the coronary venous TXB_2/6-keto-$PGF_{1\alpha}$ ratio was higher in L (Table 2). Although myocardial production of especially TXA_2 can be artificially altered by post-sampling production, large differences existed in the myocardial production rate of TXB_2 (—16 ± 98 pg/min in ML versus 1086 ± 194 pg/min in L, $p < 0.05$) and of 6-keto-$PGF_{1\alpha}$ (881 ± 176 pg/min in ML versus 2466 ± 616 pg/min L, $p < 0.05$).

Table 2. Arterial (a) and coronary venous (cv) plasma concentrations of prostaglandins before the first occlusion (PO) and during peak reactive hyperaemia of the first and sixth reperfusion periods (RP) in anaesthetized Yorkshire pigs, fed lard fat (L) or a mixture of mackerel oil and lard fat (ML)

		L ($n = 6$)			ML ($n = 7$)		
		PO	1st RP	6th RP	PO	1st RP	6th RP[d]
TXB_2	a	70 ± 7	75 ± 6	63 ± 6	28 ± 3[b]	21 ± 5[b]	51 ± 9[c]
	cv	107 ± 9	80 ± 8[c]	79 ± 9[c]	29 ± 2[b]	25 ± 2[b]	32 ± 3[b]
6-keto-$PGF_{1\alpha}$	a	83 ± 9	122 ± 11[c]	98 ± 11	31 ± 3[b]	29 ± 2[b]	48 ± 6[bc]
	cv	154 ± 4	140 ± 8	111 ± 6[c]	61 ± 6[b]	76 ± 8[b]	52 ± 3[b]
TXB_2/	a	0.85 ± 0.04	0.63 ± 0.05[c]	0.68 ± 0.08	0.88 ± 0.04	0.54 ± 0.08[c]	0.89 ± 0.06
6-keto-$PGF_{1\alpha}$	cv	0.69 ± 0.06	0.58 ± 0.07	0.71 ± 0.07	0.50 ± 0.05[b]	0.32 ± 0.02[bc]	0.62 ± 0.04

b = $p < 0.05$ versus 9% lard fat fed animals at comparable time; c = $p < 0.05$ versus PO; d = $n = 6$. All data have been expressed as means ± SEM.

During the first occlusion-reperfusion episodes no changes in arterial prostaglandin plasma levels were present except for an increase of the 6-keto-$PGF_{1\alpha}$ level in L during peak hyperaemia of the first reperfusion and an increase of TXB_2 and $PGF_{1\alpha}$ plasma levels in ML during the sixth reperfusion. The TXB_2/6-keto-$PGF_{1\alpha}$ ratios in arterial blood were lower during peak hyperaemia of the first reperfusion in both groups, but not different from preocclusion values during the sixth reperfusion. The TXB_2/6-keto-$PGF_{1\alpha}$ ratios, the TXB_2 and 6-keto-$PGF_{1\alpha}$ levels in arterial blood did not significantly correlate with the systemic vascular resistance (correlation coefficients 0.13, 0.07 and 0.01, respectively).

The coronary venous plasma levels of TXB_2 were decreased during peak hyperaemia in L but not in ML. In L, a minor production of TXB_2 occurred in the coronary vascular bed during the first and sixth reperfusion (174 ± 103 pg/min and 463 ± 203 pg/min, respectively, both $p < 0.05$ versus pre-occlusion value). In view of the fact that most, if not all, TXA_2 is produced by the

platelets, one might wonder whether the production is correctly estimated from arteriovenous concentration differences, in particular when these are small. Another complicating factor might be local productions of TXA_2 and perhaps even PGI_2. Similar to pre-occlusion, no myocardial production of TXB_2 was found in ML during reperfusion. During the first reperfusion, the coronary venous levels of 6-keto-$PGF_{1\alpha}$ were unchanged in both groups, but in L the level was decreased during the sixth reperfusion. Levels of 6-keto-$PGF_{1\alpha}$ were still significantly lower in ML, although myocardial release of PGI_2 was enhanced during the first reperfusion (2841 ± 391 pg/min; $p < 0.05$ versus pre-occlusion value). The TXB_2/6-keto-$PGF_{1\alpha}$ ratio did not change significantly in the two groups, except for a decrease in ML during the first reperfusion. The TXB_2/6-keto-$PGF_{1\alpha}$ ratio of coronary venous blood and the myocardial release of 6-keto-$PGF_{1\alpha}$ did not correlate with the coronary vascular resistance (correlation coefficients 0.07 and -0.21, respectively, and the incidence of reperfusion arrhythmias (correlation coefficients 0.08 and -0.10, respectively).

Because alterations in prostaglandin levels did not explain changes in coronary vascular resistance, we searched for other possible contributing factors, such as pH. During peak hyperaemia the differences in arterial and coronary venous pH were still doubled but in both groups the pH differences had returned to their pre-occlusion values (0.05 ± 0.01 for L and 0.04 ± 0.01 for ML) at the end of reperfusion. During reperfusion, the differences in arterial and coronary venous pH correlated with the coronary vascular resistance (correlation coefficient -0.43; $p < 0.05$).

Myocardial energy charge

As can be seen from Fig. 6, no differences were present in adenylate energy charge between the ischaemic and non-ischaemic myocardium. The ATP and total adenine nucleotide contents of the ischaemic segments were reduced to the same extent in both dietary groups (Fig. 6).

Discussion

The purpose of the study was to evaluate the acute effects of multiple short lasting ischaemic events on recovery of regional myocardial function. This appears to be of interest because recent studies showed that feeding fish oil to rats for a period of time had a protective effect on ischaemic myocardium as measured by creatine kinase leakage or the infarct size (4, 13). On the other hand, prostaglandin synthesis may be impaired by the replacement of n-6 by n-3 precursor fatty acids in the membranes. The duration of the occlusions in our experiments was only 5 min. This prevents the loss of a large number of animals through ventricular fibrillation (28). It has been shown that pigs subjected to this type of stress have an acute recovery of regional myocardial function varying from 50 to 80% (10, 22). In the present study we found that in both dietary groups, the ATP content of the ischaemic myocardium was similarly reduced, but since the energy charge remained intact, complete recovery is expected after prolonged reperfusion. After occlusions lasting 30 min, regional heart function in pigs almost completely recovers after 2 weeks (23). Because of the short duration of the ischaemic events in the present study, which does not result in ultimate loss of function, comparison with the aforementioned studies (4, 13) is not possible.

The pre-occlusion heart rate was slightly higher in ML than in L. Gudbjarnason et al. (6) derived a significant correlation between heart rate and heart phospholipid 22:6 (n-3) content in various species. Although our findings in pigs support this relationship, there is no indication of a mechanism by which heart rate would be modulated by alterations of membrane fatty acids.

Despite the differences in fatty acid composition of the platelet membrane and cardiac sarcolemmal phospholipids and the subsequent changes in prostaglandin synthesis, no differences in systemic haemodynamics were found between the two dietary groups before, during and after

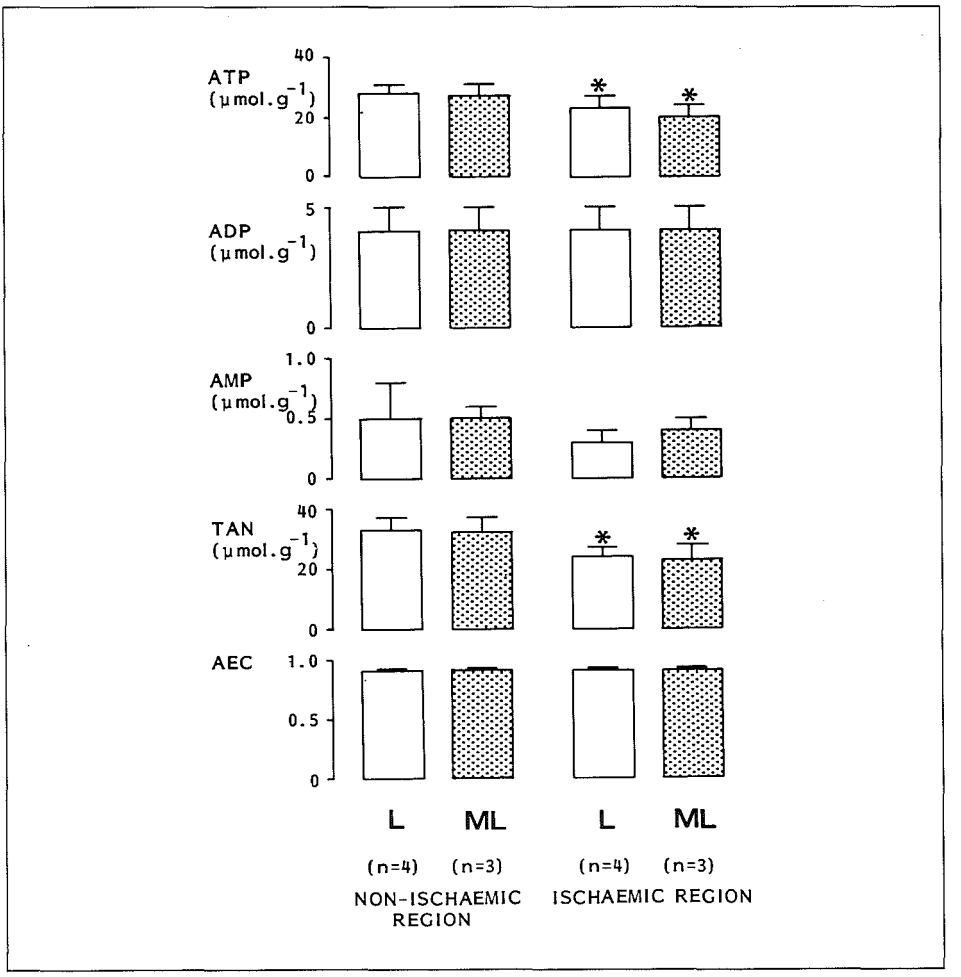

Fig. 6. Myocardial adenine nucleotide levels after 6 consecutive episodes of 5 min occlusion and 10 min reperfusion in pigs, which had been on lard fat (L) or mackerel oil + lard fat (ML) diets. From top to bottom are shown: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), total adenine nucleotides (TAN) and adenylate energy charge (AEC) which was calculated as (ATP+1/2 ADP) / TAN. ★$p<0.05$ versus non-ischaemic myocardium.

the occlusion-reperfusion periods. Slight differences, however, existed in myocardial performance of the ischaemic segment, as the reactive hyperaemic response lasted longer in ML, due to prolonged arterial vasodilatation. In our previous study using a higher daily dose of mackerel oil, we found not only a prolonged, but a more pronounced vasodilatation (10). These effects have been explained by diet-induced changes in coronary venous prostaglandin levels measured in the last reperfusion period. Recovery of systolic wall thickening was also slower in ML during the last four reperfusion periods, but was similar at the end of each reperfusion.

Although we did not add cyclooxygenase inhibitors to block prostaglandin synthesis of platelets or leukocytes, the blood samples were placed in ice to reduce (stop) this production from

occurring during separation of plasma from blood cells. Like others, we measured the myocardial release of prostacyclin (3, 15). As observed in our previous experiments, the basal coronary venous (10) and now also the arterial levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ were markedly reduced in the mackerel oil fed pigs. During the reperfusion periods no increase in coronary venous prostaglandin levels was found, which is at variance with the large stimulation previously seen (10). In that study, the animals received two-fold higher doses of mackerel oil and the coronary artery occlusion-reperfusion experiments were done after an 8 week dietary period. The arterial and coronary venous prostaglandin levels did not correlate with systemic and coronary vascular resistance, respectively. Therefore, other factors must have been more prominent in determining the vascular tone, and indeed, the arterial-coronary venous pH differences correlated much more closely with coronary vascular resistances. This does not, however, explain the prolonged hyperaemic response in ML.

The incidence of reperfusion arrhythmias did not correlate with coronary venous prostaglandin levels. For example, the levels in the three animals which encountered ventricular fibrillation were not different from those of the other animals, as has been observed by others (3). It should, however, be noted that the incidence of ventricular arrhythmias was low. The model was chosen to enable us to estimate the recovery of cardiac function rather than to evaluate the effect on arrhythmias. It is possible that more n-3 fatty acid-derived prostaglandins are produced in ML than in L, but that these can not be detected by the radioimmunoassay method used in this study. TXA_3 and PGI_3 are poor agonists and will be produced at a reduced rate, because eicosapentaenoic acid is a poor substrate for cyclooxygenase (14, 17).

We conclude that the membrane fatty acid content changes were paralleled by a reduction of the prostaglandin levels in both arterial and coronary venous blood and that no diet-related differences in cardiovascular performance and recovery of cardiac function were found in this open-chest pig model during multiple coronary artery occlusion-reperfusion periods. The lower incidence of reperfusion arrhythmias and the prolonged hyperaemic response in ML could not be correlated with changes in the coronary venous blood levels of prostaglandins.

Acknowledgements

The authors are grateful to Dr. M. Klompe, Mr. J. Endeveld and Mrs. M. Groh-Hoogenboom for their help with the biochemical analysis, Dr. A. Montfoort, Prof. Dr. W. C. Hülsmann and Prof. Dr. A. J. Vergroesen for their advice and Mr. H. Morse and Dr. M. C. Blok for the careful preparation of the diets. This study was supported by a grant from the Dutch Heart Foundation.

References

1. Bang HO, Dyerberg J (1984) Plasmalipids and lipoproteins in Greenlandic west coast eskimos. Acta Med Scand 251 (3): 351—364
2. Charnock JS, Abeywardena MY, McLennan PL (1986) Comparative changes in the fatty acid composition of cardiac phospholipids after long-term feeding of sun seed oil — or tuna fish oil — supplemented diets. Ann Nutr Metab 30, 393—406
3. Coker SJ, Parratt JR, Ledingham McA, Zeitlin IJ (1981) Thromboxane and prostacyclin release from ischaemic myocardium in relation to arrhythmias. Nature 291: 323—324
4. Culp BR, Land WEM, Lucchesi BR, Pitt R, Romson J (1980) The effect of dietary supplementation of fish oil on experimental myocardial infarction. Prostaglandins 20: 1021—1031
5. Dyerberg J, Bang HO (1979) Haemostatic function and platelet polyunsaturated fatty acids in eskimos. Lancet ii: 433—435
6. Gudbjarnason S, Oskarsdottir G, Doell B, Hallgrimson J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 25: 130—144

7. Harmsen E, de Tombe PPh, de Jong JW (1982) Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high performance liquid chromatography. J Chromatography 230: 131—136
8. Hartog JM, Verdouw PD (1986) Alleviation of myocardial ischaemia after administration of the cardioselective beta-adrenoceptor antagonist bevantolol. Cardiovasc Res 20: 264—268
9. Hartog JM, Lamers JMJ, Montfoort A et al. (1987) The effects of a mackerel-oil and a lard-fat enriched diet on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am J Clin Nutr (in press)
10. Hartog JM, Lamers JMJ, Verdouw PD (1986) The effects of dietary mackerel oil on plasma and cell membrane lipids, on hemodynamics and cardiac arrhythmias during recurrent acute ischemia in the pig. Basic Res Cardiol 81: 567—580
11. Herold PM, Kinsella JE (1986) Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am J Clin Nutr 43: 566—598
12. Hirsch PD, Hillis LD, Campbell WB, Firth BG, Willerson JT (1981) Release of prostaglandins and thromboxane into the coronary circulation in patients with ischemic heart disease. N Engl J Med 304: 685—691
13. Hock CP, Holahan M, Reibel DK (1986) Beneficial effects of dietary fish oil in acute myocardial ischemia. Circulation 74 S II: 1390 (abstract)
14. Hornstra G (1982) Dietary fats, prostanoids and arterial thrombosis. Developments in Hematology and Immunology, Vol 4. Martinus Nijhoff, The Hague, The Netherlands
15. Karmazyn M, Dhalla NS (1983) Physiological and pathological aspects of cardiac prostaglandins. Can J Physiol Pharmacol 61: 1207—1225
16. Lamers JMJ, de Jonge-Stinis JT, Hülsmann WC, Verdouw PD (1986) Reduced in vitro ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial ischaemia in anaesthetized pigs. J Mol Cell Cardiol 18: 115—125
17. Lamers JMJ, Hartog JM, Verdouw PD, Hülsmann WC (1987) Dietary fatty acids and myocardial function. Basic Res Cardiol (this volume)
18. Lantiola K, Salo MK, Metsä-Ketelä T (1986) Altered physiological responsiveness and decreases cyclic AMP levels in rat atria after dietary cod liver oil supplementation and its possible associations with increased membrane phospholipids n-3/n-6 fatty acid ratio. Biochim Biophys Acta 889: 95—102
19. Lockette WE, Webb RC, Culp BR, Pitt B (1982) Vascular reactivity and high dietary eicosapentaenoic acid. Prostaglandins 24 (5): 631—639
20. Lorenz R, Spengler U, Fischer S et al. (1983) Platelet function, thromboxane formation and blood pressure control during supplementation of the western diet with cod-liver oil. Circulation 67: 504—511
21. Montfoort A, van der Werf L, Hartog JM, Hugenholtz PG, Verdouw PD, Hülsmann WC, Lamers JMJ (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol 81: 289—302
22. Murphy ML, Kane JJ, Peng CF, Straub KD (1982) Wall motion and metabolic changes after coronary occlusion and reperfusion. J Surg Res 32: 143—149
23. Post JA, Lamers JMJ, Verdouw PD, ten Cate FJ, van der Giessen WJ, Verkleij AJ (1987) Sarcolemmal destabilization and destruction after ischaemia and reperfusion and its relation with long-term recovery of regional left ventricular function in pigs. Eur Heart J (in press)
24. Salmon JA (1978) A radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$. Prostaglandins 15: 383—397
25. Sherhag R, Kramer HJ, Düsing R (1982) Dietary administration of eicosapentaenoic and linolenic acid increases arterial blood pressure and suppresses vascular prostacyclin synthesis in the rat. Prostaglandins 23 (3): 369—382
26. Singer P, Jaeger W, Wirth M et al. (1983) Lipid and blood pressure lowering effect of mackerel diet in man. Atherosclerosis 49: 99—108
27. Van der Giessen WJ, Schoutsen B, Tijssen JGP, Verdouw PD (1986) Iloprost (ZK 36374) enhances recovery of regional myocardial function during reperfusion after coronary artery occlusion in the pig. Br J Pharmacol 87: 23—27

28. Verdouw PD, Wolffenbuttel BHR, ten Cate FJ (1983) Nifedipine with and without propranolol in the treatment of myocardial ischemia: effects on ventricular arrhythmias and recovery of regional wall function. Eur Heart J Suppl C 4: 101—108
29. Zijlstra FJ, Van Vliet HHDM, Vincent JE (1983) Thrombotic thrombocytic purpura and thromboxane B_2 levels. Thromb Res 30: 535—538

Authors' address:

P. D. Verdouw, PhD, Laboratory for Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, P.O.Box 1738, 3000 DR Rotterdam, The Netherlands

CHAPTER 10

Lipid Peroxidation in Normoxic and Ischaemic-reperfused Hearts of Fish Oil and Lard Fat Fed Pigs

Jos M. J. Lamers*, Johannes M. Hartog†, Carlo Guarnieri‡, Isabella Vaona‡, Pieter D. Verdouw† and Johan F. Koster*

Department of Biochemistry I and Thoraxcenter†, Medical Faculty, Erasmus University Rotterdam, Rotterdam, The Netherlands and Istituto di Chimica Biologica‡, Facoltà di Medicina e Chirurgia dell' Universita di Bologna, Bologna, Italy*

(*Received 2 October 1987, accepted in revised form 14 April 1988*)

J. M. J. Lamers, J. M. Hartog, C. Guarnieri, I. Vaona, P. D. Verdouw and J. F. Koster. Lipid Peroxidation in Normoxic and Ischaemic-reperfused Hearts of Fish Oil and Lard Fat Fed Pigs. *Journal of Molecular and Cellular Cardiology* (1988) **20**, 605–615. The *in situ* and *in vitro* rate of lipid peroxidation of hearts were determined in two groups of pigs which had been fed diets which differed only in fatty acid composition for 8 weeks. During the dietary period venous plasma levels of malondialdehyde and lipofuscin were not higher in pigs receiving the highly unsaturated fatty acid-containing mackerel oil than those receiving lard fat. Malondialdehyde was produced in the coronary system of the mackerel oil fed animals. After the heart was subjected to a sequence of short periods of ischaemia (5 min) and reperfusion (10 min), myocardial malondialdehyde production in the mackerel oil fed pigs did not increase. Contribution of prostaglandin synthesis products to myocardial malondialdehyde formation is probably of minor importance. Recovery of regional heart function after the ischaemic periods was similar for both dietary groups. In the phospholipids of sarcolemmal preparations isolated from the left ventricle of mackerel oil fed animals 18 : 2 n-6 and 20 : 4 n-6 were partially replaced by 20 : 5 n-3 and 22 : 6 n-3. Ischaemia–reperfusion did not alter sarcolemmal fatty acid composition and Ca^{2+} pumping ATPase activity. Sarcolemmal membrane from mackerel oil fed pigs exposed *in vitro* to a free radical generating system showed a higher malondialdehyde production than that from lard fat fed pigs. Thus, in spite of the increased susceptibility of heart membranes to free radical generated peroxidation in mackerel oil fed animals, recovery of left ventricular function was similar following multiple short-term periods of ischaemia.

Key Words: Dietary fatty acids; Polyunsaturated fatty acids; Fish oil; Lard fat; Ischaemia–reperfusion; Lipid-peroxidation; Sarcolemma; Ca^{2+} pumping ATPase; Haemodynamics; Prostaglandins.

Introduction

The direct and indirect role of oxygen radicals in the development of myocardial necrosis due to ischaemia and reperfusion has been well documented. A large body of evidence has been derived from observations that treatment with free radical scavengers (e.g. superoxide dismutase, catalase and mannitol) reduced the extent of myocardial injury (Shlafer *et al.*, 1982; Jolly *et al.*, 1984; Ferrari *et al.*, 1985; McCord, 1985) and that allopurinol-induced inhibition of xanthine oxidase, probably a major source of superoxide O_2^- in post-ischaemic tissue, can delay cell death (Manning *et al.*, 1984; Stewart *et al.*, 1985). Several reports have shown elevated endogenous generation of free radicals and their products, as malondialdehyde (MDA) and those detectable by electron spin resonance spectrometry in lyophilized tissue extracts, during myocardial ischaemia (Guarnieri *et al.*, 1980; Meerson *et al.*, 1982; Rao *et al.*, 1983). Moreover, perfusion of control hearts with free radical producing compounds leads to the development of Ca^{2+}-paradox-like myocardial injury (Koster *et al.*, 1985). Other factors which may be involved are the ischaemia-induced reduction

Please address all correspondence to: Jos M. J. Lamers, Department of Biochemistry I, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

Abbreviations: MDA, malondialdehyde; GSSG, oxidized glutathion; PUFA, polyunsaturated fatty acid; LADCA, proximal left anterior descending coronary artery; EDTA, ethylene diamine tetraacetic acid; TXB_2, thromboxane B_2; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; TXA_2, thromboxane A_2; PGI_2, prostaglandin I_2; TXB_3, thromboxane B_3; 6-keto $PGF_{2\alpha}$, 6-keto prostaglandin $F_{2\alpha}$.

0022-2828/88/070605 + 11 $03.00/0

in cellular concentration of glutathion (GSSG) and decreased activity of free radical scavengers as catalase and superoxide dismutase (Guarnieri *et al.*, 1980; Ferrari *et al.*, 1985).

Because free radical molecules contain one or more free electrons in the outer orbitals they can abstract hydrogen atoms from various intra- and extracellular molecules like proteins, nucleic acids and polyunsaturated fatty acids (PUFAs) in particular because of the evidence that support an active role of lipid peroxidation products in free-radical induced injury (Meerson *et al.*, 1982; Hess and Manson, 1984; Sevanian and Hochstein, 1985; Stam and Koster, 1985; Lucchesi and Mullane, 1986). Hydrogen abstraction from the cis–cis pentadiene center of an unsaturated fatty acid is purported to be the rate-limiting step in the auto-oxidation. The predominant and thermodynamically most stable products are allylic radicals of the PUFAs. It is therefore feasible that the nature and relative quantities of PUFAs in cardiac membranes are important in the regulation of the rate and extent of lipidperoxidation in the heart.

In the present investigation the PUFA composition of cardiac membranes was altered by a dietary intervention (Hartog *et al.*, 1986, 1987). Myocardial formation of MDA and GSSG were determined before and at the end of a sequence of brief periods of ischaemia and reperfusion. Regional systolic wall thickening measurements were used to determine whether an altered susceptibility of cardiac membranes to free radical attack leads to a change in the rate and magnitude of recovery of myocardial function. Sarcolemmal fatty acid composition were determined in ischaemic heart biopsies to examine the possible selective loss of PUFAs. A free radical-generating system, Fe^{3+}-ADP plus dihydrofumarate, was used to determine whether the cardiac sarcolemmal membrane from mackerel oil fed pigs also is more susceptible to free radical attack *in vitro*.

Materials and Methods

Experimental animals and diets

Sixteen Yorkshire piglets of either sex and 5 weeks of age (7.9 ± 0.2 kg) were housed individually in slat-bottomed cages in temperature-controlled animal quarters and divided arbitrarily into two groups. Each group followed a different diet for a period of 8 weeks, the regimen, preparation and composition of which are described in detail by Hartog *et al.* (1986, 1987). The diets mainly differed in the contents of 18 : 0, 18 : 1 n-9, 18 : 2 n-6, 20 : 5 n-3 and 22 : 6 n-3 (lard fat diet: 10, 42, 15, 0 and 0 mole%, respectively; mackerel oil diet: 1, 17, 8, 17 and 9 mole%, respectively). Before the start of the dietary period and thereafter at 2-week intervals venous blood samples were collected from 24-h fasted (to limit possible effects of dietary MDA on plasma MDA as found by Draper *et al.*, 1984 who studied cod liver oil fed rabbits) animals for measurement of plasma MDA and lipofuscin concentration.

Surgical preparation and haemodynamic measurements

After 8 weeks, the 16 animals (now weighing 20.4 ± 0.7 kg) were anaesthetized and catheterized according to Verdouw *et al.* (1986). Briefly, following exposure of the heart via a midsternal split, electromagnetic flow probes were placed around the ascending aorta and the proximal left anterior descending coronary artery (see Fig. 1). The accompanying cardiac vein was cannulated. Baseline measurements were obtained after a stabilization period of at least 30 min. Subsequently, the LADCA was clamped for 5 min and declamped for 10 min. This occlusion–reperfusion procedure was repeated five more times. The only drugs used in this study were the anaesthetics and the muscle relaxant pancuroniumbromide.

Chemical analysis of plasma and myocardial biopsies

Coronary venous blood samples were collected prior to the first coronary artery occlusion and during peak hyperaemia of the last reperfusion period (Fig. 1). At the same time arterial blood samples were taken from the ascending aorta. After separation of blood cells by centrifugation at 0 to 4°C, the plasma was frozen in liquid N_2 and stored at −80°C. The MDA content of the plasma samples was measured in the presence of 100 μM EDTA as described by Jackson *et al.* (1983). The extinc-

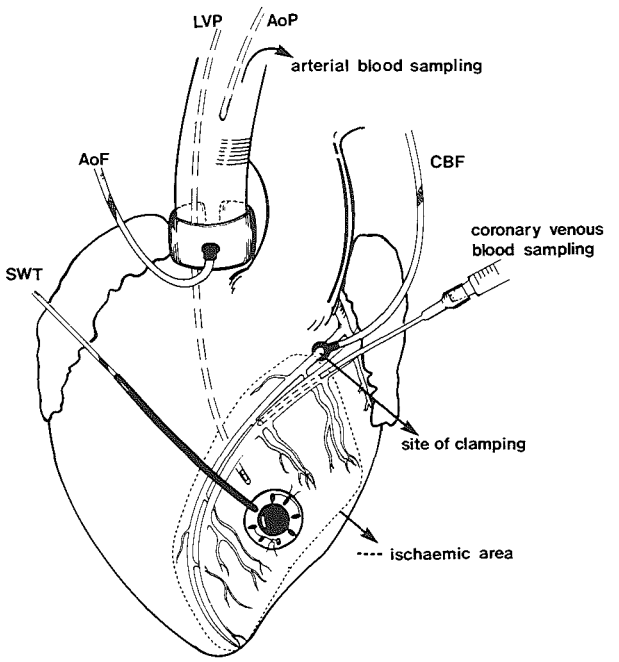

FIGURE 1. Surgical preparation and the sites of clamping of the coronary artery, blood sampling, pressure and blood flow measurements. SWT, systolic wall thickening; AoF, aorta flow; LVP, left ventricular pressure; AoP, aorta pressure; CBF, coronary blood flow.

tion coefficient of the thiobarbituric acid product was taken as 1.56×10^5 for all calculations of the MDA content. The production in nmol MDA per min was calculated from the difference in arterial and venous MDA levels multiplied by the coronary blood flow. In some of the plasma samples water-soluble fluorescent substances like lipofuscin existing in the protein fraction were also determined at excitation and emission wavelengths of 350 and 460 nm, respectively (Tsuchida *et al.*, 1985). Calibration of 100 relative fluorescence units was done against a 0.1 μg/ml quinine sulfate solution in 0.1 N sulfuric acid. Thromboxane B_2 (TXB_2) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), which are the stable products of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), respectively, were determined in plasma samples by radioimmunoassay (Hartog *et al.*, 1986). GSSG levels in plasma were determined as described by Ferrari *et al.* (1985).

After completion of the occlusion–reperfusion protocol the heart was immediately excised and cooled in ice. Ischaemic–reperfused and non-ischaemic myocardium (about 5 g each) was rapidly cut out and homogenized in buffer (0.3 M sucrose, 5 mM $MgSO_4$, 10 mM imidazole-HCl, pH 7.0) with 5 s bursts of the polytron PT_{10} at dial setting 4. Enriched sarcolemma preparations were isolated from the myocardial homogenates as previously described (Lamers *et al.*, 1984, 1986). The final membrane pellet was resuspended in 1 ml 160 mM KCl, 20 mM 4-morpholino-propane-sulfonic acid pH 7.4, frozen in liquid N_2 and stored at $-80°C$. The procedures for phospholipid extraction, subsequent phospholipid hydrolysis and formation of fatty acid methylesters and gas chromatographic separation have all been described before (Montfoort *et al.*, 1986). Ca^{2+}-stimulated Mg^{2+}-ATPase activity was determined with [γ-^{32}P]-labeled ATP as substrate and by isobutanol extraction of molybdate-complexed $^{32}P_i$ (Lamers *et al.*, 1985). The difference between activities at 0 and 0.5 or 12 μM free Ca^{2+} concentration was

taken as the Ca^{2+}-stimulated portion of the ATPase activity. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

In vitro *measurement of the rate of lipid peroxidation*

Isolated sarcolemmal vesicles (exactly 450 μg of protein) were incubated in 3 ml 0.1 mM Fe^{3+}-1 mM ADP chelate, 3.3 mM dihydrofumarate, 160 mM KCl and 20 mM MOPS, pH 7.2 at 37°C in a shaking waterbath for 60 min as described by Kramer *et al.* (1984). At intervals, aliquots of the sarcolemmal reaction mixtures were assayed for MDA. Extremely low production of MDA was monitored when one of the components Fe^{3+}-ADP or dihydrofumarate was omitted from the incubation.

Statistic treatment of the data

Results are expressed as mean ± S.E.M. Statistical significance was established by the Student's *t*-test.

Materials

Radioactive substances, [γ-^{32}P]-ATP and [^{14}C]-AMP, used for indication of Ca^{2+} pump ATPase and AMPase activity respectively, were obtained from Amersham International PLC (Amersham, UK). All other chemicals were obtained from either Merck (Darmstadt, West Germany) or Boehringer (Mannheim, West Germany).

Results

Plasma levels of MDA and lipofuscin during the dietary period

The MDA plasma concentrations of the mackerel oil fed animals tended to decrease during the 8-week feeding period (Fig. 2). Those of the lard fat fed animals followed a similar pattern after a slight increase in the first 2 weeks. In four pigs of each dietary group water-soluble fluorescent substances including lipofuscin were measured in plasma samples obtained at the end of the dietary period. The relative fluorescence per 10 μl plasma was 81 ± 12 for the lard fat fed and 38 ± 7 ($P < 0.05$) for the mackerel oil fed pigs. These fluorescent substances are likely Schiff's binding bases formed between peroxidized lipids and serum proteins (Tsuchida *et al.*, 1985). The results indicate that supplementation of the diet with mackerel oil does not result in higher levels of lipid peroxidation products in the plasma.

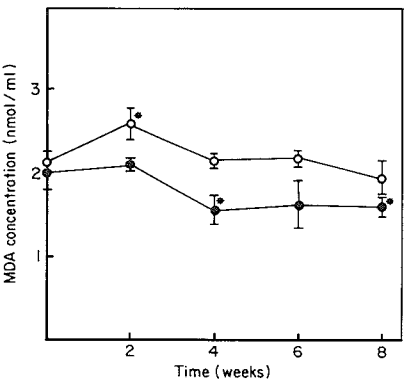

FIGURE 2. MDA concentrations in plasma of blood taken from the subclavian vein in eight pigs during the 8-week feeding period (mean ± S.E.M.). Blood samples were collected from 24-h fasted pigs that were on a mackerel oil (●) or lard fat (○) enriched diet. MDA assay has been carried out as described in the Methods section. *$P < 0.05$ vs. the pre-dietary plasma value.

MDA production by the left ventricle before and immediately after ischaemia–reperfusion

Myocardial MDA production before (19 ± 4 nmol/min) and during the last reperfusion period (52 ± 19 nmol/min) were not significantly different in the mackerel oil fed animals (Fig. 3). In the lard fat fed animals we did not observe any MDA production either before induction of ischaemia, or during the last reperfusion. At the end of the last reperfusion there was no significant production of GSSG (another indicator of oxidative injury) in either group of animals: 8 ± 45 and 30 ± 17 nmol/min for the lard fat ($n = 4$) and mackerel oil ($n = 6$) fed animals, respectively.

Prior to the first occlusion the coronary venous levels of TXB_2 (16 ± 5 and 39 ± 8 pg/ml, respectively, $P < 0.05$) and 6-keto $PGF_{1\alpha}$ (82 ± 6 and 133 ± 35 pg/ml, respectively, $P < 0.05$) were markedly lower in the

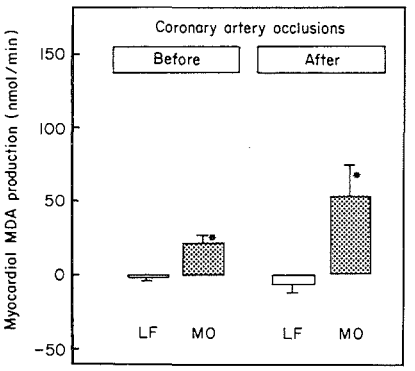

FIGURE 3. MDA production by the pig left ventricle before and after a sequence of six consecutive 5 min coronary artery occlusions interrupted by 10 min of reperfusion. Coronary arterial and venous blood samples were collected prior to ($n = 4$ LF and 5 MO) the first occlusion and during peak hyperaemia of the last reperfusion period ($n = 5$ LF and 8 MO). See Figure 1 for the sites of blood sampling and flow measurement. *$P < 0.05$ vs. the corresponding mean value obtained in the lard fat fed animals. LF, lard fat fed; MO, mackerel oil fed. Bars represent mean ± S.E.M.

mackerel oil than in the lard fat fed animals (see Hartog *et al.*, 1986). The increased coronary venous levels of TXB_2 (19 ± 7 and 77 ± 6 pg/ml, respectively, $P < 0.05$) and 6-keto $PGF_{1\alpha}$ (123 ± 7 and 371 ± 40 pg/ml, respectively, $P < 0.05$) measured during peak hyperaemia of the last reperfusion were also much lower in the mackerel oil than in the lard fat fed animals. The contribution of cyclooxygenase products of 20 : 5 n-3 (TXB_3 and 6-keto $PGF_{2\alpha}$) to the myocardial MDA formation in the mackerel oil fed animals is expected to be minor because 20 : 5 n-3 is a very poor substrate for cyclooxygenase (Lands, 1982; Lamers *et al.*, 1987).

Recovery of regional myocardial function after multiple transient periods of ischaemia

Despite the observed greater susceptibility to lipid peroxidation of hearts of mackerel oil fed pigs, the ischaemia–reperfusion-induced changes in heart rate, mean arterial blood pressure, left ventricular end-diastolic pressure and cardiac output were the same (Table 1). In both groups of animals regional systolic wall thickening was completely abolished during the occlusion periods as a consequence of the lack of any myocardial perfusion (collateral blood flow in pigs is less than 0.05 ml/min/g; Verdouw *et al.*, 1983). Recovery of function during reperfusion was partial but similar in both dietary groups (Table 1). The only diet-related difference was the more pronounced hyperaemic response in the mackerel oil fed animals during the last reperfusion period, probably caused by the observed alterations of the TXA_2/PGI_2 ratio (Hartog *et al.*, 1986).

Biochemical properties of isolated sarcolemmal preparations

There was no difference in sarcolemmal yield between the biopsies taken from non-

TABLE 1. Cardiovascular performance of lard fat and mackerel oil fed pigs subjected under anaesthesia to a sequence of six consecutive coronary artery occlusions (5 min) interrupted by 10 min of reperfusion

	Lard fat fed group ($n = 8$)		Mackerel oil fed group ($n = 8$)	
	Baseline	End of last reperfusion	Baseline	End of last reperfusion
HR (beats/min)	110 ± 9	83 ± 6[a]	107 ± 15	99 ± 11
MAP (mmHg)	86 ± 5	60 ± 3[a]	94 ± 5	71 ± 5[a]
LVEDP (mmHg)	7.7 ± 0.6	9.1 ± 0.9	8.7 ± 1.3	10.3 ± 1.7
CO (l/min)	1.8 ± 0.2	1.0 ± 0.1[a]	1.8 ± 0.2	1.2 ± 0.1[a]
CBF (ml/min)	31 ± 5	32 ± 7	32 ± 5	50 ± 10[a,b]
SWT (%)	37 ± 3	29 ± 4[a]	28 ± 3	21 ± 2[a]

HR, heart rate; MAP, mean arterial blood pressure; LVEDP, left ventricular end-diastolic pressure; CO, cardiac output; CBF, left anterior descending coronary artery flow; SWT, systolic wall thickening; n, the number of pigs.

[a] $P < 0.05$ vs. corresponding baseline value; [b] the ischaemia-induced changes in the mackerel oil fed animals is significantly different ($P < 0.05$) from those in the lard fat fed animals. All data are presented as mean ± S.E.M.

TABLE 2. Yield and purities of sarcolemma isolated from biopsies of the left ventricle of lard fat and mackerel oil fed pigs subjected under anaesthesia to a sequence of six consecutive coronary artery occlusions (5 min) interrupted by 10 min of reperfusion

	Lard fat fed group		Mackerel oil fed group	
	C ($n = 8$)	I–R ($n = 8$)	C ($n = 8$)	I–R ($n = 8$)
Yield (%)[a]	0.38 ± 0.04	0.45 ± 0.05	0.47 ± 0.04	0.51 ± 0.06
AMPase (RSA)[b]	10.7 ± 0.9	13.3 ± 2.4	12.6 ± 1.8	12.4 ± 2.4

[a] Yield of sarcolemma protein is expressed as percentage of total homogenate protein.

[b] RSA of 5′-nucleotidase (AMPase) is calculated by dividing the measured specific activity (in nmol/min/mg) of sarcolemma through that measured in the corresponding homogenate fraction.

C, control segment; I–R, segment subjected to occlusion–reperfusion; *n*, number of pigs. All data are presented as mean ± S.E.M.

ischaemic and ischaemic–reperfused regions of lard fat and mackerel oil fed animals (Table 2). No diet-related differences were also observed between the purities of the sarcolemmal preparations, which were estimated using the marker enzyme AMPase. The results indicate that the differences in diet and short-term periods of ischaemia–reperfusion had no effect on subcellular fractionation patterns.

As expected, feeding of mackerel oil induced a marked shift in the membrane PUFA composition as 18 : 2 n-6 and 20 : 4 n-6 were partially replaced by 20 : 5 n-3 and 22 : 6 n-3 (Table 3). This causes the mean number of double bonds per mole fatty acid (double bond index) to increase from 1.28 ± 0.06 ($n = 8$) in the lard fat fed pigs to 1.85 ± 0.06 ($n = 8$, $P < 0.05$) in the mackerel oil fed animals. The sum of PUFAs increased slightly ($P < 0.05$) from 33.5 ± 2.2 to 39.1 ± 1.6 mole % of the total fatty acid pool. The results in Table 3 also show that, except for a minor component fatty acid 22 : 5 n-3,

TABLE 3. Fatty acid composition of sarcolemma isolated from biopsies of the left ventricle of lard fat and mackerel oil fed pigs subjected under anaesthesia to a sequence of six consecutive coronary artery occlusions (5 min) interrupted by 10 min of reperfusion

	Lard fat fed group		Mackerel oil fed group	
Fatty acids	C ($n = 8$)	I–R ($n = 7$)	C ($n = 8$)	I–R ($n = 8$)
14 : 0	3.8 ± 0.9	3.4 ± 0.6	4.2 ± 0.6	0.8 ± 0.5
DMA 16 : 0	2.9 ± 0.7	1.6 ± 0.5	3.9 ± 1.0	3.2 ± 0.8
16 : 0	25.7 ± 0.9	24.7 ± 1.0	26.3 ± 0.9	25.7 ± 1.2
DMA 18 : 0	1.4 ± 0.4	1.2 ± 0.3	2.1 ± 0.5	2.1 ± 0.5
18 : 0	10.9 ± 0.5	10.5 ± 0.7	10.7 ± 0.6	10.8 ± 0.9
18 : 1 n-9	21.8 ± 2.2	20.7 ± 1.9	13.6 ± 1.2[a]	13.7 ± 1.3
18 : 2 n-6	15.7 ± 1.4	17.5 ± 1.1	6.2 ± 0.4[a]	6.5 ± 0.9
20 : 4 n-6	13.2 ± 1.4	14.4 ± 1.2	6.1 ± 0.3[a]	6.2 ± 0.2
20 : 5 n-3	0	0.2 ± 0.1	16.9 ± 1.0[a]	16.3 ± 0.6
22 : 4 n-3	2.9 ± 0.5	2.9 ± 0.8	3.7 ± 0.6	5.3 ± 1.2
22 : 5 n-3	0.5 ± 0.2	1.2 ± 0.1[b]	1.4 ± 0.2[a]	1.4 ± 0.2
22 : 6 n-3	1.2 ± 0.2	1.3 ± 0.2	4.9 ± 0.3[a]	5.1 ± 0.3

DMA, dimethylated acetals.

[a], $P < 0.05$ vs. that obtained in the lard fat fed animals; [b], $P < 0.05$ vs. data from control segment.

C, control segment; I–R, segment subjected to occlusion–reperfusion; *n*, number of pigs. All data are presented as mean ± S.E.M.

TABLE 4. Ca^{2+} pumping ATPase activities of sarcolemma isolated from biopsies of the left ventricle of lard fat and mackerel oil fed pigs subjected under anaesthesia to a sequence of six consecutive coronary artery occlusions (5 min) interrupted by 10 min of reperfusion

	Lard fat fed group		Mackerel oil fed group	
	C ($n = 6$)	I–R ($n = 6$)	C ($n = 6$)	I–R ($n = 6$)
Activity[a] (nmol/min/mg)				
Basal	46.0 ± 5.2	52.8 ± 6.9	47.7 ± 10.1	46.0 ± 5.2
Ca^{2+} stimulated	61.3 ± 8.3	76.3 ± 7.8	102.9 ± 10.6[b]	109.1 ± 14.2[b]
Affinity for Ca^{2+}				
Activity ratio (%)	43.6 ± 7.6	41.1 ± 3.8	43.4 ± 5.1	43.6 ± 7.6

[a] The activity ratio represents the activity stimulated by 0.5 μM Ca^{2+} as a percentage of the activity stimulated by the maximum Ca^{2+} concentration (12 μM). This percentage is taken as a rough parameter for the affinity of the enzyme for Ca^{2+} ions.

C, control segment; I–R, segment subjected to occlusion–reperfusion; *n*, number of pigs.

[b] $P < 0.05$ vs. the activity measured in a corresponding sample of the lard fat fed animals.

All data are presented as mean ± S.E.M.

no change in PUFA composition occurs after the recurrent ischaemia–reperfusion periods.

The maximal activity and the Ca^{2+} affinity of the sarcolemmal Ca^{2+} pumping ATPase, from non-ischaemic and ischaemic–reperfused myocardium did not differ at the end of the experimental protocol (Table 4). The significantly higher Ca^{2+} pumping ATPase activity of the membranes of the mackerel oil fed pigs may be related to altered fluidity properties of the phospholipid bilayer due to a higher degree of unsaturation of the membrane fatty acids (Table 3).

The fatty acids with higher degree of unsaturation in cardiac sarcolemma of mackerel oil fed animals are expected to be more readily peroxidized as indeed follows from the results on *in situ* myocardial MDA production (Fig. 3). The relative vulnerability to exogenously evoked free radicals of *in vitro* incubated cardiac sarcolemma preparations from the two dietary groups was also examined. The free radicals were generated during autoxidation of dihydrofumaric acid in the presence of Fe^{3+}-ADP. In the mackerel oil fed pigs MDA formation started after a lag phase of about 20 min, and had a much higher rate in the sarcolemma fraction in which relatively high portions of 20 : 5 n-3 and 22 : 6 n-3 were incorporated (Fig. 4 and Table 3). The lag phase may be caused by the presence of larger quantities of vitamin E in the sarcolemma due to the dietary supplementation. Hammer and Wills (1978) demonstrated that dietary supplementation of vitamin E markedly reduced MDA formation in rat liver microsomes (measured in the presence of ascorbate) after lard fat as well as herring oil diets. In that study we can not evaluate the effect of dietary vitamin E supplementation on the duration of the lag phase because information on the time course of MDA production is lacking.

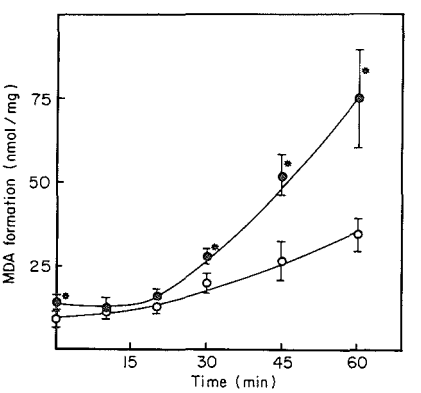

FIGURE 4. MDA production by isolated sarcolemmal membranes that were incubated *in vitro* with Fe^{3+}-ADP plus dihydrofumarate. Sarcolemmal vesicles were isolated from non-ischaemic myocardium of pigs fed mackerel oil (●) or lard fat (○) for 8 weeks. Further details of sarcolemmal isolation procedure, incubation conditions and MDA assay are given in the Methods. Each point represents the mean (S.E.M.) of determinations in five pigs. *$P < 0.05$ vs. sarcolemma isolated from lard fat fed pigs.

Discussion

With reintroduction of molecular oxygen to the hypoxic/ischaemic myocardium, there may be a burst of highly destructive oxygen free radicals from the activated free radical producing systems. Peroxidative breakdown of membrane PUFAs could be a consequence of action of these oxygen free radicals. Das *et al.* (1986) showed that in isolated *in situ* porcine hearts the free radical scavengers superoxide dismutase and catalase improved recovery of cardiac function and reduced myocardial MDA production during reperfusion. In the present study, recovery of regional myocardial function was similar for both dietary groups, but MDA production was only detectable in the mackerel oil fed animals. Recently, larger amounts of thiobarbituric acid-reactive substances have been found in heart tissue from rats fed with cod liver oil for 14 to 21 days, results which are in agreement with those of the present investigation (Herbert and Wills, 1987). The arterial and coronary venous concentrations of MDA in the present study vary between 2 and 4 nmol/ml which are similar to data reported by Liedtke *et al.* (1984). Das *et al.* (1986) reported 100 to 200 times higher (200 to 800 nmol/ml) coronary systemic concentrations of MDA for pig hearts on an extracorporal circulation. It is therefore likely that considerable scavenging of free radicals occurs by components supplied by the systemic circulation (e.g. ferritin-Fe^{2+} complex and Cu^{2+}-ceruloplasmin).

MDA was not produced by the left ventricle of the lard fat fed animals which contrasts the significant production in the mackerel oil fed animals. This was an unexpected finding in view of the similar PUFA content of the isolated sarcolemmal membranes (33.5 ± 2.2 vs. 39.1 ± 1.6 in the lard fat versus mackerel oil fed pigs) and the high content of 20 : 4 n-6 (Table 3). However, a significant production of MDA was observed in the *in vitro* experiments with exposure of cardiac sarcolemma from lard fat fed pigs to a free radical generating system. Three carbon dialdehyde may arise from fatty acid hydroperoxide when an unsaturated bond resides β–γ to the peroxide-bearing carbon (Sevanian and Hochstein, 1985; Stam and Koster, 1985). These cyclic peroxides may be the precursors of MDA and other volatile products. To cyclize to compounds bearing a five-membered ring also requires a nonconjugated system residing β–γ to the peroxyradical. Hence, such reactions leading to MDA require three or more double bonds for the fatty acid. Hammer and Wills (1978) determined the rate of lipid peroxidation in the presence of ascorbate in suspensions of liver endoplasmic reticulum membranes isolated from rats fed on herring oil or lard fat diet. The 3.5-fold higher rate of MDA formation as seen in the fish oil fed animals compares favorably with the present observations.

A number of cyclic peroxides (or endoperoxides) produced from PUFAs bear a remarkable resemblance to prostaglandins—notably prostaglandin G_2. The free radical-induced cyclization reaction in PUFAs is, in fact, a chemical equivalent to the enzymatically controlled reactions catalyzed by cyclooxygenase (Sevanian and Hochstein, 1985; Stam and Koster, 1985). Decomposition of cyclic peroxides formed by cyclooxygenase can also yield MDA. In the mackerel oil fed animals 3- to 4-fold lower concentrations of TXB_2 and 6-keto $PGF_{1\alpha}$ were measured in the coronary vein both before and after ischaemia. This reduction is most likely caused by replacement of the precursor 20 : 4 n-6 by 20 : 5 n-3 within the membrane (Table 3). Although knowledge of the production rates of TXB_2 and 6-keto $PGF_{1\alpha}$ would offer a more definite proof, it is likely that the observed myocardial MDA production in the mackerel oil fed pigs prior to the first occlusion and at peak hyperaemia originates from products of non-enzymatic lipid peroxidation.

The diets in the present study were supplemented with similar amounts of vitamin E and selenium to prevent development of yellow fat disease due to dietary fish oil (Ruiter *et al.*, 1978; Hartog *et al.*, 1987). During the entire dietary period plasma MDA and lipofuscin content were not higher in mackerel oil than in the lard fat fed animals. This could be related to this supplementation of diets with natural antioxydantia. That rabbits receiving large amounts of fish oil supplemented with tocopherols had much higher plasma MDA concen-

trations implies a species difference in the endogenous scavenging capacity (Thiery and Seidel, 1987).

The sarcolemmal membrane, relatively poor in tocopherols, is probably more susceptible to lipid peroxidation than, e.g., the sarcoplasmic reticulum (Kramer *et al.*, 1984; Guarnieri *et al.*, 1985; Stam and Koster, 1985). However, the PUFA composition of sarcolemma did not change during recurrent ischaemia–reperfusion (Table 3). The absence of these proves that attack of generated free radicals and phospholipase A_2 on PUFAs in membrane phospholipid is accompanied by active reacylation. It has been shown that for the production of 1 nmol MDA about 9 nmoles PUFA are used (Hammer and Wills, 1978). We observed a mean MDA production of 52 nmol/min during the last ischaemic–reperfusion period. The great cardiac vein drains blood from approximately 40 g wet wt of left ventricular tissue which contains a total of 200 mg sarcolemmal protein. If the MDA production rate remains constant during recurrent ischaemia–reperfusion periods (total of 60 min reperfusion time), the total sarcolemmal PUFA breakdown will be 0.05 μmoles/mg membrane protein. Such a high breakdown should be detectable by measurement of mole % of PUFAs as sarcolemma-enriched membranes contain about 0.5 to 0.8 μmol phospholipid-bound PUFA per mg protein (unpublished). Meerson *et al.* (1982) proposed that lipid hydroperoxides may form so-called transmembrane clusters by lateral diffusion which serve as ion permeability channels. Other investigators have shown that membrane-localized enzymes (e.g. Ca^{2+} pumping ATPase and Na^+ pumping ATPase) are affected by lipid peroxidation processes as a consequence of altered annulus phospholipid or as a result of interaction of lipid peroxide (radicals) with membrane proteins (Hess *et al.*, 1981; Jones *et al.*, 1983; Okabe *et al.*, 1983; Hess and Manson, 1984; Kramer *et al.*, 1984; Kim and Akera, 1987). Sarcolemmal Ca^{2+} pumping ATPase, however, remained stable after recurrent ischaemia–reperfusion in either dietary group. It is possible that the heart should be subjected to longer periods of ischaemia to demonstrate changes in sarcolemmal membrane Ca^{2+} transport functions, although previously we also showed sarcolemmal Na^+ pumping ATPase activity to remain unaltered even after 3 h of ischaemia (Lamers *et al.*, 1986).

In spite of an increased susceptibility to free radical generated peroxidative damage of heart membranes of the mackerel oil fed pigs, the recovery of regional left ventricular function was similar to that of lard fat fed pigs during recurrent myocardial ischaemia–reperfusion. In recent studies performed with rats fed cod liver oil the loss of creatine kinase was examined following *in situ* coronary ligation (Hock *et al.*, 1987) and low flow ischaemia using the Langendorff procedure (Karmazyn *et al.*, 1987). Only in the *in situ* model dietary fish oil reduced creatine kinase release (36%) after 6 h of ischaemia. An explanation could be that dietary supplementation of marine lipids to experimental animals produces, in addition to the harmful effects of increased membrane lipid peroxidation, some beneficial effects through an unknown mechanism.

Acknowledgements

This work was supported by the Dutch Heart Foundation. Dr A. Montfoort is thanked for advice, Mrs J. T. De Jonge-Stinis and Miss L. van der Werf for technical assistance and Miss P. H. Vegter for the assistance in the preparation of this manuscript.

References

Das DK, Engelman RM, Rousou JA, Breyer RH, Otani H, Lemeshow S (1986) Pathophysiology of superoxide radical as potential mediator of reperfusion injury in pig heart. Basic Res Cardiol **81**: 155–166.

Draper HH, Polensek L, Hadley M, McGirr LG (1984) Urinary malondialdehyde as an indicator of lipid peroxidation in the diet and in the tissues. Lipids **19**: 836–843.

Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O (1985) Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defenses against oxygen toxicity. J Moll Cell Cardiol **17**: 937–945.

Guarnieri C, Flamigni F, Caldarera CM (1980) Role of oxygen in the cellular damage induced by reoxygenation of hypoxic hearts. J Mol Cell Cardiol **12**: 797–808.
Guarnieri C, Ventura C, Georgountzos A, Muscari C, Budini R (1985) Involvement of superoxide radicals in adrenochrome formation stimulated by arachidonic acid in bovine heart sarcolemmal vesicles. Biochim Biophys Acta **838**: 355–360.
Hammer CT, Wills ED (1978) The role of lipid components of the diet in the regulation of fatty acid composition of the rat liver endoplasmic reticulum and lipid peroxidation. Biochem J **174**: 585–593.
Hartog JM, Lamers JMJ, Verdouw PD (1986) The effects of dietary mackerel oil on plasma and cell membrane lipids, on hemodynamics and cardiac arrhythmias during recurrent acute ischemia in the pig. Basic Res Cardiol **81**: 567–580.
Hartog JM, Lamers JMJ, Montfoort A, Becker AE, Klompe M, Morse H, Ten Cate FJ, Hülsmann WC, Hugenholtz PG, Verdouw PD (1987) Comparison of mackerel-oil and lard-fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am J Clin Nutr **46**: 258–266.
Herbert KE, Wills ED (1987) Platelet function and tissue lipid peroxidation in rats fed polyunsaturated fatty acids. Biochem Soc Transact **15**: 410–411.
Hess ML, Manson NH (1984) Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion damage. J Mol Cell Cardiol **16**: 969–985.
Hess ML, Okabe E, Kontos HA (1981) Proton and free oxygen radical interaction with the calcium transport system of cardiac sarcoplasmic reticulum. J Mol Cell Cardiol **13**: 767–772.
Hock CE, Holahan MA, Reibel DK (1987) Effect of dietary fish oil on myocardial phospholipids and myocardial ischemic damage. Am J Physiol **252**: H554–H560.
Jackson MJ, Jones DA, Edwards RHT (1983) Lipid peroxidation of skeletal muscle: an *in vitro* study. Biosci Rep **3**: 609–618.
Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR (1984) Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase. Circ Res **54**: 277–285.
Jones DP, Thor H, Smith MT, Jewell SA, Orrenius S (1983) Inhibition of ATP-dependent microsomal Ca^{2+} sequestration during oxidative stress and its prevention by glutathion. J Biol Chem **258**: 6390–6393.
Karmazyn M, Horackova M, Murphy MG (1987) Effects of cod liver oil on fatty-acid composition and calcium transport in isolated adult rat ventricular myocytes and on the response of isolated hearts to ischemia and reperfusion. Can J Physiol Pharmacol **65**: 201–209.
Kim MS, Akera T (1987) O_2 free radicals: cause of ischemia–reperfusion injury to cardiac Na^+-K^+-ATPase. Am J Physiol **252**: H252–H257.
Koster JF, Slee RG, Essed CE, Stam H (1985) Studies on cumene hydroperoxide-induced lipid peroxidation in isolated perfused rat heart. J Mol Cell Cardiol **17**: 701–708.
Kramer JH, Mak IT, Weglicki WB (1984) Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipid peroxidation. Circ Res **55**: 120–124.
Lamers JMJ, Stinis JT, Ruigrok TJC (1984) Biochemical properties of membranes isolated from calcium depleted rabbit hearts. Circ Res **54**: 217–226.
Lamers JMJ, Cysouw KJ, Verdouw PD (1985) Slow calcium channel blockers and calmodulin. Effect of felodipine, nifedipine, prenylamine and bepridil on cardiac sarcolemmal calcium pumping ATPase. Biochem Pharmacol **34**: 3837–3843.
Lamers JMJ, De Jonge-Stinis JT, Hülsmann WC, Verdouw PD (1986) Reduced *in vitro* ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial ischemia in anaesthetized pigs. J Mol Cell Cardiol **18**: 115–125.
Lamers JMJ, Hartog JM, Verdouw PD, Hülsmann WC (1987) Dietary fatty acids and myocardial function. Bas Res Cardiol **82** [Suppl. 1]: 209–221.
Lands WEM (1982) Control of prostaglandin biosynthesis. Progress Lipid Res **20**: 875–883.
Liedtke AJ, Mahar CQ, Ytrehus K, Mjos OD (1984) Estimates of free-radical production in rat and swine hearts: method and application of measuring malondialdehyde levels in fresh and frozen myocardium. Basic Res Cardiol **79**: 513–518.
Lowry OH, Rosebrough NJ, Fare AL, Randall RJ (1951) Protein measurement with the Folinphenol reagent. J Biol Chem **193**: 265–275.
Lucchesi BR, Mullane KM (1986) Leukocytes and ischemia-induced myocardial injury. Ann Rev Pharmacol Toxicol **26**: 201–224.
Manning AS, Coltart DJ, Hearse DJ (1984) Ischemia and reperfusion-induced arrhythmias in the rat: effects of xanthine oxidase inhibitors with allopurinol. Circ Res **55**: 545–548.
Meerson FZ, Kagan VE, Kozlov YP, Belkina IM, Arkhipenko YV (1982) The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. Basic Res Cardiol **77**: 465–485.
McCord JM (1985) Oxygen derived free radicals in postischemic tissue injury. New Engl J Med **312**: 159–163.
Montfoort A, van der Werf L, Hartog JM, Hugenholtz PG, Verdouw PD, Hülsmann WC, Lamers JMJ (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol **81**: 289–302.
Okabe E, Hess ML, Oyama M, Ito H (1983) Characterization of free radical-mediated damage of canine cardiac sarcoplasmic reticulum. Arch Biochem Biophys **225**: 164–177.

Rao PS, Cohen MV, Mueller HS (1983) Production of free radical and lipid peroxidation in early experimental myocardial ischemia. J Mol Cell Cardiol **15**: 713–716.
Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM (1978) The influence of dietary mackerel oil on the condition of organs and on blood lipid composition in the young growing pig. Am J Clin Nutr **31**: 2159–2166.
Sevanian A, Hochstein P (1985) Mechanisms and consequences of lipid peroxidation in biological systems. Ann Rev Nutr **5**: 365–390.
Shlafer M, Kane MF, Wiggins VY, Kirsh MM (1982) Possible role for cytotoxic oxygen metabolites in the pathogenesis of cardiac ischemic injury. Circulation **66** [Suppl. I]: I–85–92.
Stam H, Koster JF (1985) Fatty acid peroxidation in ischemia. In: *Prostaglandins and Other Eicosanoids in the Cardiovascular System*, edited by K Schrör. Basel, Karger, pp. 131–148.
Stewart JR, Crute SL, Loughlin V, Hess ML, Greenfield LJ (1985) Prevention of free radical-induced myocardial reperfusion injury with allopurinol. J Thorac Cardiovasc Surg **90**: 68–72.
Thiery JM, Seidel D (1987) Fish oil feeding results in an enhancement of cholesterol-induced atherosclerosis in rabbits. Atherosclerosis **63**: 53–56.
Tsuchida M, Miura T, Mizutari K, Mizutani K, Aibara K (1985) Fluorescent substances in mouse and human sera as a parameter of *in vivo* lipid peroxidation. Biochim Biophys Acta **834**: 196–204.
Verdouw PD, Wolffenbuttel BHR, Van der Giessen WJ (1983) Domestic pigs in the study of myocardial ischemia. Eur Heart J **4** [Suppl. C]: 61–67.
Verdouw PD, Hartog JM, Duncker DJ, Roth W, Saxena PR (1986) Cardiovascular profile of pimobendan, a benzimidazole-pyridazinone derivative with vasodilating and inotropic properties. Eur J Pharmacol **126**: 21–30.

CHAPTER 11

Does platelet aggregation play a role in the reduction in localized intimal proliferation in normolipidemic pigs with fixed coronary artery stenosis fed dietary fish oil?

J.M. Hartog [1], J.M.J. Lamers [2], C.E. Essed [1], W.P. Schalkwijk [1] and P.D. Verdouw [1]

[1] *Laboratory for Experimental Cardiology, Thoraxcenter, and* [2] *Department of Biochemistry I, Erasmus University Rotterdam, Rotterdam (The Netherlands)*

(Received 3 June, 1988)
(Revised, received 2 November, 1988)
(Accepted 7 November, 1988)

Summary

In order to investigate the effect of fish oil on intimal proliferation of coronary arteries with a fixed stenosis normolipidemic piglets received a basic diet to which either 9% (w/w) lard (L, n = 8) or 4.5% (w/w) lard and 4.5% (w/w) mackerel oil (ML, n = 8) was added for 4 months. Stenosis was applied by implanting a 4.0 × 2.0 mm (i.d.) Teflon constrictor around the left anterior descending coronary artery (LADCA) (o.d. 2.7 ± 0.1 mm). During the dietary period ADP-induced platelet aggregation in whole blood was higher in L than in ML. Partial replacement of 20 : 4 $n-6$ by 20 : 5 $n-3$ fatty acids in the platelet membranes of ML may have altered platelet aggregation by changes in eicosanoid synthesis. The plasma cholesterol and triglyceride levels did not change in L, but decreased in ML. At the end of the 4-month dietary period the animals were again anesthetized and regional myocardial perfusion (radioactive labelled microspheres) and systolic segment length shortening (SLS) were measured while the hearts were paced at 160 pulses/min. Perfusion and SLS of the non-LADCA nourished segment were similar for L and ML. However, transmural flow to the LADCA perfused myocardium was impaired in both groups, but the deficiency in endocardial perfusion was considerably larger in L than in ML, resulting in a larger loss of SLS in the former. Remote (2–3 cm from the site of the constrictor) luminal encroachment was minimal (< 2%) in both groups, but at the site of the constrictor there was significant encroachment in both groups which was higher in L (62 ± 7%) than in ML (11 ± 4%). It is thought that in these normolipidemic pigs the reduction in platelet aggregation may play a role in the smaller intimal proliferation of the fish oil-fed animals.

Key words: Fish oil; Platelet aggregation; Coronary artery perfusion; Plasma lipids; Coronary artery constriction; Intimal proliferation; Prostaglandin; (Pig)

Introduction

Recent clinical trials have stressed the importance of lowering plasma cholesterol in reducing

Correspondence to: P.D. Verdouw, PhD, Laboratory for Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

the risk of developing ischemic heart disease [1,2]. Experimental evidence suggests that eicosapentanoic (EPA) and docosahexanoic (DHA) acids lower blood cholesterol and triglyceride levels [3–8]. The high contents of these fatty acids in fatty marine fish may therefore explain the lower incidence of coronary heart disease in populations consuming these fish [9–12]. The slower development of atherosclerosis in fish oil-fed rhesus monkeys is consistent with this hypothesis [13].

EPA and DHA not only modify plasma lipids but they also affect platelet aggregation, blood viscosity and possibly arterial blood pressure and formation of prostaglandins and leukotrienes [14–18]. These effects are of interest because in 2 epidemiological studies consumption of fish reduced mortality from coronary artery disease without affecting cholesterol and triglyceride levels [10,11]. Moreover, the addition of 30 ml cod liver oil to atherogenic diets fed to pigs, reduced the severity of the atherosclerotic lesions without affecting serum lipids [9]. In this last study platelet fatty acid composition was altered, serum thomboxane B_2 levels reduced and the interaction of platelets with the abraded vessel wall inhibited.

These factors could account for the lower incidence of atherosclerosis in these animals. Furthermore, negative effects of dietary fish oil consumption have also been reported as cholesterol-induced atherosclerosis was more severe in rabbits when their standard diet was supplemented daily with 0.4 g EPA [20]. In these rabbits levels of plasma cholesterol did not change but those of plasma triglycerides tripled. It appears that the role of regular fish oil intake on the progression of atherosclerosis is still debatable. Moreover, if such an action exists it is questionable whether lowering of plasma lipid levels is the prime factor responsible. Therefore more information is needed on intimal thickening of the arterial wall caused by a combination of factors such as mechanical disruption of endothelium and mural thrombosis while changes in plasma lipids are minimal.

Several investigators have reported that partial constriction of a coronary artery leads to cyclic variations in blood flow due to platelet plug formation [21,22]. The combination of locally high blood flow and wall shear forces causes hemolysis and denudation of the vessel wall leading to primary and secondary platelet aggregation. The formation of mural thrombi is believed to initiate proliferation of smooth muscle cells migrating into the intimal layers [23]. In the present study we evaluated the effect of fish oil-induced changes on coronary artery proliferation in normolipidemic pigs with a chronic stenosis. Normolipidemic pigs were used to minimize the effects on plasma lipid composition.

Methods

Animals

Eight castrated male and 8 female weanling Yorkshire piglets (5 weeks of age; 7.7 ± 0.2 kg) were housed individually in slat-bottomed cages in temperature-controlled animal facilities and divided arbitrarily into 2 groups, each consisting of 4 castrated male and 4 female pigs.

Diets

Both groups of animals received the same basic diet (fat content $< 2\%$, w/w) to which either 9% lard (w/w; group L) or 4.5% mackerel oil and 4.5% lard (w/w; group ML) was added (Hope Farms, Woerden, The Netherlands). A consequence of the fat supplementation was that both EPA (8%, w/w) and DHA (5%, w/w) were present in the total fatty acid pool of the diet of ML, but absent in that of L. Full details of the composition of the diets have been described elsewhere [7,8,24].

Implantation of coronary artery constrictor

After the animals (now weighing 20 ± 1 kg (L) and 21 ± 1 kg (ML)) had been on their diets for 2 months, they were pretreated with intramuscular injections of 300 000 U procaine penicillin G and 300 000 U bezathine penicillin G (Duplocilline®, Gist Brocades, Delft, The Netherlands) and anesthetized with 30 mg/kg ketamine i.m. (Aescoket®, Aesculaap BV, Boxtel, The Netherlands), incubated and connected to a respirator for artificial ventilation with a mixture of oxygen and nitrous oxide (1 : 2) to which halothane (Fluothane®, Macclesfield, U.K.) was added. After the thorax was opened via an incision in the left third intercostal space and the proximal left anterior descending coronary artery was dissected free

over a length of 10 mm to place a 4.0 × 2.0 mm (i.d.) Teflon® ring around the vessel (o.d. 2.7 ± 0.1 mm). The diameter of the constrictor was such that the minimal narrowing of the lumen did not impede coronary blood supply at the time of placement. After closure of the thorax the animals were allowed to recover and the dietary regimen was continued for 2 more months.

Seven animals died during the post-implantation period. Three animals of L and 2 animals of ML died suddenly after 5–7 weeks. In each of these animals a thrombus was found in the LADCA (examination within 30 min after sudden death). One animal of L died of cardiac failure after 8 weeks and 1 animal (ML) was killed because of postoperative infection after 2 weeks. This last animal was excluded from further study.

Plasma lipids

Plasma levels of triglycerides and total cholesterol were determined in fasted animals before the start of the dietary period and 8 weeks later using methods described earlier [7,8].

Chemical analysis of platelet membranes

At the time of implantation of the constrictor blood (50 ml) was collected via a catheter located in the external jugular vein from 3 male animals of each group. Washed platelet suspensions were prepared from these samples, homogenized and the total phospholipid fractions extracted and separated from other lipids by thin-layer chromatography. Transmethylation, extraction of methyl esters and subsequent gas chromatographic separation have all been described [25].

Platelet aggregation of whole blood

A 5-ml heparinized (200 IU/ml) blood sample was collected from each animal during implantation of the constrictor. After a 15-min incubation time at room temperature, aggregation tests were performed either spontaneously or after the addition of collagen (1 μg/ml) and ADP (1 and 5 μM), for 10 min [26].

Cardiac function during the experimental period

Two-dimensional and M-mode echocardiograms were recorded with a 5 MHz transducer (21200 A Ultrasound Transducer, Hewlett-Packard, Palo Alto, CA, U.S.A.) before the start of the dietary period, at the time of placement of the constrictor and 4, 6 and 8 weeks (weeks 12, 14 and 16 of the dietary period, respectively) while the animals were sedated with ketamine (30 mg/kg i.m.). The images were recorded on videotape (Panasonic VHS-Pro, type AG-6200, Matsushita Electrical Industrial Co., Ltd., Japan). Three consecutive heart beats were selected at the end of the respiratory cycle. The M-mode recordings of the anterior and posterior left ventricular wall of these beats were projected on a videoscreen and the end-diastolic (EDT) and end-systolic wall thickness (EST) were determined using a digital tablet (Summagraphics MM 1201, Summagraphics Co., Fairfield, CT, U.S.A.). The systolic wall thickening was calculated as:

$$SWT(\%) = (EST - EDT)/EDT \times 100.$$

Hemodynamic evaluation at the end of the experimental period

Two months after placement of the constrictor (week 16 of the dietary period), the surviving animals were anesthetized and instrumented using standard techniques [27]. Regional segment length changes were measured by sonomicrometry (Triton Technology, San Diego, CA, U.S.A.) by implanting one pair of ultrasonic crystals (5 MHz) in the area perfused by the left anterior descending coronary artery and another pair in the adjacent myocardium. From the tracings regional systolic segment length shortening (SLS) was calculated as described earlier [28]. Regional myocardial blood flows were determined with radioactive labelled microspheres using the reference sample technique [29]. To this end the left atrium was cannulated for the injection of a batch (2×10^6) of microspheres (15 ± 1 μm (SD)) which were labelled with ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Se (NEN Chemicals GmbH, Dreieich, F.R.G.). A pacing catheter was positioned in the coronary sinus. After the preparation had been stable for at least 30 min the heart was paced at 160 pulses/min. Five minutes later all systemic hemodynamic and regional myocardial function data were recorded and a batch of microspheres injected.

In order to determine what fraction of the myocardial blood flow in the post-stenotic myocardium was due to collateral blood flow the LADCA was clamped distal to the site of the constrictor and another batch of microspheres was injected. The flow data obtained with this last measurement were considered to be the collateral fractions. After this last measurement the animals were killed, the hearts excised and treated as described earlier before radioactivity was counted in a gamma-scintillation counter (Packard, model 5986) equipped with a multichannel pulse height analyzer (Conrac) using suitable windows for discriminating the different isotopes used [29].

Quantification of stenosis

The proximal left anterior descending coronary artery, including the constrictor, was excised and put in 10% formaldehyde within 30 min after the animals had died suddenly or been killed. Tissue was prepared by dissecting through the constrictor, such that the contours of the vessel at the site of constriction were visualized. Sections were cut at 7 μm thickness and stained with hematoxylin and elastic tissue stains. The sections were projected on a videoscreen and the outer contours, internal elastic lamina and endothelial lining of the vessels were traced. The intimal lesion area was calculated as the area between the endothelial lining of the lumen and the internal elastic lamina. The luminal encroachment was calculated by dividing the intimal lesion area by the surface of the area surrounded by the internal elastic lamina. The media was calculated as the area between the internal elastic lamina and the outer vessel contours using the method described by Weiner et al. [19].

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). The data of the 2 groups are compared using Student's *t*-test and analysis of variance. Statistical significance is accepted at $P < 0.05$ (two-tailed).

Results

Plasma lipids

Cholesterol (from 2.56 ± 0.42 to 2.33 ± 0.10 mM) and triglycerides (from 0.64 ± 0.10 to 0.68 ± 0.10 mM) did not change significantly in L during the first 2 months of the dietary period. In ML, however, cholesterol decreased from 2.48 ± 0.28 to 1.69 ± 0.11 mM and triglycerides from 0.52 ± 0.04 to 0.30 ± 0.04 mM (both $P < 0.05$). In an earlier study we have shown that continuation of the diets for another 8 weeks does not further affect plasma lipid levels [7].

Platelet fatty acid composition

Table 1 shows the fatty acid composition of the total phospholipid fraction of the platelets from L and ML at the time of implantation of the constrictor. Partial replacement of 20:4 $n-6$ by 20:5 $n-3$ fatty acid had occurred in ML analogous to earlier findings [24].

Platelet aggregation

Fig. 1 illustrates the platelet aggregation data in the blood samples collected after 2 months. No spontaneous aggregation occurred. In M and L, the maximal aggregation induced by collagen (1 μg/ml) was not significantly different. However, the aggregation induced by ADP (1 and 5 μM) in L ($29 \pm 7\%$ and $81 \pm 5\%$ of maximal aggregation, respectively) was significantly higher than in ML ($8 \pm 2\%$ and $67 \pm 13\%$ of maximal aggregation,

TABLE 1

FATTY ACID COMPOSITION (mol%) OF PLATELET HOMOGENATE PHOSPHOLIPIDS IN PIGS FED A 9% LARD (L) OR A 4.5% MACKEREL OIL AND 4.5% LARD (ML) DIET FOR 2 MONTHS

n = number of animals; DMA = dimethylated acetals.

Fatty acid	L (n = 3)	ML (n = 3)
14:0	0.7±0.1	1.2±0.2
DMA 16:0	1.3±0.2	2.0±0.3
16:0	20.0±0.2	22.3±0.3
DMA 18:0	1.7±0.7	0.8±0.1
18:0	18.1±2.0	16.0±0.3
18:1 $n-9$	22.8±1.3	21.0±0.8
18:2 $n-6$	7.7±0.9	7.0±0.1
20:4 $n-6$	16.4±2.0	8.1±0.3 *
20:5 $n-3$	0.4±0.2	10.6±0.3 *
22:4 $n-6$	2.4±0.2	1.4±0.4 *
22:5 $n-3$	0.9±0.1	1.9±0.3 *
22:6 $n-3$	0.8±0.2	1.3±0.2
others	6.8±1.0	6.7±1.5

* $P < 0.05$ vs. 9% lard fed animals.

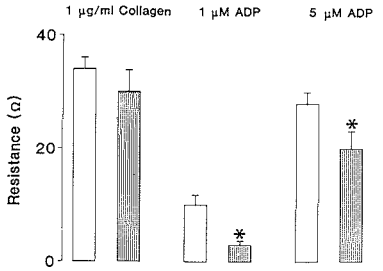

Fig. 1. Platelet aggregation of lard (□, n = 6) and mackerel oil (▨, n = 5) fed animals. The data were obtained after an 8-week dietary period. Data are presented as mean ± SEM. * $P < 0.05$ vs. data of lard fed animals.

respectively). For each pig the aggregation induced by ADP is based on the maximal value found with 1 μg/ml collagen.

Regional myocardial function during the dietary period

Before implantation of the constrictor the myocardial wall thickness (MWT) at end-diastole (EDT) as well as end-systole (EST) of both the posterior and the anterior wall increased with time independent of the diet (Fig. 2). These changes

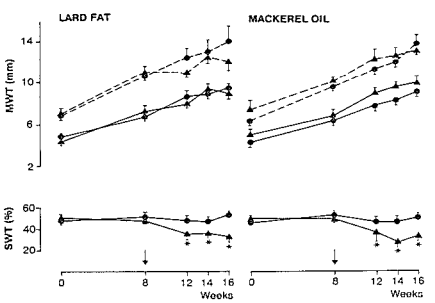

Fig. 2. The end-diastolic (———) and end-systolic (- - - - - -) myocardial wall thickness (MWT) and systolic wall thickening (SWT) of the anterior (▲) and posterior (●) wall of lard (L, n = 8) and mackerel oil (ML, n = 8) fed pigs with a chronically implanted 2.0 mm constrictor around the left anterior descending coronary artery during the dietary period. The arrow denotes the time of implantation of the constrictor. The last measurements in L were obtained in 4 and in ML in 5 animals due to sudden death after implantation of the constrictor. * $P < 0.05$ vs. data of posterior wall.

had no effect on systolic wall thickening (SWT). Four weeks after implantation percentage systolic wall thickening of the anterior segment had declined to 72 ± 12% of the pre-implantation value in L and to 72 ± 13% in ML. At the end of the dietary period there was a further decrease to 62 ± 9% of the pre-implantation value for L and 63 ± 7% for ML.

Heart rates of the slightly sedated but still spontaneously breathing animals of L were 166 ± 10, 142 ± 11 and 110 ± 9 beats/min before and after 8 and 16 weeks into the dietary period, respectively. The heart rates of the animals belonging to ML were very similar as values of 161 ± 11, 129 ± 9 and 108 ± 12 beats/min, respectively, were recorded.

Hemodynamics at the end of the experimental period

To eliminate the effects of heart rate on regional myocardial performance all hearts were paced at 160 pulses/min. Mean arterial blood pressure and the peak rate of rise in left ventricular pressure were similar for both groups but cardiac output was 23% lower in L than in ML and left ventricular filling pressure 33% higher in L than in ML (both $P < 0.05$) (Fig. 3).

Transmural perfusion and function of the non-ischemic posterior myocardial wall segments of L and ML did not differ and the data agree closely with those reported for conscious pigs [26,27].

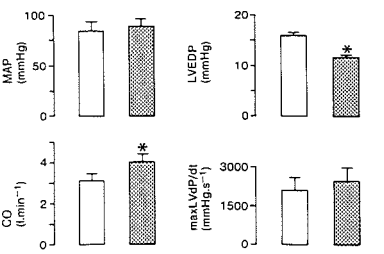

Fig. 3. Systemic hemodynamics of lard (□, n = 4) and mackerel oil (▨, n = 5) fed pigs with a constrictor implanted around the left anterior descending coronary artery. The measurements were made while the animals were anesthetized and the hearts paced at 160 pulses/min. MAP = mean arterial blood pressure; LVEDP = left ventricular end-diastolic pressure; CO = cardiac output; maxLVdP/dt = maximum rate of rise in left ventricular pressure. Data are presented as mean ± SEM. * $P < 0.05$ vs. data in lard fed animals.

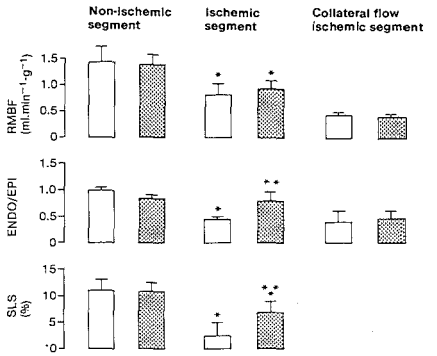

Fig. 4. Regional myocardial blood flow (RMBF) and its distribution over the subendocardial and subepicardial layers (ENDO/EPI) and segment length shortening (SLS) of lard (□, n = 4) and mackerel oil (▨, n = 5) fed pigs. For further details see legend of Fig. 2. * $P < 0.05$ vs. data of non-ischemic segment of same dietary group. ** $P < 0.05$ vs. data of lard fed group.

Transmural perfusion of the anterior wall segments, which were perfused by the left anterior descending coronary artery distal to the constrictor, was also very similar for both groups, but considerably less than for the non-ischemic segments (Fig. 4). In spite of similar transmural perfusion values of these post-stenotic ischemic segments a striking difference existed between the distribution of flow over the subendo- and subepicardial layers. In L the decrease in perfusion af-

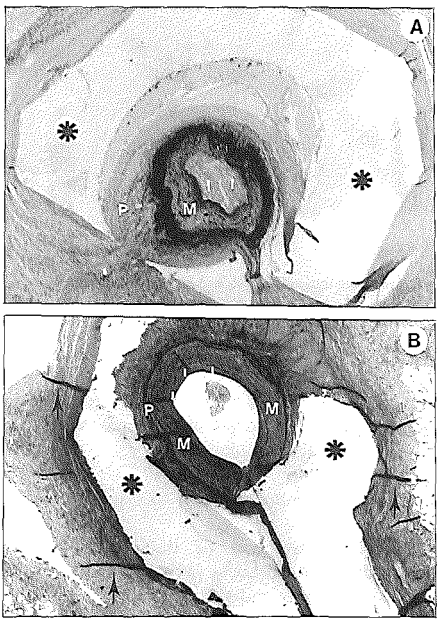

Fig. 5. Cross sections of the left anterior descending coronary artery taken at the site of chronically implanted constrictor of lard (A) and mackerel oil (B) fed pigs. Sections were cut at 7 μm thickness and stained with elastic tissue stains. Arrows indicate staining artifacts due to cutting through the Teflon constrictor. Parts of the constrictor are visible as indicated (*). The location of intimal (I) and medial layers (M) are indicated. P = periadventitial tissue.

TABLE 2

LUMINAL ENCROACHMENT AT THE SITE OF THE CHRONICALLY IMPLANTED CONSTRICTOR IN THE LEFT ANTERIOR DESCENDING CORONARY ARTERY OF 9% LARD (L) AND 4.5% MACKEREL OIL PLUS 4.5% LARD (ML) FED PIGS

	L (n = 8)		ML (n = 7)	
	Mean ± SEM	Range	Mean ± SEM	Range
Cross-sectional area (mm^2)				
Vessel	2.89 ± 0.10	2.22–3.14	2.70 ± 0.13	2.22–3.14
Lumen	0.26 ± 0.06	0.05–0.55	0.54 ± 0.13 *	0.18–0.99
Media	2.21 ± 0.14	1.41–2.68	2.12 ± 0.11	1.78–2.58
Intima lesion	0.43 ± 0.07	0.16–0.73	0.05 ± 0.02 *	0.01–0.10
Encroachment (%)				
Lumen	62 ± 7	37–93	11 ± 4 *	1–26

* $P < 0.05$ vs. 9% lard fed animals; n = number of animals.

fected in particular the subendocardial layers, whereas in ML the flow reduction affected both layers equally. The systolic segment length shortening in ML exceeded that of L (Fig. 4). When perfusion measurements of the post-stenotic zones of L and ML were repeated after the left anterior descending coronary artery was completely occluded distally to the site of the stenosis the microsphere data revealed that the residual collateral blood flow was similar for both myocardial zones (Fig. 4).

Luminal encroachment at the site of stenosis

Luminal encroachment was minimal 2–3 cm distal to the constrictor ($1.8 \pm 0.3\%$ for L and $1.7 \pm 0.2\%$ for ML). However, all animals showed significant intimal proliferation at the site of the constrictor (Fig. 5). Luminal encroachment was higher in L ($62 \pm 7\%$) than in ML ($11 \pm 4\%$) due to a marked difference in the cross-sectional area of the intimal lesion (Table 2).

Discussion

Critical interactions between four types of cells – monocytes, platelets, endothelium and smooth muscle cells – are believed to be involved in the development of atherosclerosis [30]. Each of the interacting cells can produce growth factors and these contribute to the characteristic cellular proliferation of the developing atheroma. Endothelium of hyperlipidemic animals permits monocyte adhesion and migration into the vessel wall after which these cells assume the shape of foam cells in fatty streaks. They may also promote growth factor formation in the endothelial cells [23,31]. Platelets, that are part of the thrombi which initially cover the intimal defects, secrete granule contents containing platelet-derived growth factor. The latter stimulates smooth muscle cell migration from the media to the intima [30]. Several factors – hyperlipidemic-induced changes in membrane viscosity, increased wall shear forces, wall stress and hypertension – contribute to the development of endothelial injury. The prostanoid metabolism is probably involved in the molecular basis of many of these events [3,5,6].

The effects of dietary fish oil upon atherosclerosis may be mediated by (1) reduction of plasma cholesterol, (2) alteration of prostanoid metabolism (e.g., formation of leukotrienes by monocytes and endothelial cells), (3) production of platelet cell thromboxane A_2 and endothelial cell prostacyclin, (4) reduction of viscosity due to altered membrane fluidity of the erythrocytes, (5) production of endothelium-dependent relaxation, (6) decreased neutrophil aggregation, and (7) reduced accumulation of mast cells in the coronary arteries [32]. In the present study, we therefore used a chronic stenosis of the coronary artery in which repeated adherence and degranulation of platelets over the 2-month period is probably the major factor in producing mitosis of the smooth muscle cells [21–23].

There are several models for the study of platelet–vessel wall interaction, such as the aorta loop [33], intra-arterial protheses [34], and electrically-induced arterial thrombosis [35]. Arterial constriction (40–95% luminal narrowing), either by a plastic ring [21,22] or a thread [36], has been used before, but these studies were all short lasting. In the study by Gertz et al. [36] endothelial damage was more prominently present on the proximal than on the distal slope of the vessel immediately adjacent to the thread. At the point of maximal constriction, platelets were attached to subendothelial tissues and microthrombi (platelets with some fibrin and erythrocytes) protruded into the vascular lumen. It is therefore not surprising that we found a marked intimal proliferation at the site of the constrictor. As we did not investigate intimal proliferation at the vessel wall immediately proximally and distally to the constrictor, we have no information about local differences in luminal encroachment.

Luminal encroachment was higher in the lard than in the fish oil fed animals. Because the constrictor produced a fixed stenosis it is unlikely that factors such as endothelial-dependent relaxations in response to released platelet products, increased neutrophil aggregation and accumulation of mast cells in the coronary arteries and release of leukotrienes, all affecting vascular permeability and coronary artery constriction, are involved.

Fish oil feeding slightly reduced plasma lipid levels. However, because we used young normolipidemic pigs, known for their low plasma lipid levels, it is unlikely that this played a large role in

the modification of the degree of intimal proliferation produced by fish oil. This assumption is further supported by the findings that fish oil suppressed the development of atherosclerosis in pigs fed atherogenic (high cholesterol) diets without affecting plasma lipids. In that study [19] platelet fatty acid composition was altered, serum thromboxane B_2 levels reduced, platelet aggregation and the interaction of platelet with the abraded vessel wall inhibited. We also demonstrated that coronary venous plasma concentrations of prostaglandin derivatives of thromboxane A_2 and prostacyclin were markedly reduced by dietary fish oil which was in agreement with the observed reduction in 20 : 4 $n-6$ content and the increase in 20 : 5 $n-3$ content in platelet membranes [24]. In the present study we found again a partial replacement of arachidonic acid for EPA in the total phospholipid fraction of platelet membranes, which points towards similar changes in prostanoid concentrations. The reduction in ADP-induced platelet aggregation in whole blood (Fig. 1) is in agreement with such alterations in thromboxane B_2 levels.

In both groups wall thickness at the end of diastole of the posterior wall increased gradually during the 4 months. The increase in myocardial muscle mass and the lower heart rate as the animals grow older may be responsible for this observation. There was no change in percentage systolic wall thickening because the wall thickness at end-systole also increased. The changes in anterior wall thickness were parallel to those in the posterior wall before implantation of the constrictor, but thereafter percentage and absolute systolic wall thickening became less than that of the posterior wall. Radioactive microspheres and regional systolic segment length shortening were used to determine whether proliferation of the endothelial layer also affected myocardial blood flow and function. When the hearts were stressed by raising the heart rate to 160 beats/min the myocardium, especially the subendocardium of L, nourished by the partially constricted coronary artery was hypoperfused and did not function normally. The reason for the apparent beneficial effect of fish oil on the distribution of myocardial blood flow cannot be explained by differences in systemic hemodynamic variables such as heart rate, left ventricular filling pressure and arterial blood pressure [37]. Further investigations are needed to determine whether changes in viscosity may have played a role.

Since subendocardial blood flow is the major determinant of regional function [38,39] it is not surprising that segment length shortening was more severely impaired in L than in ML. The microsphere data also revealed that placement of the constrictor led to the development of functional collaterals which is consistent with earlier observations [40].

We conclude that in pigs the chronic stenosis resulted in intimal proliferation at the site of the constrictor but that the diet determined the extent of this encroachment. Inhibition of platelet aggregation may be responsible for the lower amount of intimal proliferation in the mackerel oil fed pigs.

Acknowledgements

The assistance of Henny Vegter in the preparation of this manuscript is gratefully acknowledged. This study was supported by a grant 86–086 from the Netherlands Heart Foundation.

References

1 The Lipid Research Clinics Program: Research Clinics Coronary Primary Prevention Trial. Results I. Reduction in incidence of coronary heart disease. J. Am. Med. Assoc., 251 (1984) 351.
2 The Lipid Research Clinics Program: The Lipid Research Clinics Coronary Primary Prevention Trial. Results II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. J. Am. Med. Assoc., 251 (1984) 365.
3 Metha, J., Lopez, L.M. and Wargorich, T., Eicosapentanoic acid: its relevance in atherosclerosis and coronary artery disease. Am. J. Cardiol., 59 (1987) 155.
4 Herold, P.M. and Kinsella, J.E., Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. Am. J. Clin. Nutr., 43 (1986) 566.
5 Norum, K.R. and Drevon, C.A., Dietary $n-3$ fatty acids and cardiovascular diseases. Arteriosclerosis, 6 (1986) 352.
6 Goodnight, S.H., Harris, W.J., Connor, W.E. and Illingworth, R.D., Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. Arteriosclerosis, 2 (1982) 87.
7 Hartog, J.M., Verdouw, P.D., Klompe, M. and Lamers, J.M.J., Dietary mackerel oil in pigs: effect on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. J. Nutr., 117 (1987) 1371.

8 Hartog, J.M., Lamers, J.M.J., Montfoort, A., Becker, A.E., Klompe, M., Morse, H., ten Cate, F.J., van der Werf, L., Hülsmann, W.C., Hugenholtz, P.G. and Verdouw, P.D., Comparison of the effects of mackerel oil and lard fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am. J. Clin. Nutr., 46 (1987) 258.

9 Dyerberg, J. and Bang, H.O., A hypothesis on the development of acute myocardial infarction in Greenlanders. Scand. J. Clin. Lab. Invest., 42 (1982) 7.

10 Kagawa, Y., Nishizawa, M., Suzuki, M., Miyataka, T., Hamamoto, T., Goto, K., Montonaga, E., Izumikawa, H., Hirata, H. and Ebihara, A., Eicosapolyenoic acid of serum lipids of Japanese islanders with low incidence of cardiovascular diseases. J. Nutr. Sci. Vitaminol., 28 (1982) 441.

11 Kromhout, D., Bosschieter, E.B. and de Lezenne Coulander, C., The inverse relation between fish consumption and 20 year mortality from coronary heart disease. N. Engl. J. Med., 312 (1985) 1205.

12 Dyerberg, J., Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. Nutr. Rev., 44 (1986) 125.

13 Daris, H.R., Bridenstine, R.T., Vesselinovitch, D. and Wissler, R.W., Fish-oil inhibits development of atherosclerosis in rhesus monkeys. Arteriosclerosis, 7 (1987) 441.

14 Brox, J.H., Killie, J.E., Gunne, S. and Nørdoy, A., The effect of cod-liver oil and corn oil on platelets and vessel wall in man. Thromb. Haemost., 46 (1981) 604.

15 Goodnight, S.H., Harris, W.S. and Connor, W.E., The effect of dietary ω-3 fatty acids on platelet composition and function in man: a prospective, controlled study. Blood, 58 (1981) 880.

16 Brox, J.H., Killie, J.E., Osterud, B., Holme, S. and Nørdoy, A., Effects of cod-liver oil on platelets and coagulation in familial hypercholesterolemia (type IIA). Acta Med. Scand., 213 (1983) 137.

17 Sanders, T.A.B. and Hochland, M.C., A comparison of the influence on plasma lipids and platelet function of supplements of ω-3 and ω-6 polyunsaturated fatty acids. Br. J. Nutr., 50 (1983) 521.

18 Driss, F., Vericel, E., Lagarde, M., Dechavanne, M. and Darcet, P., Inhibition of platelet aggregation and thromboxane synthesis after intake of small amount of eicosapentaenoic acid. Thromb. Res., 36 (1984) 389.

19 Weiner, B.H., Ockene, J.S., Levine, P.H., Cuénoud, H.F., Fisher, M., Johnson, B.F., Daoud, A.S., Jarmolych, J., Hosmer, D., Johnson, M.H., Natala, A., Vaudreuil, C. and Hoogasian, J.J., Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. N. Engl. J. Med., 315 (1986) 841.

20 Thiery, J. and Seidel, D., Fish-oil feeding results in an enhancement of cholesterol-induced atherosclerosis in rabbits. Atherosclerosis, 63 (1987) 53.

21 Folts, J.D., Crowell, E.D. and Rowe, G.G., Platelet aggregation in partially obstructed vessels and its elimination with aspirin. Circulation, 54 (1976) 365.

22 Aiken, J.W., Gorman, R.R. and Shebuski, R.J., Prevention of blockage of partially obstructed coronary arteries with prostacyclin correlates with inhibition of platelet aggregation. Prostaglandins, 17 (1979) 483.

23 Ross, R., Glomset, J.A. and Kariya, B., A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA, 71 (1974) 1207.

24 Hartog, J.M., Lamers, J.M.J., Achterberg, P.W., Van Heuven-Nolsen, D., Nijkamp, F.P. and Verdouw, P.D. The effects of dietary mackerel oil on the recovery of cardiac function after acute ischemic event in the pig. Bas. Res. Cardiol., 82 Suppl. 1 (1987) 223.

25 Montfoort, A., Van der Werf, L., Hartog, J.M., Hugenholtz, P.G., Verdouw, P.D., Hülsmann, W.C. and Lamers, J.M.J., The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidyl-ethanolamine. Bas. Res. Cardiol., 81 (1986) 289.

26 Cardinal, D.C. and Flower, R.J., The electronic aggregometer: a novel device for assessing platelet behaviour in blood. J. Pharmacol. Methods, 3 (1980) 135.

27 Verdouw, P.D., Sassen, L.M.A., Duncker, D.J., Schmeets, I.O.L., Rensen, R.J. and Saxena, P.R., Nicorandil-induced changes in the distribution of cardiac output and coronary blood flow in pigs. Naunyn-Schmiedeberg's Arch. Pharmacol., 336 (1987) 352.

28 Duncker, D.J., Saxena, P.R. and Verdouw, P.D., The effects of nisoldipine alone and in combination with beta-adrenoceptor blockade on systemic haemodynamics and myocardial performance in conscious pigs. Eur. Heart J., 8 (1987) 1332.

29 Saxena, P.R. and Verdouw, P.D., 5-Carboxamide tryptamine, a compound with high affinity for 5-hydroxytryptamine$_1$-binding sites, dilates arterioles and constricts arteriovenous anastomoses. Br. J. Pharmacol., 84 (1985) 533.

30 Ross, R., The pathogenesis of atherosclerosis. An update. N. Engl. J. Med., 314 (1986) 488.

31 Schwartz, S.M., Cellular mechanism in atherosclerosis. Theories and therapies. Ann. NY. Acad. Sci., 454 (1986) 320.

32 Yagonisawa, A. and Lefer, A.M., Vasoactive effects of eicosapentanoic acid on isolated vascular smooth muscle. Bas. Res. Cardiol., 82 (1987) 197.

33 Hornstra, G. and Vendelmans-Starrenburg, A., Induction of experimental arterial occlusive thrombi in rats. Atherosclerosis, 17 (1973) 369.

34 Pearl, F. and Friedman, M., Experimental coronary thromboatherosclerosis in the dog. Arch. Pathol., 77 (1964) 370.

35 van der Giessen, W.J., Mooi, W., Rutteman, A.M., van Vliet, H.H.D.M., Slager, C.J. and Verdouw, P.D., A new model for coronary thrombosis in the pig: preliminary results with thrombolysis. Eur. Heart J., 4 (1983) 69.

36 Gertz, S.D., Uretsky, G., Wajnberg, R.S., Navot, N., Gotsman, M.S., Endothelial cell damage and thrombus formation after partial arterial constriction: relevance to the role of coronary artery spasm in the pathogenesis of myocardial infarction. Circulation, 63 (1981) 476.

37 Feigl, E.O., Coronary physiology. Physiol. Rev., 63 (1983) 1.

38 Gallagher, K.P., Kumuda, T., Koziol, J.A., McKnown, M.D., Kemper, W.S. and Ross, J. Jr., Significance of regional wall thickening abnormalities relative to transmural myocardial perfusion in anesthetized dogs. Circulation, 62 (1980) 1266.

39 Vatner, S.F., Correlation between acute reductions in myocardial blood flow and function in conscious dogs. Circ. Res., 47 (1980) 201.

40 Millard, R.W., Induction of functional collaterals in the swine heart. Bas. Res. Cardiol., 76 (1981) 468.

CHAPTER 12

ATHEROSCLEROSIS, A LITERATURE REVIEW

12.1 Historical notes

Although atherosclerosis is considered to be a "welfare" disease and is the largest single cause of death in Western society [1], it was also common in the antiquity. In the beginning of this century Shattock (1909) examined microscopic sections of the aorta of King Menephtah, the reputed Pharaoh of the Exodes and found senile calcification of the aorta [2]. A few years later Ruffer reported calcified arterial lesions in several other mummies [3]. The first written report, however, dates already from over 400 years ago as Fallopius (1575) described a "degeneration of arteries into bone". Over a century later Cowper (1700) was probably the first to remark that the changes in the arterial wall could affect blood flow as he noted that "the passage of blood is impeded in thickened arteries" [4]. Senac (1749) and Morgagni (1761) described coronary artery lesions in individuals with cardiac complaints [5]. That these two phenomena were indeed related was established by Edward Jenner some 10 years later [5].

Rokitansky (1844) thought that blood constituents were deposited on the vessel wall [6], however, Risse (1933) and Virchow (1956) stated that the material was deposited under the subendothelial surface [7, 8].

Albrecht von Haller (1755) applied the term "atheroma" to the arterial lesions [4]. Crawford (1960) has defined atherosclerosis as "the widely prevalent arterial lesion characterized by patchy thickening of the intima, the thickenings comprising accumulations of fat and layers of collagen like fibres both being present in widely varying proportions" [9]. Pickering (1963) literally translated the term atherosclerosis directly from the Greek as "porridge-like hardness" [10]. It has become clear that atherosclerotic process affects all layers of the vessel wall and therefore Adams (1964) defined atherosclerosis as "a multifocal proliferative and degenerative condition that affects the lumen, intima and inner part of the media both of large elastic arteries and certain muscular arteries in the senescent individual" [11].

12.2 Risk factors

Most epidemiological surveys have shown significant correlations between plasma total cholesterol and the incidence of coronary heart disease [12-16]. Coronary heart

disease proves to be more common in the population of countries with mean plasma cholesterol exceeding 5 mmol/l (e.g. central and northern Europe, USA and Australia [12, 14]. On the other hand atherosclerotic disease is rare in countries where average cholesterol levels are lower than 4 mmol/l (e.g. in the Orient) [12, 17, 18]. In the Multiple Risk Factor Intervention Trial no such treshold was found, and the relationship was curvilinear [19]. Pathological studies show a relationship between the grade of atherosclerosis and the plasma cholesterol level [20].

In man most of the plasma cholesterol is carried in the low density lipoprotein (LDL), and that in particular high levels of LDL-cholesterol may be atherogenic has been demonstrated most dramatically in patients with familial hypercholesterolemic LDL receptor deficiency [21, 22].

The high density lipoprotein (HDL), another major carrier of cholesterol, plays an important role in the transport of cholesterol from peripheral tissues to the liver where it can be catabolized. Therefore, it is believed that an inverse relationship exists between HDL-cholesterol and atherosclerosis [14]. Most retrospective studies report a univariate relation between plasma triglyceride level and coronary heart disease [23-25], but plasma triglyceride levels appear to be of limited value as an independent predictor of coronary heart disease [26]. However, when the plasma cholesterol level is below 5.7 mmol/l, plasma triglyceride level becomes an independent predictor of risk, after adjustment for all other risk factors [27].

Platelet activity may be another factor in the process of atherosclerosis. When the endothelium is injured platelets interact with or adhere to the subendothelial connective tissue to release their granulae contents [28]. Platelets contain at least two mitogens, epidermal growth factor [29, 30] and platelet derived growth factor [31-34], and other releasable platelet constituents such as platelet factor 4, beta-thromboglobuline and products of the lipoxygenase pathway [34]. Enhanced aggregability of blood platelets by smoking, cathecholamines, turbulence and hemolysis will lead to degranulation of the platelets releasing the aforementioned factors.

Other factors which also predispose for atherosclerosis are: age, male sex, race, hypertension [19, 20, 35-39], smoking [40-43], diabetes mellitus [38, 44], physical inactivity [45-48], stress and behaviour [49, 50], increased blood viscosity [51, 52], elevated thromboxane synthesis [53, 54], high arachidonic acid levels combined with low eicosapentaenoic and linoleic acid levels of platelet membrane phospholipids [54, 55], and low linoleic acid levels of adipose tissue [55].

12.3 Current views of the pathogenesis of atherosclerosis

Brown and Goldstein have modified the Virchow theory into the lipid infiltration theory [22, 56, 57]. The lipid content of atherosclerotic lesions is mainly composed of cholesterol, cholesteryl esters and phospholipids. Cholesteryl esters present in early atherosclerotic lesions are located within foam cells [58-60]. These foam cells

originate from macrophages and smooth muscle cells [61-63]. LDL binds to the LDL-receptors or scavenger receptors and cholesteryl esters are accumulated in the foam cells, leading to progression of the atherosclerotic lesion [22, 64-70]. As the lesion grows into the atherosclerotic plaque, a fissure in the latter leads to hemorrhage which in turn provokes rapid (further) aggregation of platelets and thrombosis [71].

Ross has postulated that endothelial injury leads to leakage of serum components into the subendothelial tissue and subsequent cellular responses [64, 72]. Platelet aggregation with release of prostanoids and mitogenic factors will be initiated, leading to migration and proliferation of smooth muscle cells [28-34, 73-75]. This sequence of events will result in the eventual formation of an atherosclerotic plaque.

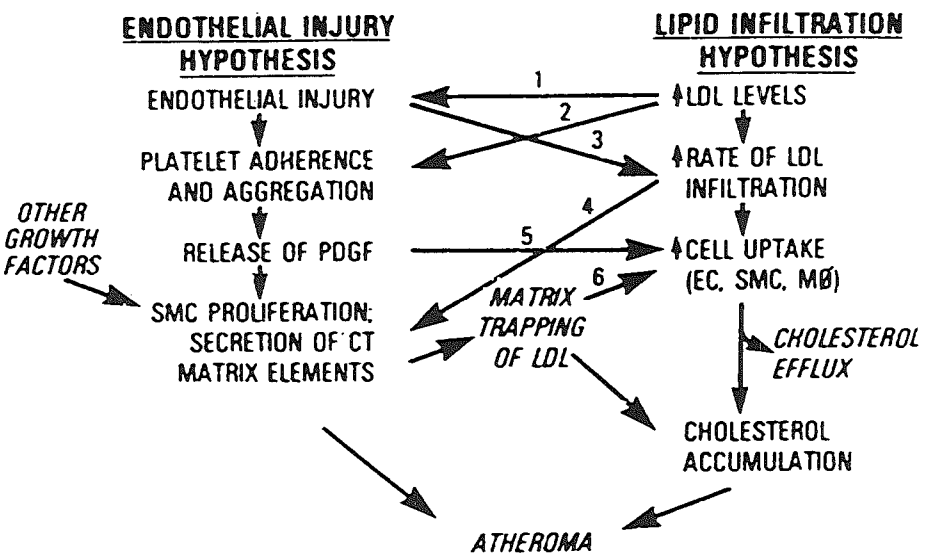

Figure 1. A proposed unified hypothesis linking the endothelial injury "school" and the lipid infiltration "school". See text. With permission from Steinberg (108).

The combination of the lipid infiltration and endothelial injury-hypothesis, enables us to best understand the complex processes in atherosclerosis [76]. There may be at least six interactions between the two theories (*Figure 1*):

1. Elevated LDL levels may damage endothelial cells [64, 77, 78]. The lipid infiltration is accompanied by growth factors released from the different cells [72].

2. Hyperlipoproteinemia has been reported to exert a proaggregatory action on blood platelets [79-81], which might initiate the release of platelet derived growth factor (PDGF).
3. Injury to endothelium will increase the infiltration rate of lipoproteins, cellular uptake and degradation at any LDL plasma level [82].
4. High LDL plasma levels can stimulate the growth of smooth muscle cells [83].
5. Growth factors increase the expression of the LDL receptors on smooth muscle cells, thereby increasing the rate of lipid uptake [84].
6. Stimulation of smooth muscle cell growth may cause the deposition of large amounts of certain intercellular matrix materials, such as glycosaminoglycans which trap LDL in the subintimal space [85].

As shown in *Figure 1* there are several input channels that converge to what may be a common final path. The severity of the atherosclerotic lesion may depend on how many of these independent input channels are operating and on what level [76]. Thus, the process of atherosclerosis is multicausal and cannot be defined in a simple model.

12.4 Possible effects of dietary n-3 PUFA's on atherosclerosis

In the preceeding chapters we have shown that n-3 PUFA's possess several actions which may interfere in the atherosclerotic process (*figure 1*) as fish oil has been shown to:

1. lower plasmacholesterol and plasmatriglyceride (chapter 4, 5 and 6)
2. shift the PGI/TXA ratio to anti-aggregatory (chapter 8 and 9)
3. decrease platelet aggregation and intimal proliferation (chapter 11)

In the next chapter we therefore studied the effect of dietary fish oil on regression of atherosclerosis in swine.

12.5 Induction and regression of experimental atherosclerosis

Swine develop atherosclerotic lesions (*Figures 2 and 3*) that closely resemble the arterial lesions in human beings with the first early lesions being found at 6 months of age [86, 87]. Over the years they develop advanced lesions with complications [88, 89].

Atherosclerosis in swine can be markedly accelerated and intensified by feeding atherogenic diets with 2% or more cholesterol and bile salts. Under these conditions the first lesions are seen after 7 weeks and develop into impressive fibrous plaques within 6 months. These lesions also resemble human atherosclerotic lesions [90], but complications like arterial occlusion or thrombosis are not seen within the first year [91]. Endothelial damage, induced by balloon abrasion, ionizing radiation or

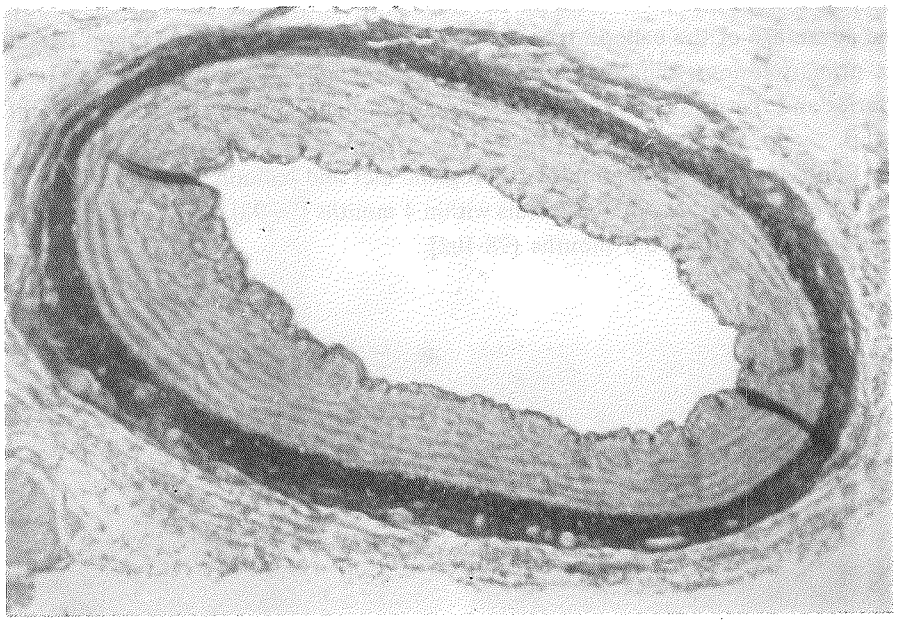

Figure 2. Transverse section of a pig coronary artery stained with elastic tissue stains, showing an early atherosclerotic lesion.

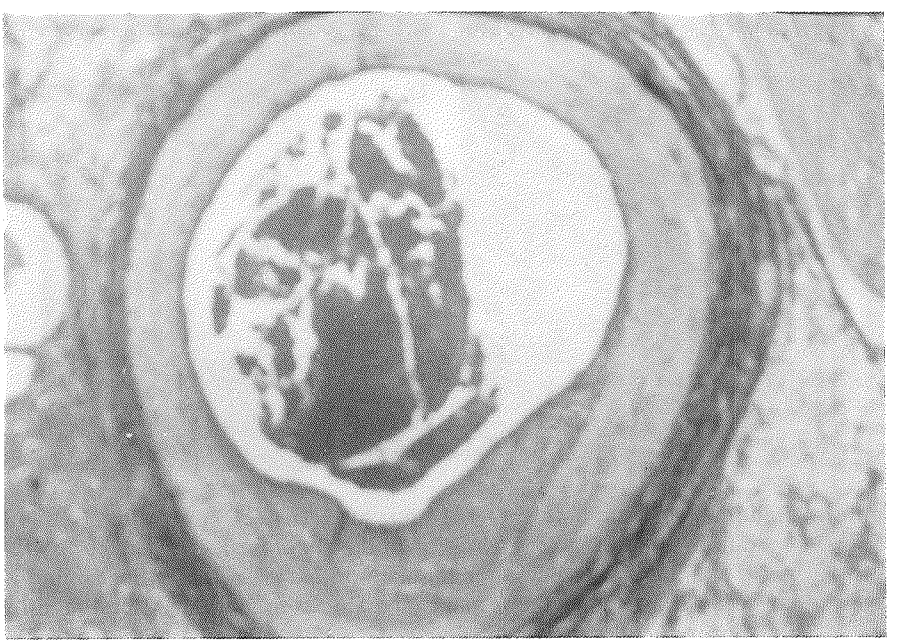

Figure 3. Transverse section of a pig coronary artery stained with elastic tissue stains showing moderate fibromuscular thickening.

chemical factors, also accelerates the development of atherosclerotic lesions [92-95]. The advantage of the balloon technique over the X ray-irradiation method is that the primary lesions, similar to that in man, are mainly in the proximal coronary arteries and that irradiation effects on the myocardium and lung tissue are absent [94].

Following the combination of atherogenic diet and balloon abrasion (*figure 4*) pigs develop moderate atherosclerosis within 4 months [96-98], and extreme severe atherosclerosis within 6-8 months [98-102].

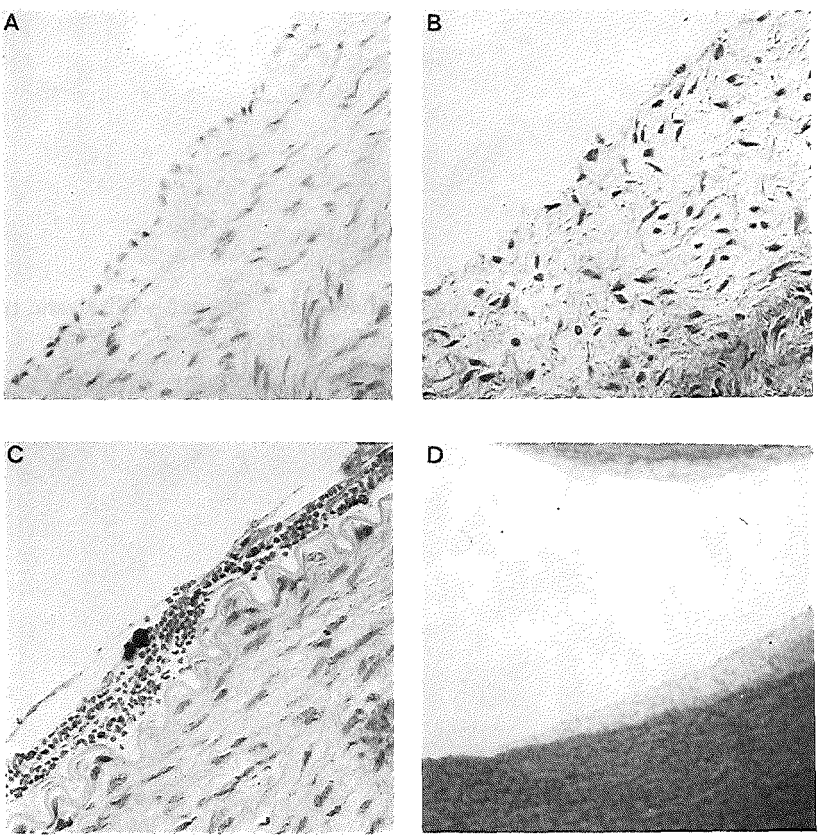

Figure 4. Endothelial lining before (A), 1 hour after (B), 24 hours after (C) and 4 months (D) after balloon abrasion of the abdominal aorta (hemotoxyllin eosin staining). One day after the balloon abrasion the intimal lining is covered with blood platelets and a trhombus is organized. After 4 months the fibromuscular thickening had led to flat atheromatous plaques.

In animal models regression of atherosclerosis can be demonstrated by withdrawal of the atherogenic diet by a low-cholesterol diet and/or other interventions [59, 96, 97, 99, 101, 103-106]. Depending on the quantification method used, regression of atherosclerotic lesions is already detectable 6 weeks after the start of the intervention [101].

12.6 Quantification of atherosclerosis

Several methods are available to quantify atherosclerosis: angiographic description of the lesions, topography of vessel surface involvement with e.g. Sudan IV staining, biochemical analysis of vessel wall and morphometric measurements of vessel sections.

Coronary artery lesions can best be quantified by angiographic description or morphometrical measurements including lumen area and lesion area, and calculation of the ideal lumen area and luminal encroachment [90]. Angiography is not very sensitive for the detection of early or extended lesions [98].

Table 1
Quantification of coronary artery atherosclerosis by morphometrical methods in pigs following coronary artery balloon abrasion and atherogenic diets consisting of >30% fat, >2% cholesterol and >1% sodium cholate for 4 to 6 months.

	lesion area (mm^2)		luminal encroachment (%)	
	4 months	8 months	4 months	8 months
Weiner et al. (98)	0.25	2.75	8	49
Weiner et al. (102)	-	1.84	-	44

Table 1 gives the data on morphometrical lesion measurements in studies on coronary artery atherosclerosis at different time intervals. In control animals with a 9% lard fat low cholesterol diet (n=6) the lesion area is 0.09 ± 0.03 mm^2 and the luminal encroachment 5 ± 1% after 4 months (unpublished data from our laboratory).

Lesions in the aorta or femoral arteries can be quantified by angiography, description of lesions, topography of the surface involvement according to type of lesion (non lesion, flat lesion, raised lesion or complicated lesion) or staining, and biochemical analysis of the different kinds of lesions.

Table 2
Quantification of abdominal aorta surface involvement in atherosclerosis by Sudan IV staining in pig following balloon abrasion of the abdominal aorta and atherogenic diets consisting of >30% fat, >2% cholesterol and >1% sodium cholate for 4 to 6 months.

	surface involvement (%)	
	4 months	6 months
Daoud et al. (96)	21	--
Daoud et al. (99)	--	43

Table 2 gives the data of comparable studies on abdominal aortic involvement after Sudan IV staining at different time intervals. In mash fed control animals the vessel wall involvement was less than 1% after 4 months [96].

Table 3
Quantification of cholesterol contents of abdominal aortic atherosclerotic lesions in pigs following balloon abrasion of the abdominal aorta and atherogenic diets consisting of >30% fat, >2% cholesterol and >1% sodium cholate for 4 to 6 months.

	cholesterol (µg/mg dry weight)					
	total		free		ester	
	4months	6months	4months	6months	4months	6months
Fritz et al. (97)	5	--	4	--	11	--
Fritz et al. (100)	--	90	--	--	--	--
Fritz et al. (101)	--	108	--	34	--	75

Table 3 gives the data of cholesterol contents of raised abdominal aortic lesions in comparable studies at different time intervals. In mash fed control animals the total cholesterol content of non lesions was 3 ug/mg dry weight after 4 months and 1 ug/mg dry weight after 6 months. The free and esterified cholesterol contents were 1 ug/mg dry weight after 4 and 6 months [97, 101]. In all of these studies the pigs were fed a >30% fat, >2% cholesterol and >1% sodium cholate containing atherogenic diet and the coronary artery concerned and abdominal aorta were abraded by balloon technique at the beginning of the period. From these data it is clear that in this experimental set up, atherosclerosis is a very dynamic process [96-98].

REFERENCES

1. Report of the working group of Arteriosclerosis of the National Heart, Lung and Blood Institute. Vol. 2, Washington DC: Government Printing Office. DHEW publication NIH 1981; 82-2035.
2. Shattock SG. Royal society of medicine. Pathological section. Lancet I 1909; 318-9.
3. Ruffer MA. On arterial lesions found in Egyptian mummies. J Pathol Bact 1911; 15: 453-62.
4. Long E. Development of our knowledge of atherosclerosis. In: Blumenthal HT (ed). Cowdry's Arteriosclerosis. Springfield, Charles C Thomas, 1967; 5-20.
5. Morgan AD. The pathogenesis of coronary occlusion. Blackwell, Oxford, 1956.
6. Rokitansky C. Handbuch der Pathologischen Anatomie. Vol. 2. Vienna, Braunmuller und Seidel, 1844.
7. Risse A. In: Cowdry EV (ed). Arteriosclerosis. New York, Mac Millan, 1933.
8. Virchow R. Phlogose und Thrombose in Gefass System. Gesammelte Abhandlungen zur wissenschaftlichen Medizin. Frankfurt, Meidinger, 1956; 458.
9. Crawford T. Some aspects of the pathology of atherosclerosis. Proc Royal Soc Med 1960; 53: 9-12.
10. Pickering G. Arteriosclerosis and atherosclerosis. Am J Med 1963; 34: 7-18.
11. Adams CWM. Arteriosclerosis in man, other mammals and birds. Biol Rev 1964; 39: 372-423.
12. Keys A (ed). Coronary heart disease in seven countries. Circulation 1970; 41 (SI): I-1-211.
13. Kannel WB, Castelli W, Gordon T. Serum cholesterol, lipoproteins, and risk of coronary heart disease: The Framingham study. Ann Intern Med 1971; 74: 1-12.
14. Pooling Project Research Group. Relationship of blood pressure, serum cholesterol, smoking habit, relative weight and ECG abnormalities to the incidence of major coronary events: final report of the pooling project. J Chronic Dis 1978; 31: 201-306.
15. Goldbourt V, Holtzman E, Neufeld HN. Total and high density lipoprotein cholesterol in the serum and risk of mortality: Evidence of a treshold effect. Br Med J 1985; 290: 1239-43.
16. Stamler J, Wentworth D, Neaton J. Is the relationship between serum cholesterol and risk of death from coronary heart disease continuous and graded? JAMA 1986; 256: 2823-8.
17. Kimura N. Analysis of 10. 000 postmortem examinations in Japan. In: Keys A, White PD (eds). World trends in cardiology: I. Cardiovascular epidemiology. New York, Hoeker-Harper, 1956; 159.
18. McGill HC (ed). The geographic pathology of atherosclerosis. Baltimore, Williams & Wilkins Co, 1968; 41.
19. Multiple Risk Factor Trial Research Group. Multiple risk factor intervention trial. Risk factor changes and mortality results. JAMA 1982; 248: 1465-77.
20. Solberg LA, Strong JP. Risk factors and atherosclerotic lesions: A review of autopsy studies. Arteriosclerosis 1983; 3: 187-98.
21. Brown MS, Goldstein JL. Lipoprotein receptors in the liver: control signals for plasma cholesterol traffic. J Clin Invest 1983; 72: 743-7.

22. Goldstein JL, Brown MS. The LDL pathway and its relation to atherosclerosis. Ann Rev Biochem 1977; 46: 897-930.
23. Goldstein JL, Hazzard WR, Schrott HG, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. I. Lipid levels in 500 survivors of myocardial infarction. J Clin Invest 1973; 52: 1533-43.
24. Gotto AM, Gorry GA, Thompson JR. Relationship between plasmalipid concentrations and coronary artery disease in 496 patients. Circulation 1977; 56: 875-83.
25. Branner D, Altman S, Loebl K, Schwartz S, Levin S. Serum cholesterol and triglycerides in patients suffering from ischemic heart disease and in healthy subjects. Atherosclerosis 1977; 28: 197-204.
26. Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease: new perspectives based on the Framingham Study. Ann Intern Med 1979: 90: 85-91.
27. Cambien F, Jaqueson A, Richard JL, Warnet JM, Ducimetiere P, Claude JR. Is the level of serum triglyceride a significant predictor of coronary death in normocholesterolemic subjects? The Parvis prospective study. Am J Epid 1986; 124: 624-32.
28. Baumgartner HR. Platelet interaction with vascular structures. Thromb Diath Haemorrh 1972; S51: 161-76.
29. Oka Y, Orth DN. Human plasma epidermal growth factor / beta-urogastrone is associated with blood platelets. J Clin Invest 1983; 72: 249-59.
30. Bower-Pope DF, Ross R. Is epidermal growth factor present in human blood? Interference with the radio-receptor assay for epidermal growth factor. Biochem Biophys Res Commun 1983; 114: 1036-41.
31. Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci USA 1974; 71: 1207-10.
32. Kohler N, Lipton A. Platelets as a source of fibroblast growth-promoting activity. Exp Cell Res 1974; 87: 297-301.
33. Grotendorst GR, Chang T, Seppa HEJ, Kleinman HK, Martin GR. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J Cell Physiol 1982; 113: 261-6.
34. Deuel TF, Senior RM, Chang D, Griffin GL, Heinrickson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophil elastase. Proc Natl Acad Sci USA 1981; 78:4584-7.
35. Kannel WB, Schwartz MD, McNamara PM. Blood pressure and risk of coronary heart disease: The Framingham study. Dis Chest 1969; 56: 43-52.
36. Kannel WB, Wolf PA, Verter J, McNamara PM. Epidemiological assessment of the role of blood pressure in stroke. J Am Med Ass 1970; 214: 301-10.
37. Paul O. The risks of mild hypertension: A ten year report. Br Heart J 1971; 33: 116-21.
38. Stamler J, Berckson DM, Lindberg HA. Risk factors: Their role in the etiology and pathogenesis of the atherosclerotic diseases. In: Wissler RS, Geer JC (eds). The pathogenesis of atherosclerosis. Baltimore, Williams and Wilkins Co, 1972; 41-119

39. Rhoads GG, Blackwelder WC, Stemmerman GN, Hayashi T, Kagan A. Coronary risk factors and autopsy findings in Japanese-American men. Lab Invest 1978; 38: 304-11.
40. Crawford T. The Pathology of ischemic heart disease. London, Butterworths, 1972; 57-9.
41. McGill HC jr. Atherosclerosis: problems in pathogenesis. In: Paoletti R, Gotto AM (eds). Atherosclerosis Reviews Vol. 2. New York, Raven Press, 1977; 27-65.
42. Doll R, Hill AB. Mortality in relation to smoking. Ten years observations of British doctor. Br Med J 1964; 1: 1399-1410 and 1460-67.
43. Dawber TR, Kannel WB, Stokes J, Kagan A, Gordon T. Some factors associated with the development of coronary heart disease. Six years follow-up experience in the Framingham study. Am J Publ Health 1959; 49: 1349-56.
44. Bradley RF, Partamanian JO. Coronary heart disease in the diabetic patient. Med Clin North Am 1965; 49: 1093-1104.
45. Morris JN, Everitt MG, Pollard R, Chave SPW. Vigorous exercise in leisure-time: protection against coronary heart disease. Lancet 1980; II: 1207-10.
46. Paffenbarger RS, Hyde RT, Wing AL, Hsieh CC. Physical activity, all-cause mortality, and longevity of college alumni. N Engl J Med 1986; 314: 605-13.
47. Tram ZV, Weltman A, Glass CV, Mood DP. The effects of exercise on blood lipids and lipoproteins: a meta-analysis of studies. Med Sci Sports Exerc 1983; 15: 393-402.
48. Dufaux B, Assmann G, Hollman W. Plasma lipoproteins and physical activity: a review. Inst J Sports Med 1982; 3: 123-38.
49. Rosenman RH, Brand RJ, Scholtz RI, Friedman M. Multivariate prediction of coronary heart disease in the Western Collaborative Group Study. Am J Cardiol 1976; 37: 903-10.
50. Rosenman RH, Brand RJ, Jenkins CD, Friedman M, Straus R, Wurm M. Coronary heart disease in the Western Collaborative Group study: final follow-up experience of 8. 5 years. JAMA 1975; 233: 872-7.
51. Dormandy JA, Hoare E, Colley J, Arrowsmith DE, Dormandy TL. Clinical haemodynamic rheological and biochemical findings in 126 patients with intermittent claudication. Br Med J 1973; 4: 576-81.
52. Rainer C, Kawanishi DT, Chandraratna AN, Bauersachs RM, Reid CL, Rahimtoola SH, Meiselman HJ. Changes in blood rheology in patients with stable angina pectoris as a result of coronary artery disease. Circulation 1987; 76: 15-20.
53. Fitzgerald DJ, Roy L, Catella F, Fitzgerald GA. Platelet activation in unstable coronary disease. N Engl J Med 1986; 315: 938-9.
54. Prisco D, Rogasi PG, Matucci M, Abbata R, Gensini GF, Serneri GGN. Increased thromboxane A_2 generation and altered membrane fatty acid composition in platelets from patients with active angina pectoris. Thromb Res 1986; 44: 101-12.
55. Wood DA, Riemersma RA, Butler S, Thromson M, Macintyre C, Elton RA, Oliver MF. Linoleic and eicosapentaenoic acids in adipose tissue and platelets and risk of coronary heart disease. Lancet I 1987; 177-83.

56. Goldstein JL, Kita T, Brown MS. Defective lipoprotein receptors and atherosclerosis: lessons from an animal counterpart of familial hypercholesterolemia. N Engl J Med 1983; 309: 288-96.
57. Brown MS, Goldstein JL. How LDL receptors influence cholesterol and atherosclerosis. Sci Am 1984; 251: 58-66.
58. Rapp JH, Connor WE, Lin DS, Inahara T, Porter JM. Lipids of human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression. J Lipid Res 1983; 24: 1329-35.
59. St Clair RW. Atherosclerosis regression in animal models: current concepts of cellular and biochemical mechanisms. Prog Cardiovasc Dis 1983; 26: 109-32.
60. St Clair RW. Metabolism of the arterial wall and atherosclerosis. In: Paoletti R, Gotto AM jr (eds). Atherosclerosis Reviews Vol. 1. New York, Raven Press, 1976; 61-117.
61. Ross R, Glomset JA. The pathogenesis of atherosclerosis. N Engl J Med 1976; 295: 369-77, 420-5.
62. Fowler S, Shio H, Haley HJ. Characterization of lipid laden aortic cells from cholesterol-fed rabbits. Lab Invest 1979; 41: 372-8.
63. Schaffner T, Tayler K, Bartucci EJ, Fischer-Dzoga K, Beeson JH, Glagov S, Wissler RW. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. Am J Pathol 1980; 100: 57-80.
64. Ross R. Atherosclerosis: A problem in the biology of aterial wall cells and their interactions with blood components. Arteriosclerosis 1981; 1: 293-311.
65. Goldstein JL, Anderson RGW, Brown MS. Coated pits, coated vesicles, and receptor mediated endocytosis. Nature 1979; 279: 679-84.
66. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding sites on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Natl Acad Sci USA 1979; 76: 333-7.
67. Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RGW. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J Cell Biol 1979; 82: 597-613.
68. Brown MS, Basa SK, Falak JR, Ho YK, Goldstein JL. The scavenger cell pathway for lipoprotein degradation: Specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J Supramol Struct 1980; 13: 67-81.
69. Haberland ME, Fogelman AM, Edwards PA. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. Proc Natl Acad Sci USA 1982; 79: 1712-6.
70. Van der Schroeff JG, Havekes L, Emeis JJ, Wijsman M, Van der Meer H, Vermeer BJ. Morphological studies on the binding of low-density lipoproteins and acylated low-density lipoproteins to the plasma membrane of cultured monocytes. Exp Cell Res 1983; 145: 95-103.
71. Benditt EP, Benditt JM. Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci USA 1973; 70: 1753-6.
72. Ross R. The pathogenesis of atherosclerosis - an update. N Engl J Med 1986; 314: 488-500.
73. Childs CB, Proper JA, Tucker RF, Moses HL. Serum contains a platelet derived growth factor. Proc Natl Acad Sci USA 1983; 79: 5312-6.

74. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor beta in human platelets: identification of a major storage site, purification, and characterization. J Biol Chem 1983; 258: 7155-60.
75. Tucker RF, Shipley GD, Moses HL, Holley RW. Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. Science 1984; 226: 705-7.
76. Steinberg D. Lipoproteins and atherosclerosis. A look back and a look ahead. Arteriosclerosis 1983; 3: 283-301.
77. Hendriksen T, Eversen SA, Carlander B. Injury to human endothelial cells in culture induced by low density lipoproteins. Scan J Clin Lab Invest 1979; 39: 361-8.
78. Ross R, Harker L. Hyperlipidemia and atherosclerosis. Science 1976; 193: 1094-100.
79. Shattil SJ, Anaya-Galindo R, Bennet J, Colman RW, Cooner RA. Platelet hypertensitivity induced by cholesterol incorporation. J Clin Invest 1975; 55: 636-43.
80. Carvalho AC, Colman RW, Lees RS. Platelet function in hyperlipoproteinemia. N Engl J Med 1974; 290: 434-8.
81. Insel PA, Nirenberg P, Turnhall J, Shattil SJ. Relationships between membrane cholesterol, alpha-adrenergic receptors and platelet functions. Biochemistry 1978; 17: 5269-74.
82. Carew TE, Pittman RC, Marchaud ER, Steinberg D. Aortic endothelium is an active site of low density lipoprotein degradation in vivo in normal rabbits. Clin Res 1983; 31: 382A.
83. Fischer-Dzoga K, Fraser RA, Wissler RW. Stimulation of proliferation in stationary primary cultures of monkey and rabbit aortic smooth muscle cells. I. Effects of lipoprotein fractions of hyperlipemic serum and lymph. Exp Mol Pathol 1976; 24: 346-59.
84. Chalt A, Ross R, Albers J, Bierman F. Platelet derived growth factor stimulates low density lipoprotein receptor activity. Proc Natl Acad Sci USA 1980; 77: 4084-8.
85. Ross R, Klebanoff SJ. The smooth muscle cell. I. In vivo synthesis of connective tissue proteins. J Cell Biol 1971; 50: 159-71.
86. Rowsel HC, Mustard JF, Downie HG. Experimental atherosclerosis in swine. Ann NY Acad Sci 1965; 127: 743-62.
87. Luginbuhl H, Jones JE. The morphology and morphogenesis of atherosclerosis in aged swine. Ann NY Acad Sci 1965; 127: 763- 79.
88. Getty R. The gross and microscopic occurrence and distribution of spontaneous atherosclerosis in the arteries of swine. In: Roberts JC jr, Strauss R (eds). Comparative Atherosclerosis. New York, Harper, 1965; 11-20.
89. Gresham GA, Howard AN. Comparative pathology of spontaneously occuring and experimentally induced atherosclerotic lesions. Methods Archiev Exp Pathol 1966; 1: 314-36.
90. Wissler RW, Vesselinowitch D. Differences between human and animal atherosclerosis. In: Schetler G, Weizel A (eds). Atherosclerosis III. New York, Springer-Verlag, 1974; 319-29.
91. Florentin RA, Nam SC, Daoud AS, Jones R, Scott RF, Morrison ES, Kim DN, Lee KT, Thomas WA, Dodds WJ, Miller KD. Dietary- induced atherosclerosis

in miniature swine. Exp Mol Pathol 1968; 8: 263-301.
92. Lee KT, Jarmolych J, Kim DN, Grant C, Krasney JA, Thomas WA, Bruno AM. Production of advanced coronary artery atherosclerosis, myocardial infarction and sudden death in swine. Exp Mol Pathol 1971; 15: 170-90.
93. Nam SC, Lee WM, Jarmolych J, Lee KT, Thomas WA. Rapid production of advanced atherosclerosis in swine by a combination of endothelial injury and cholesterol feeding. Exp Mol Pathol 1973; 18: 369-79.
94. Lee WM, Lee KT. Advanced coronary atherosclerosis in swine produced by combination of balloon-catheter injury and cholesterol feeding. Exp Mol Pathol 1975; 23: 491-9.
95. Scott RF, Thomas WA, Lee WM, Reiner JM, Florentin RA. Distribution of intimal smooth muscle cell masses and their relationship to early atherosclerosis in the abdominal aorta's of young swine. Atherosclerosis 1979; 34: 291-301.
96. Daoud AS, Jarmolych J, Augustyn JM, Fritz HE, Singh JH, Lee KT. Regression of advanced atherosclerosis in swine. Arch Pathol Lab Med 1976; 100: 372-9.
97. Fritz KE, Augustyn JM, Jarmolych J, Daoud AS, Lee KT. Regression of advanced atherosclerosis in swine. Chemical studies. Arch Pathol Lab Med 1976; 100: 380-5.
98. Weiner BH, Oakene JS, Jarmolych J, Fritz KE, Daoud AS. Comparison of pathologic and angiographic findings in a porcine preparation of coronary atherosclerosis. Circulation 1985; 72: 1081-6.
99. Daoud AS, Jarmolych J, Augustyn JM, Fritz HE. Sequential morphologic studies of regression of advanced atherosclerosis. Arch Pathol Lab Med 1981; 105: 233-9.
100. Fritz KE, Daoud AS, Augustyn JM, Jarmolych J. Morphological and biochemical differences among grossly-defined types of swine aortic atherosclerotic lesions induced by a combination of injury and atherogenic diet. Exp Mol Pathol 1980; 32: 61-72
101. Fritz KE, Augustyn JM, Jarmolych J, Daoud AS. Sequential study of biochemical changes during regression of swine aortic atherosclerotic lesions. Arch Pathol Lab Med 1981; 105: 240-6.
102. Weiner BH, Ockene JS, Levine PH, Cuenoud HF, Fisher M, Johnson BF, Daoud AS, Jarmolych J, Hosmer D, Johnson MH, Natale A, Vaudreuil C, Hoogasian JJ. Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. New Engl J Med 1986; 315: 841-6.
103. Manilow MR, Blaton V. Regression of atherosclerotic lesions. Arteriosclerosis 1984; 4: 292-5.
104. Jarmolych J, Daoud AS, Fritz KE, Augustyn JM, Singh JK, Kim DN. Morphological effects of moderate diet and clofibrate on swine atherosclerosis. Arch Pathol Lab Med 1978; 102: 289-93. 105. Augustyn JM, Fritz KE, Daoud AS, Jarmolych J, Lee KT. Biochemical effects of moderate diet and clofibrate on swine atherosclerosis. Arch Pathol Lab Med 1978; 102: 294-7.
106. Kim DN, Schmee J, Ho HT, Thomas WA. The "turning off" of excessive cell replicative activity in advanced atherosclerotic lesions of swine by a regression diet. Atherosclerosis 1988: 71: 131-42.

CHAPTER 13

MACKEREL OIL AND ATHEROSCLEROSIS IN PIGS

L.M.A. Sassen [1], J.M. Hartog [1], J.M.J. Lamers [2], M. Klompe [3], L.J. Van Woerkens [1] and P.D. Verdouw [1].

[1] Laboratory for Experimental Cardiology (Thoraxcentre), [2] Department of Biochemistry I and [3] Oogziekenhuis, Erasmus University Rotterdam, Rotterdam, The Netherlands

Summary

In 35 pigs atherosclerosis was induced by balloon abrasion and a diet containing 2% (w/w) cholesterol and 7% (w/w) lard fat. After 4 months of induction 9 animals were killed (I) for analysis of the extent of atherosclerosis, while the diet of the other 26 pigs was changed to a low (w/w) cholesterol diet containing either 9% (w/w) lard fat (L), 9% (w/w) fish oil (F) or 4.5% (w/w) lard fat and 4.5% (w/w) fish oil (LF). This diet was continued for three months to induce regression of atherosclerosis. The cholesterol-rich diet increased plasma total cholesterol, but did not affect plasma triglycerides. Low-cholesterol feeding decreased plasma total cholesterol in all 3 groups, but triglycerides only in LF and F. Lipid infiltration of the aortic wall was similar in I, L, LF and F. In the denudated coronary arteries of I mean luminal encroachment was 11 ± 2 %. This was similar in L (13 ± 4 %) but significantly lower ($P<0.05$) in LF (6 ± 2 %) and in F (3 ± 1 %). In the non-abraded coronary arteries of I mean luminal encroachment was 1.3 ± 0.3 %. For F and LF similar values were found, but in L there was an increase to 11 ± 3 % during low-cholesterol feeding. ADP-induced platelet aggregation was lower in LF and F than in L. Thromboxane A_2 production was only reduced in F, while the production of the weak thromboxane A_3 agonist was larger in F than in LF. It is concluded that fish oil retards the progression of and causes regression of coronary atherosclerosis.

Introduction

Anatomical evidence of regression of coronary atherosclerosis has been demonstrated after combined colestipol-niacin therapy in non-smokers who have undergone coronary bypass surgery [1]. Lowering of LDL-cholesterol and the concomitant increase in HDL-cholesterol appear to be the two major factors associated with the reduction in the number of lesions in the native coronary arteries of these patients. Armstrong et al. showed that in non-human primates coronary atherosclerosis regressed both morphologically [2] and biochemically [3] after 40 months of low-cholesterol feeding following an induction period of 17 months. Clarkson et al. [4] found that in rhesus monkeys cholesterol-induced coronary atherosclerosis regressed after total cholesterol levels were decreased from 15 mM to 5 mM, but still progressed in half of the animals in which cholesterol was reduced to only 7.5 mM (normal values 3.4-5.4 mM [5]). Regression of atherosclerosis has also been shown in pigs on removal of cholesterol from the diet [6-8]. Weiner et al. [9] have shown that fish oil added to an atherogenic diet can retard the formation of plaques in coronary arteries of pigs but it is not known whether marine oil can also cause regression of atherosclerosis.

The purpose of the present study was to assess the effect of regular intake of mackerel oil on the regression of atherosclerosis. We have previously shown that the amounts of fish oil used in the present study markedly reduce levels of plasma cholesterol and triglycerides in normolipidemic pigs, whereas lard fat did not exert these effects [10, 11]. In the mackerel oil fed pigs prostanoid formation and aggregation of platelets were also lower than in the lard fat fed pigs [10, 11] .

Material and Methods

Induction of atherosclerosis

Blood samples for measurement of blood lipids were obtained from overnight fasted castrated male Yorkshire piglets (five weeks of age, 9.4 ± 0.2 kg, n=48). The animals were then started on a diet containing 7% (w/w) lard fat and 2% (w/w) cholesterol (Hope Farms, Woerden, The Netherlands, see *Tables 1 and 2*). Initially the animals received 200 g daily, but the portions were increased with 200 g each month as the animals gained weight. After 2 weeks the endothelium of the left anterior descending coronary artery and descending aorta was removed by the balloon abrasion technique [6-7, 9]. Hitherto the overnight fasted piglets (now weighing 11.0 ± 0.2 kg) were pretreated with an intramuscular injection of 300, 000 U procain-penicillin-G and 300, 000 U benzathin-penicillin G (Duplocilline^{R}, Gist Brocades, Delft, The Netherlands), anaesthetized by inhalation of a mixture of oxygen and nitrous oxide (1:2) and 1% halothane (Fluothane^{R}, Macclesfield, UK) and

connected to a respirator for artificial ventilation. Following an intravenous injection of 5000 IU of heparin (Thromboliquine^{R}, Organon Teknika B.V., Boxtel, the Netherlands) the animals were catheterized via the left external carotid artery. For denudation of the left anterior descending coronary artery a 4 F Fogarty embolectomy catheter (Edwards Laboratories, Santa Ana, California, USA) was introduced under the guidance of fluoroscopy. The balloon was inflated with 0.3 ml air and pulled back quickly [12]. This procedure was repeated twice to ensure effective denudation of the coronary artery. The descending aorta was catheterized with a 7F Fogarty embolectomy catheter and the balloon was subsequently inflated with 3 ml air and pulled back to the aortic arch. This procedure was also repeated twice. After the carotid artery was stitched, the incision was closed in one layer, the animals were allowed to recover and the dietary period was continued. The efficacy of this abrasion technique was evaluated in a few animals prior to the study (*figure 1*).

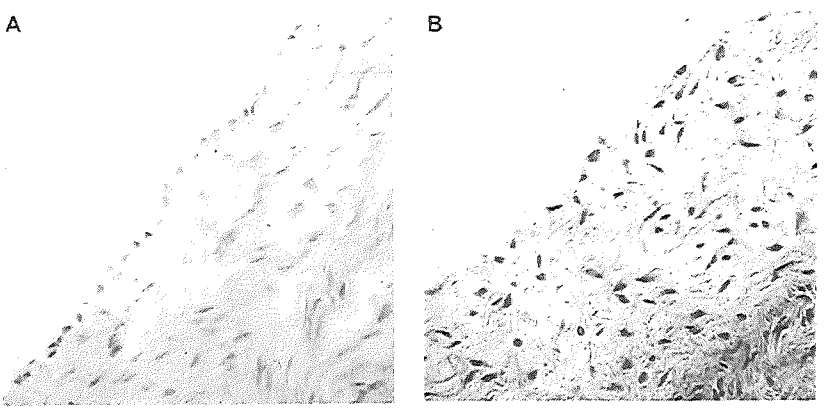

Figure 1
The efficacy of the abrasion technique. An example of a coronary artery with intact endothelium is shown on the left hand side (A), while the example on the right hand side (B) was taken from one of the animals that died suddenly one day after the procedure.

Thirteen animals encountered sudden death during the immediate post-operative period. Autopsy showed ruptures of the coronary artery in five animals and perforation of the left ventricle in two animals. No coronary artery lesions or thrombosis were found in the six other animals.

Extent of atherosclerosis after 4 months of high-cholesterol feeding

Fourteen weeks after balloon denudation (total dietary period 4 months), nine randomly chosen animals (I) were sacrificed to establish the severity of atherosclerosis (see below).

Diets after four months of high-cholesterol feeding

The remaining animals (n=26) were arbitrary divided over three groups and their diet was changed to low cholesterol but with a different fatty acid composition for each group.

Table 1
Composition of the atherosclerotic induction diet and the three low-cholesterol diets

	Induction diet	Post-induction diets		
Ingredients	I	L	LF	F
Corn (extruded)	32	32	32	32
Wheat (extruded)	18	18	18	18
Soybean meal	14	14	14	14
Wheat middling	9	9	9	9
Dehydrated skinned milkpowder	14	14	14	14
$CaHPO_4.H_2O$	1.3	1.3	1.3	1.3
$CaCOO_3$	1.1	1.1	1.1	1.1
NaCl, iodinized	0.3	0.3	0.3	0.3
MgO	0.05	0.05	0.05	0.05
$MgSO_4$	0.05	0.05	0.05	0.05
$KH_2PO_4.2H_2O$	0.36	0.36	0.36	0.36
Choline chloride 50% (wt/wt)	0.18	0.18	0.18	0.18
Vitamin and trace element mixes[1]	0.7	0.7	0.7	0.7
Lard fat	7.27	9.10	4.55	--
Fish oil	--	--	4.55	9.10
Mixed tocopherols	0.03	0.03	0.03	0.03
Cholesterol	1.83	0.01	0.02	0.03

[1]Vitamin and trace element mixes supply the following per 100 g diet: retinol 1400 IU; cholecalciferol 140 IU; alpha-tocopherol 8 mg; menadione 0.2 mg; thiamin hydrochloride 1.8 mg; riboflavin 1.8 mg; pyridoxine HCl 1.4 mg; niacin 3.6 mg; vitamin C coated 20 mg; D-calcium pantothenate 3.6 mg; folic acid 0.4 mg; cyanocobalamin 0.004 mg; biotin 0.1 mg; inositol 4.5 mg; iron subcarbonate (57% Fe) 9.1 mg; $FeSO_4.H_2O$ (30% FE) 14 mg; $Cu_2(OH)_2CO_3$ (55%Cu) 2.3 mg; ZnO (78% Zn) 11 mg; MnO (62%Mn) 9.1 mg; $Na_2Se_3.5H_2O$ (45%Se) 0.08 mg; $Ca(IO_3)_2$ (65% I) 0.2 mg; $CoCO_3$ (47% Co) 0.09 mg; The composition (g/100 g) is on a as-fed basis.

Table 2
Fatty acid composition (mole %) of the induction diet and the three post-induction diets

	Induction diet	Post-induction diets		
Fatty acid	I	L	LF	F
14:0	2	2	5	7
16:0	24	24	21	18
16:1	2	3	6	8
18:0	9	10	6	1
18:1	41	42	29	17
18:2 n-6	16	15	11	8
18:3	1	1	1	1
20:1	1	1	4	6
20:5 n-3	-	-	8	17
22:1	-	-	2	4
22:6 n-3	-	-	5	9
24:1	-	-	1	1
others	4	2	1	3

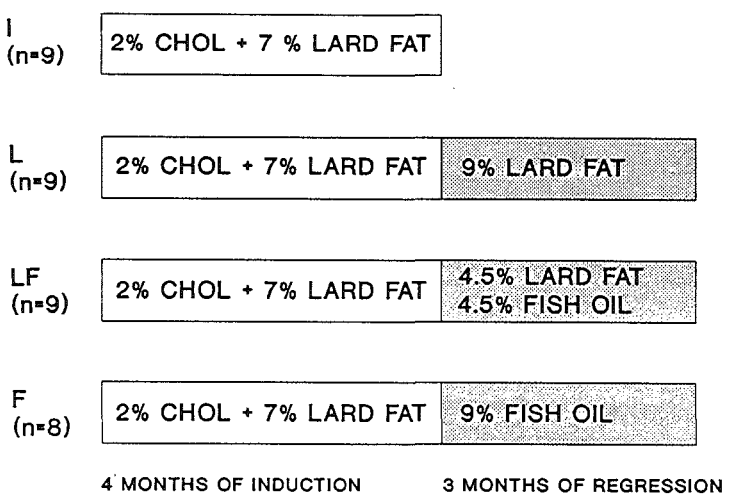

Figure 2
Schematic overview of the dietary groups. Each group received a low fat basal diet to which 9% (w/w) fat was added. The 13 animals which died immediately following the endothelial denudation are not included.

Nine animals (L) received 9% (w/w) lard fat, nine animals (LF) 4.5% (w/w) lard fat and 4.5% (w/w) mackerel oil (AS Johan C Martens and Co, Bergen, Norway) and eight animals received 9% (w/w) of mackerel oil (F) for 3 months. These diets will be called post-induction diets (*Tables 1 and 2*). For convenience the experimental groups have been schematically presented in *figure 2.*

Plasma lipids

Plasma levels of triglycerides [13], total [14] and HDL-cholesterol [15] were determined before the start of, and at one month intervals during the dietary period. Collection of blood samples occurred after an overnight fast by puncturing the jugular vein. During the first 2 months, samples were taken from conscious animals, but after the third month the animals were first sedated with 30 $mg.kg^{-1}$ ketamine i.m. (Aescoket^{R}, Aesculaap BV, Boxtel, the Netherlands).

Platelet aggregation

After 7 months (4 months of induction and 3 months of post-induction) a 5 ml blood sample was collected and placed in tubes containing 1000 IU heparin. After a 15 minutes incubation time at room temperature, whole blood aggregation tests were performed after the addition of 2 $\mu g.ml^{-1}$ collagen or 5 uM ADP for 10 minutes in an electrical aggregometer [16]. For measurement of the prostanoid production the aggregation-reaction was stopped after ten minutes of stimulation with 2 $\mu g.ml^{-1}$ collagen by placing the blood sample on ice. After centrifugation (at 2000 g for 5 minutes) the plasma was stored at -80°C. The levels of the thromboxane A (TXA) derivatives HHT and HHTE were determined by high pressure liquid chromatography [17]. HHT (12 L-hydroxy-5, 8, 10-heptadecatrienoic acid) and HHTE (12 L-hydroxy-5, 8, 10, 14-heptadecatetraenoic acid) are stable products which are, through the cyclo-oxygenase pathway, derived from arachidonic (20:4 n-6) and eicosapentaenoic acid (20:5 n-3), respectively, and reflect TXA_2 and TXA_3 formation [17-20]. The platelets were counted and TXA production was calculated on basis of the platelet concentration.

Chemical analysis of the aortic vessel wall

Samples (40-200 mg) were excised from lesion (abdominal) and non-lesion (ascending) areas of the aorta, dissected free of adventitia and directly frozen in liquid nitrogen and stored at -80°C until analysis. In principle the method of Bligh and Dyer [21] was used for lipid extraction. Briefly, tissue samples were homogenized in chloroform / methanol/distilled water (4:10:5, v/v/v) with a polytron PT 10 (half-setting). After centrifugation at 1500 g_{max} for 5 min and washing the pellet by rehomogenization in 1.9 ml of the same solvent, the 2 supernatants were combined and supplemented with 1.5 ml chloroform and 1.5 ml water. After

vigorous mixing the upperphase was discarded and, when necessary, the intermediate solid material dissolved by dropwise addition of methanol. Subsequently the mixture was dried under nitrogen at 37°C and the residue dissolved in 0.2 ml 2-propranolol. Cholesterol, triglyceride and phospholipid contents were measured enzymatically (GHOD-PAP and GPO-PAP, Boehringer, Mannheim, FRG; and PAP 150 of Biomerieux, Cherbourieres, Les Bains, France). In the delipidized extracts protein [22] and DNA [23] contents were measured.

Grading of coronary artery atherosclerosis

From the coronary arteries biopsies were taken at 5 mm intervals. All biopsies from the heart and aorta were immediately fixed in 10% formalin. Tissue samples were routinely processed for light microscopy: embedded in paraffin, cut at 7 um thickness and stained with haematoxylline azophloxine and recorsine fuchsine. The sections were projected on a video screen and the outer contours, external and internal elastic lamina, and endothelial lining of the vessels were traced using an integrated image analysis system (IBAS-2000, Kontron, Oberkochen, FRG). The surface between the endothelial lining of the lumen and the internal elastic lamina was taken as the lesion area [24]. The encroachment was defined as the ratio (x 100%) of the surface of the lesion area and the corrected (by circular shape factor) surface of the area surrounded by the internal elastica lamina [24, 25]. The media area was the difference between the surfaces circumscribed by the internal and external elastic lamina.

Statistical analysis

All data are described as mean ± standard error of the mean (SEM). The data were analyzed statistically using the paired and unpaired Student's t-tests and a one way analysis of variance with repeated measurements followed by the Newman-Keul procedure for multiple comparisons of mean values. Statistical significance was accepted at $P < 0.05$.

Results

Experimental animals

After 4 months the animals weighed 55 ± 1 kg. During the 3 months of low cholesterol feeding the animals increased their weight to 91 ± 3 kg(L), 88 ± 4 kg(LF) and 94 ± 3 kg(F). There were no differences in the weight gain of the 3 groups during these 3 months.

Plasma lipids

During the four months of 2% (w/w) cholesterol feeding plasma levels of total cholesterol doubled, primarily due to a tripling of the VLDL- + LDL-cholesterol content as HDL-cholesterol levels increased by less than 30% (*figure 3*).

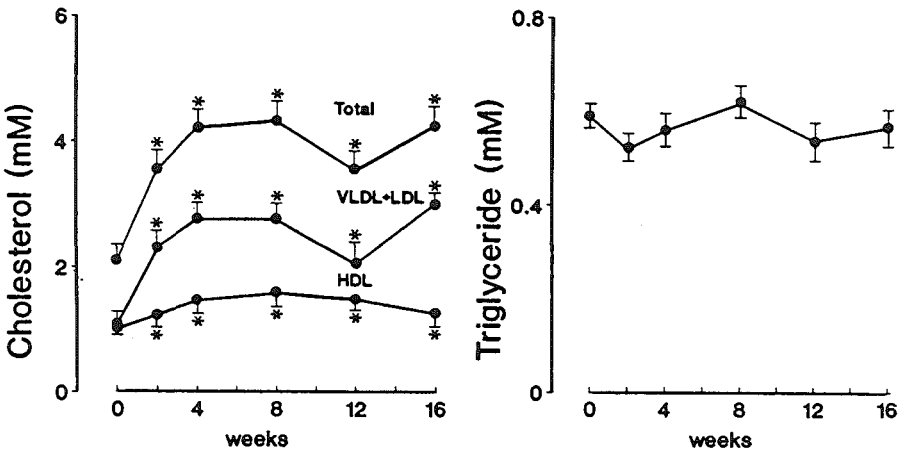

Figure 3
Plasma lipid levels during the 4 month induction period.
* = $p<0.05$ versus pre-atherogenic diet value.

A consequence of these changes was that the ratio between HDL-cholesterol and total cholesterol decreased from 0.50 ± 0.01 mm to 0.31 ± 0.01 mm ($P<0.05$). Levels of plasma triglycerides (0.59 ± 0.03 mM) were not affected by this diet (*figure 3*).

During the 3 months of low-cholesterol feeding total cholesterol decreased in all 3 groups, the changes being the most pronounced in the fish oil fed animals (-1.28 ± 0.33 mM in L, -2.37 ± 0.34 mm in LF and -2.35 ± 0.35 mm in F, *figure 4*). The largest changes already occurred during the first weeks after the animals had changed from the high- to the low-cholesterol diet. Although HDL-cholesterol also decreased in all 3 groups, there was an increase in the HDL-total cholesterol ratio (0.48 ± 0.03 in L, 0.44 ± 0.02 in LF and 0.40 ± 0.01 in F). The plasma triglyceride levels were significantly reduced in the first 6 weeks in LF and F, but returned to those measured in L after 12 weeks.

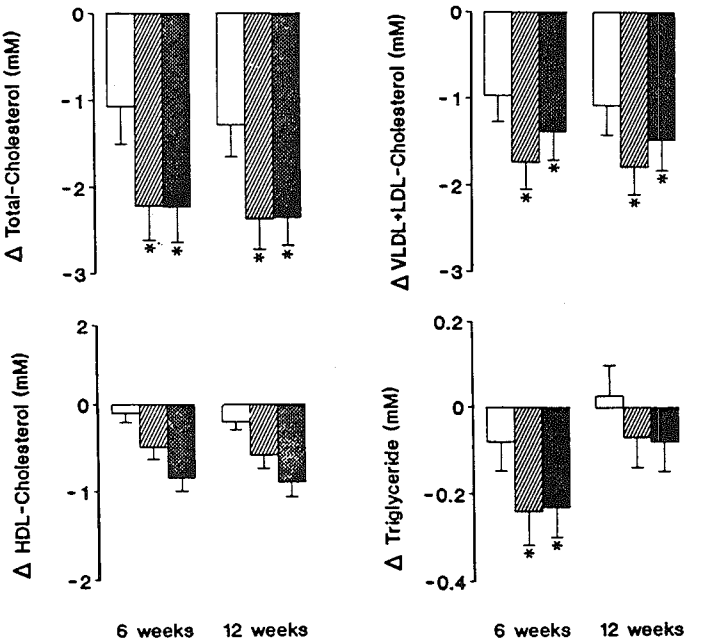

Figure 4
Changes in plasma lipid levels during the 3 month post-induction period on low-cholesterol diets. □ L = 9% lard fat, (n=8); ▨ LF = 4.5% lard fat and 4.5% fish oil, (n=9); ■ F = 9% fish oil, (n=9). * = $P<0.05$ versus L.

Platelet aggregation and prostaglandin production

Collagen-stimulated platelet aggregation was similar in L, LF and F (not shown), but the ADP-stimulated aggregation was lower in LF and F than in the animals which received lard fat only (*figure 5*).

The total amount of thromboxane A derivatives, measured after 10 min of stimulation with collagen, was similar in L (62 ± 5 pg/10^9 platelets), LF (67 ± 6 pg/10^9 platelets) and F (58 ± 7 pg/10^9 platelets). HHT production was lower in F (49 ± 6 pg/10^9 platelets) than in LF (55 ± 6 pg/10^9 platelets) and L (62 ± 5 pg/10^9 platelets). None of the animals belonging to L produced HHTE (*figure 5*), which is in accordance with the absence of eicosapentanoic acid in the platelet membrane [26]. Not all fish oil fed animals produced HHTE (*figure 5*). A large production of HHTE was found in 4 of the 7 animals of F but only 2 of 9 animals of LF produced some HHTE.

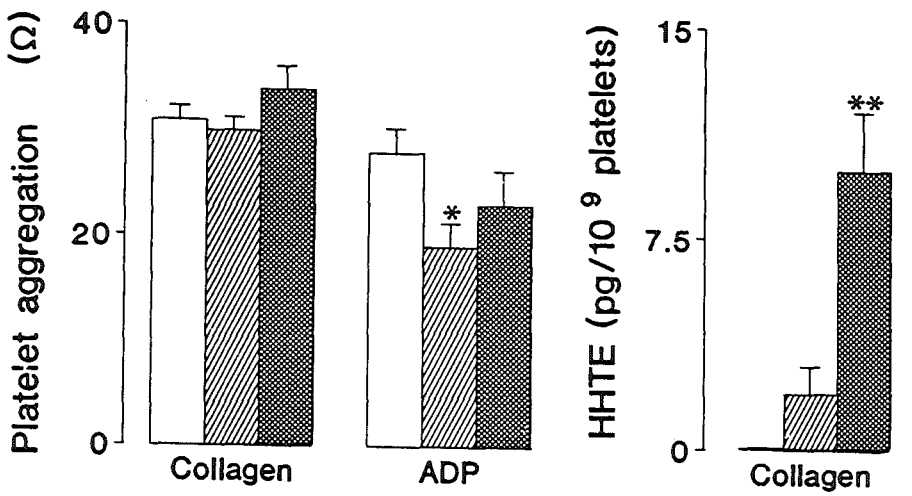

Figure 5
Collagen (2 ug/ml)- and ADP (5uM)-induced platelet aggregation in the 3 post-induction groups after 3 months of low cholesterol feeding. For measurement of the prostanoid production the collagen-induced aggregation was stopped after 10 minutes. HHTE was not produced in the animals which received only lard fat. □ L = 9% lard fat, (n=8); ■ LF = 4.5% lard fat and 4.5% fish oil, (n=9); ■ F = 9% fish oil, (n=9). * = P<0.05 versus L; x = P<0.05 versus F.

Lipid infiltration in the abraded and the non-abraded segments of the aortic wall

After 4 months of cholesterol feeding (I) all lipid concentrations in the lesions of the abdominal aorta were higher than in the non-lesions of the ascending aorta (*Table 3*).

During the following 3 months of low-cholesterol feeding, there were no changes in the lipid contents of the non-lesions in any of the 3 post-induction groups. Cholesterol and phospholipid infiltration in the lesions tended to increase in all 3 groups during low-cholesterol feeding. On the other hand, triglyceride contents of the lesion and the non-lesion areas did not differ at the end of the post-induction period. Expression of lipid contents as umol/g protein and DNA gave similar differences between the lesion and the non-lesion areas of I and the three post-induction groups (not shown).

Table 3
Lipids, protein and DNA contents of the aortic vessel wall

		Induction Group	Post-induction Groups		
		I (n=6)	L (n=6)	LF (n=7)	F (n=7)
Abdominal Aorta (abraded)					
cholesterol	(μmol/g)[a]	4.07 ± 0.46*	5.10 ± 0.60*	5.54 ± 0.66*	4.83 ± 0.47*
triglyceride	(μmol/g)	3.05 ± 0.64*	1.92 ± 0.45	2.19 ± 0.34	2.05 ± 0.31
phospholipids	(μmol/g)	3.71 ± 0.27*	4.56 ± 0.30*	4.37 ± 0.50*	4.06 ± 0.17*
protein	(mg/g)	160 ± 14	168 ± 15	185 ± 20	208 ± 30
DNA	(mg/g)	1.91 ± 0.25	2.45 ± 0.20	2.03 ± 0.14	2.15 ± 0.10
Ascending Aorta (non abraded)					
cholesterol	(μmol/g)	2.82 ± 0.36	2.63 ± 0.13	2.60 ± 0.12	2.82 ± 0.11
triglyceride	(μmol/g)	1.35 ± 0.28	1.38 ± 0.23	1.68 ± 0.43	1.67 ± 0.73
phospholipids	(μmol/g)	2.92 ± 0.07	2.91 ± 0.10	2.91 ± 0.17	2.92 ± 0.19
protein	(mg/g)	166 ± 3	235 ± 42	222 ± 23	228 ± 31
DNA	(mg/g)	2.45 ± 0.13	2.18 ± 0.29	2.71 ± 0.40	2.67 ± 0.52

For the composition of the diets see tables 1 and 2. a = All units per g wet weight.
* = $P<0.05$ versus ascending aorta in the same group.

Luminal encroachment of the abraded and non-abraded coronary arteries

Mean luminal encroachment of the abraded left anterior descending coronary artery was 11 ± 2 % and of the non-abraded left circumflex coronary artery 1.3 ± 0.3 % after 4 months of 2% (w/w) cholesterol feeding (I, *Table 4*). During the post-induction period, feeding with only lard fat had no effect on the mean luminal encroachment of the abraded coronary arteries, but addition of fish oil caused a dose-dependent decrease. In the non-abraded left circumflex coronary artery mean luminal encroachment increased to 11 ± 3 % in L. Addition of fish oil to the low-cholesterol diet inhibited this increase (*Table 4*).

Table 4
Luminal encroachment of the abraded and non-abraded coronary arteries.

		Induction Group	Post-induction Groups		
		I (n=9)	L (n=8)	LF (n=9)	F (n=9)
LADCA (abraded)					
lesion area	(mm^2)	$0.22 \pm 0.03^{*}$	0.27 ± 0.03	$0.10 \pm 0.02^{*+x}$	$0.09 \pm 0.02^{x+}$
encroachment	(%)	$11 \pm 2^{*}$	13 ± 2	$6 \pm 1^{*+x}$	$3 \pm 1^{+xo}$
media	(mm^2)	0.86 ± 0.06	0.90 ± 0.08	0.83 ± 0.08	0.97 ± 0.05
LCXCA (non-abraded)					
lesion area	(mm^2)	0.03 ± 0.01	$0.30 \pm 0.06^{+}$	0.02 ± 0.01^{x}	0.05 ± 0.02^{x}
encroachment	(%)	1.3 ± 0.3	$11 \pm 3^{+}$	0.9 ± 0.3^{x}	1.1 ± 0.4^{x}
media	(mm^2)	0.94 ± 0.08	1.08 ± 0.11	0.84 ± 0.08	0.82 ± 0.12

For the composition of the diets see Tables 1 and 2.
* = P<0.05 versus non-abraded vessel of the same group;
+ = P<0.05 versus I; x = P<0.05 versus L; o = P<0.05 versus LF; LADCA = left anterior descending coronary artery; LCXCA = left circumflex coronary artery.

Discussion

In the present study addition of 2% (w/w) cholesterol to the 21 energy% fat (15 mol% linoleic acid) containing diet increased plasma cholesterol from 2 mM to 4 mM. Weiner et al. added 2% (w/w) cholesterol and 1% sodiumcholate to a 30 energy% fat (mainly saturated fatty acids) containing diet causing increases up to 14 mM during an induction period of 8 months [24]. This partly explains the lower mean luminal encroachment (11%) in the abraded coronary arteries of the present study compared to that found by Weiner et al. (44%). The total cholesterol content of the abdominal aortic lesions in the present study was 4.1 μmol/g wet weight, which is about 27 μmol/g dry weight. Fritz et al. [6] found a cholesterol content of about 38 μmol/g dry weight in the aortic wall with mean plasma levels of cholesterol of 14 mM. Other factors contributing to the difference in luminal encroachment in the coronary arteries and lipid infiltration in the aortic wall may be the difference in the duration of the induction period and performance of the abrasion technique.

Three months of low-cholesterol feeding lowered the plasma cholesterol levels (*figure 4*). In the animals with fish oil added to the diet (F and LF) these decreases

were more pronounced than in the animals in which only lard fat (L) was added, which is consistent with earlier observations in normolipidemic pigs [10, 11, 19]. The reduction in ADP-induced platelet aggregation was less in F than in LF. The data on platelet aggregation in F are not in agreement with the changes in HHT and HHTE production, which were measured in vitro after the aggregation reaction. However in the in vivo situation the effects of fish oil on the synthesis of prostacyclin I_2 and I_3 by endothelial cells should also be taken into account [19, 27-29].

At the end of the induction period the lesions in the abdominal aorta contained more cholesterol than the non-lesions in the ascending aorta. Three months of low cholesterol feeding did not affect the lipid contents of the lesions and non-lesions in either group. Further studies are needed on the localization (intra- or extracellularly) and precipitation forms of cholesterol(ester) for definite proof of lack of regression [30].

Addition of fish oil to the low-cholesterol diet decreased luminal encroachment in the abraded coronary arteries, whereas addition of lard fat only inhibited further intimal proliferation. In the non-abraded coronary arteries intimal proliferation was negligible after 4 months, but developed during the following 3 months when only lard fat was added to the low-cholesterol diet. Although in L cholesterol levels were lower during lard fat feeding than during high-cholesterol feeding they were still higher than in low-cholesterol fed pigs of the same age [31]. This is consistent with the findings of Clarkson et al. [4], who also found progression of lesions in half of the animals whose cholesterol levels remained elevated as compared to baseline values whereas in the animals with normal levels of cholesterol, lesions did regress. Lesions did not develop in the non-abraded arteries of the pigs with fish oil added to the diet, proving that fish oil can also prevent progression of coronary atherosclerosis.

In LF and F the return to normal cholesterol levels most likely is involved in the regression of coronary artery sclerosis, but it cannot explain the dose dependent effect. The results on platelet aggregation also cannot elucidate this dose-dependency, because platelet aggregation was only reduced in LF. Several factors such as leukotrienes, tissue plasminogen activator, platelet derived growth factor, interleukin-1 and tumor necrosis factor, have also been implicated in the development (or regression) of atherosclerosis and are also modulated by fish oil (for review see references 32, 33).

Lipid infiltration could not be determined in the coronary arteries, as the samples were used for the measurement of luminal encroachment. If the results obtained in the aorta can be extrapolated to the smaller blood vessels there appears to be a discrepancy between these two variables, which are both frequently used for the grading of the severity of atherosclerosis. An additional complication is that widening of a lumen at the site of the lesion not necessarily implies a regression of the atherosclerotic process [8]. Further studies on the constitution of the plaques

are needed to assess whether the data on intimal thickening in the present investigation represent a true regression of the atherosclerotic process.

Acknowledgements:

We are grateful to Mrs. M.C. Dubelaar for the biochemical analysis of the aortic biopsies.
This study was supported by grant 86-086 from the Netherlands Heart Foundation.

REFERENCES

1. Blankenhorn DH, Nessim SA, Jofnson RL, Sanmarco, Azen SP, Cashin-Hemphill L. Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts. JAMA 1987; 257: 3233-3240.
2. Armstrong ML, Warner ED, Connor WE. Regression of coronary atheromatosis in rhesus monkeys. Circ Res 1970; 27:59-67.
3. Armstrong ML, Megan MB. Lipid depletion in atheromatous coronary arteries in rhesus monkeys after regression diets. Circ Res 1972; 30: 675-680.
4. Clarkson TB, Bond MG, Bullock BC, McLaughlin, Sawyer JK. A study of atherosclerosis regression in Mamaca mulatta. Exp Mol Pathol 1984; 41: 96-118.
5. Eggen DA, Strong JP, Newmann WP, Malcom GT, Restrepo C. Regression of experimental atherosclerotic lesions in rhesus monkeys consuming a high saturated fat diet. Arteriosclerosis 1987; 7: 125-134.
6. Fritz KE, Augustyn JM, Jarmolych J, Daoud AS. Sequential study of biochemical changes during regression of swine aortic atherosclerotic lesions. Arch Pathol Lab Med 1981; 105: 240-246.
7. Daoud AS, Jarmolych J, Augustyn JM, Fritz KE, Singh JH, Lee KT. Regression of advanced atherosclerosis in swine. Arch Pathol Lab Med 1976; 100: 372; 372-379.
8. St Clair RW. Atherosclerosis regression in animal models: current concepts of cellular and biochemical mechanisms. Prog Cardiovasc Dis 1983; 26 (2): 109-132.
9. Weiner BH, Ockene JS, Levine PH et al. Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. New Engl J Med 1986; 315: 841-846.
10. Hartog JM, Lamers JMJ, Montfoort A et al. Comparison of mackerel-oil and lard-fat enriched diets on plasmalipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am J Clin Nutr 1987; 46: 258-266.
11. Hartog JM, Verdouw PD, Klompe M, Lamers JMJ. Dietary mackerel oil in pigs: Effect on plasmalipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. J Nutr 1987; 117: 1371-1378.
12. Nam SC, Lee WM, Jarmolych J, Lee KT, Thomas WA. Rapid production of advanced atherosclerosis in swine by a combination of endothelial injury and cholesterol feeding. Exp Mol Path 1973; 18: 369-379.

13. Fossati R, Prenoipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 1982; 280: 2077-2080.
14. Siedel J, Schlumberger H, Klosse S, Ziegenhorn J, Wahlefeld AW. Improved reagent for the enzymatic determination of serum cholesterol. J Clin Chem Biochem 1981; 19: 838-839.
15. Warnick GR, Albers JJ. A comprehensive evaluation of the heparin-manganese precipitation procedure for estimation high density lipoprotein cholesterol. J Lipid Res 1978; 19: 65-76.
16. Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behaviour in blood. J Pharmacol Meth 1980; 3: 135-158.
17. Hornstra G, Christ-Hazelhof E, Haddeman E, Ten Hoor F, Nugteren DH. Fish oil feeding lowers thromboxane and prostacyclin production by rat platelets and aorta and does not result in the formation of prostaglandin I_3. Prostaglandins 1981; 21: 727-738.
18. Hanley SP, Bevan J, Cockbill SR, Heptinstall S. Differential inhibition by low-dose aspirin of human venous prostacyclin synthesis and platelet thromboxane synthesis. Lancet I 1981;969-971.
19. Lamers JMJ, Hartog JM, Verdouw PD, Hülsmann WC. Dietary fatty acids and myocardial function. Basic Res Cardiol 1987; (Suppl.I): 209-221.
20. Gryglewski RJ, Salomon JA, Ubatuba FB, Weatherly BC, Moncada S, Vane JR. Effects of all cis-5, 8, 1, 14, 17 eicosapentaenoic acid and PGH3 on platelet aggregation. Prostaglandins 1979; 18(3): 453-478.
21. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911-918.
22. Jacobs EE, Jacob M, Sanad DR, Bradley LB. Uncoupling of oxidative phosphorylation by cadmium ion. J Biol Chem 1966; 223: 147-156.
23. Geriotti G. A microchemical determination of desoxyribonucleic acid. J Biol Chem 1952; 198: 297-303.
24. Weiner BH, Ockene JS, Jarmolych J, Fritz KE, Daoud AS. Comparison of pathologic and angiographic findings in a porcine preparation of coronary atherosclerosis. Circulation 1985; 72: 1081-1086.
25. Polimeni PI, Cunningham P, Otten MD, McCrea D. Morphometric quantification of atherosclerotic plaques by computer-assisted image-analysis of histographs. Comp and Biomed Res 1987; 20: 113-124.
26. Hartog JM, Lamers JMJ, Verdouw PD. The effects of dietary mackerel oil on plasma and cell membrane lipids, on the hemodynamics and cardiac arrhythmias during recurrent acute ischemia in the pig. Basic Res Cardiol 1986; 81: 567-580.
27. Hartog JM, Lamers JMJ, Achterberg PW, van Heuven-Nolsen D, Nijkamp FD, Verdouw PD. The effects of dietary mackerel oil on the recovery of cardiac function after acute ischaemic events in the pig. Basic Res Cardiol 1987; (Suppl.1): 223-234.
28. Fischer S, Weber PC. Thromboxane A_3 (TXA_3) is formed in human platelets after dietary eicosapentaenoic acid (C20:5 w3). Bioch Biophys Res Comm 1983; 116(3): 1091-1099.
29. Fischer S, Weber PC. Prostaglandin I_3 is formed in man after dietary eicosapentaenoic acid. Nature 1984; 307: 165-168.
30. Small DM, Progression and regression of atehrosclerotic lesions. Insights from lipid physical biochemistry. Arteriosclerosis 1988; 8: 103-129.

31. Khan MA, Earl FL, Farber TM et al. Elevation of serum cholesterol and increased fatty streaking in egg yolk: Lard fat castrated miniature pigs. Exp Mol Pathol 1977; 26: 63-74.
32. Leaf A, Weber PC. Cardiovascular effects of n-3 fatty acids. New Engl J Med 1988; 318: 549-557.
33. Metha J, Lopez LM, Wargorich T. Eicosapentaenoic acid: its relevance to atherosclerosis and coronary artery disease. Am J Cardiol 1987; 59: 155-159.

CHAPTER 14

EPICRISIS

The experimental data described in this thesis show that dietary n-3 polyunsaturated fatty acids (PUFA's) exert some actions which may be beneficial in the prevention and treatment of cardiovascular disease.

We arrived at this conclusion by comparing the effects of fish oil diets with a control diet enriched with lard fat. The control as well as the fish oil diet supplied sufficient amounts of n-6 PUFA's. Hence, the diets used in our studies only differed in fatty acid composition.

As summarized in chapter 1 fish oil has a strong hypotriglyceridemic and a moderate hypocholesterolemic effect in man. In our studies in pigs (chapters 4 and 5) the hypocholesterolemic effect was, at variance with the hypotriglyceridemic effect, dose dependent. Comparing our results with those of Ruiter et al. [1], we conclude that in normolipidemic pigs the hypotriglyceridemic effect reaches a maximum at a daily dose of 150 mg EPA/kg bodyweight, but that the hypocholesterolemic effect is only observed at much higher doses (*table 1*).

Table 1
Effects of dietary n-3 PUFA's on plasma cholesterol (CHOL) and triglyceride (TG) levels in randomized controlled studies in normolipidemic pigs.

REFERENCE	grams EPA/kg/day	PERIOD IN WEEKS	CONTROL	change in % CHOL	change in % TG
Ruiter et al. (1)	0.15	4	OLIVE OIL	+3	-44*
Hartog et al. (chapter 5)	0.30	16	LARD FAT	-29*	-54*
Hartog et al. (chapter 4)	0.60	8	LARD FAT	-47*	-60*

* = $p < 0.05$ versus control group

The lowering of triglycerides occurred almost exclusively in VLDL, but that of cholesterol was observed in all lipoproteins (chapter 6). Based on the decrease of lipoprotein lipase activity it is reasonable to assume that fish oil reduces VLDL secretion and that the reduction of cholesterol content of the other lipoproteins is a

secondary phenomenon. Increased clearance of VLDL and of faecal bile-salts by fish oil have also been reported, but most other reports indicate that fish oil decreases VLDL synthesis (chapters 1 and 6). In hypercholesterolemic pigs (chapter 13) plasma triglyceride levels did not change, but the fall in plasma cholesterol was accelerated and increased by fish oil in the regression period. In that study hypercholesterolemia was induced by high cholesterol feeding prior to the introduction of fish oil containing low cholesterol diets. The plasma cholesterol is mainly carried in the LDL particle [2]. Therefore the hypocholesterolemic effect of the fish oil is less than in the normolipidemic pigs (final plasma cholesterol level 1.7 versus 1.3 mM; chapters 4 and 13).

Plasma cholesterol is a major risk factor for atherosclerosis (chapter 12). Fish oil can retard the development of atherosclerotic lesions by its cholesterol lowering properties. However, the mean plasma cholesterol levels (5.6 mM) of the Eskimos [3] do not differ from people of similar age living in the USA (5.4 mM) [4], or The Netherlands (5.7 mM) [5]. Vegans and vegetarians in Western countries have even lower plasma cholesterol levels (4.3 and 4.9 mM, respectively) than Eskimo's [3, 6, 7]. Therefore, it is likely that other than the hypocholesterolemic effects of the n-3 PUFA's play also a role in the prevention of ischemic heart disease.

In our studies (chapters 9, 10 and 11) the main fish oil PUFA's eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were incorporated in cardiac, sarcolemma and platelet membrane phospholipids. At the same time arachidonic acid content of the phospholipids was lowered, which confirms other reports (chapter 1). Although the myocardial sarcolemma membrane composition and double bond index were markedly changed in the fish oil fed pigs (chapters 8, 9 and 10), minor effects were seen in the functional properties of isolated membrane vesicles. For example the activities of the 5' nucleotidase and Ca^{2+} pumping ATPase after Ca^{2+} stimulation were slightly higher, and adenylate cyclase was more sensitive to isoproterenol stimulation in the fish oil fed group (chapters 3 and 10). The latter finding is interesting in view of the observations of Gudbjarnason et al. made some 10 years ago [8]. They found an increased mortality in cod liver oil fed rats induced by catecholamines (1-5 mg/kg norepinephrine s.c.). We could not confirm Gudbjarnason's data in a similar study, using a daily dose of 1-4 mg/kg norepinephrine suspended in PEG 400 s.c. in order to slow down the release (chapter 7). The differences in mode of cathecholamine administration may have influenced the mortality rates. Administration of norepinephrine via an implanted osmotic pump resulted in no mortality at all, but also less pronounced effects on fatty acid composition of the phospholipids (chapter 7). It should be noted, however, that in a more recent study with a daily dose of 1 mg/kg isoproterenol s.c. Gudbjarnason et al. were unable to reproduce their own observations [9].

During the dietary period plasma malondialdehyde (MDA) and lipofuscin, as a measure of in vivo lipidperoxidation, were not increased in the fish oil (0.6 g EPA/kg

body weight/day) fed pigs (chapter 10). Isolated sarcolemmal membranes of fish oil fed pigs exposed to a free radical generating system showed an increased MDA production (chapter 10). The addition of natural antioxidants like vitamin E and selenium to the diets probably reduced the rate of peroxidation of the PUFA's in vivo. Indeed, morphological examination of the fish oil fed pigs gave no signs of lipid peroxidation in organ tissues (chapter 4 and 5), except for some in the subcutaneous fat tissue. The pigs receiving 0.6 g EPA/kg bodyweight/day for 8 weeks had an increased lipofuscin content in the subcutaneous fat, which indicates a slight vitamin E deficiency (LHJC Danse, personal communication). For this reason the vitamin E content of the diets was increased when we investigated the effects of fish oil on atherosclerosis (chapter 13). Here we found no signs of yellow fat disease. The finding that the heart produced MDA in fish oil fed pigs, but not in lard fat fed pigs was surprising. Experimentally induced ischemia-reperfusion, did not affect the rate of this MDA production in the fish oil fed pigs (chapter 10). This observation is important as free radicals are believed to play a crucial role in the development of damage in the ischemic, reperfused, or infarcted myocardium.

Cardiac function during normoxia was not affected by dietary fish oil (chapters 4, 5, 8 and 9). During multiple short-lasting intermittent occlusions the hyperemic response was prolonged in the fish oil fed animals (chapters 8 and 9), probably by changes in the thromboxane-prostacyclin ratio (TXA/PGI). In one study the incidence of reperfusion arrhythmias was slightly lowered after fish oil feeding (chapter 9). The mechanism of this effect can not be deducted from this study as this ischemia-reperfusion model was designed to measure the recovery of cardiac function after repeated ischemic events and not to study the mechanism of reperfusion arrhythmias. Several other investigators have reported on the protective effects of dietary fish oil on infarct size [10-12]. Changes in TXA/PGI ratio may be responsible for these beneficial effects of fish oil consumption in these experiments [10-12].

In pigs with fixed coronary artery stenosis the cardiac function was better preserved in the fish oil fed animals during a pacing stress test (chapter 11). Myocardial blood flow to the jeopardized region was also better preserved, as was the distribution of the myocardial bloodflow to the subendothelial layers. The latter could not be explained by differences in systemic hemodynamic parameters and further studies are required to confirm this observation. The improved suendocardial blood flow was also not caused by an increased collateral blood flow (chapter 11). Examination of the stenosed vessels showed less intimal proliferation in the fish oil fed pigs.

The TXA generation was decreased after fish oil consumption (chapters 8, 9 and 13), while the PGI production was decreased to a lesser extent (chapter 8 and 9). Others have reported no effect on PGI prodution while TXA synthesis was decreased (chapter 1). Differences in doses and subsequent phospholipid EPA

content may account for these controversial effects. The TXA_3 generation by platelets, shown in chapter 13, was only a small fraction (<15%) of the total TXA generation. The changes in prostanoid concentrations may have an important impact on the platelet-vessel wall interaction. The conclusions of our studies and other reports (chapter 1) are summarized in *figure 1.*

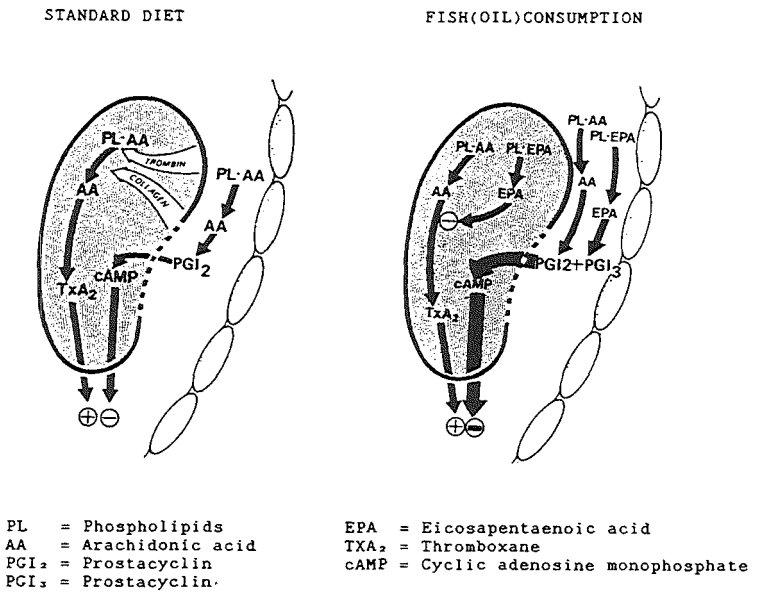

Figure 1 Prostanoid synthesis and platelet function

Platelet aggregation induced by low doses of ADP was decreased after fish oil consumption (chapters 11 and 13). In the hypercholesterolemic pigs the platelet aggregation was not affected in both fish oil (0.2 and 0.4 g EPA/kg bodyweight/day) treated groups (chapter 13). Our measurements were performed in whole blood. At present this method has not been used on this issue by others yet, but it seems preferrable as it is a more direct method (chapter 1). In vivo methods would be most suitable, but are not available.

In the pigs with the fixed coronary artery stenosis fish oil attenuated the extent of intimal proliferation (chapter 11). We believe that in this modified arterial thrombosis model wall shear forces at the site of the constrictor have produced local hemolysis and denudation of the vessel wall. ADP released by the damaged erythrocyts and collagen of the denuded vessel wall have led to platelet adherence to the vessel wall, platelet aggregation was initiated which ultimately led to the formation of a mural thrombus. TXA and granular products like platelet derived

growth factor are released leading to vasoconstriction, secondary platelet aggregation and intimal proliferation. Fish oil may have attenuated this process primarily by reduction of the TXA synthesis, rather than by lowering the plasma cholesterol levels, as these were also low in the lard fat fed animals (chapter 11). Nearly 30% of the patients undergoing percutaneous transluminal coronary angioplasty (PTCA) suffer restenosis of the coronary artery by thrombosis and/or intimal proliferation within six months after the procedure [13, 14]. According to the "response to injury theory" by Ross [15] endothelial damage and flow turbulences initiate platelet aggregation and release of mitogenic factors, leading to migration of smooth muscle cells and monocyts and subsequent intimal proliferation. Therefore, fish oil might have a beneficial in patients undergoing PTCA procedures. Indeed, fish oil has prevented restenosis in a few studies on PTCA patients [16, 17, 18]. As in the aforementioned studies [16, 17] the plasmacholesterol levels were not affected by the fish oil supplements the inhibition of platelet vessel wall interaction is probably the major contribution to the prevention of restenosis in these patients. These studies, however, need to be confirmed in more controlled investigations.

The lesions of coronary arteries and abdominal aorta found in pigs after a 4 months induction period (chapter 13) were comparable to the data reported by Weiner et al. and Fritz et al. as shown in *table 1 and 3* in the overview of chapter 12. However, more marked lesions are found after a longer induction period (chapter 12).

In the atherosclerotic pigs fish oil consumption caused a marked regression of luminal encroachment of the abraded left anterior descending coronary artery, but had no effect on the minor lesions found in the also abraded abdominal aorta (chapter 13). The cholesterol lowering effect of dietary fish oil may have played a major role in this regression. The platelet aggregation was decreased by the low dose of fish oil, but was not by the high dose of fish oil. The data on TXA generation were not in agreement with the data on platelet aggregation in this study. Both measurements (platelet aggregation and TXA generation) were, however, performed in vitro. The physiological role of endothelium and its product PGI should also be taken into account, as the TXA/PGI ratio is the main determinant of in vivo platelet aggregation. In other experiments it was shown that the TXA/PGI ratio was increased after fish oil consumption (chapters 8 and 9). Therefore, the in vitro aggregation tests may mask the in vivo situation. There seems to be a discrepancy between the effects on luminal encroachment of the coronary arteries and lipid content of the aortic wall after fish oil consumption (chapter 13). Futher long-lasting studies may help to clarify this issue. Perhaps the three month regression period is to short to establish an effect on the lipid content of the arterial lesions (chapter 12).

It has been established that ischemia and reperfusion damage of myocardial cells is caused by free radical generation, intracellular Ca^{2+} overload and phospholipid breakdown (chapter 7 and 10). In our experiments we found no effect on in

vivo MDA production (chapter 10). The in vitro Ca^{2+} stimulated Ca^{2+} pumping ATPase activity was slightly higher in the fish oil fed pigs, while the basal activity was not affected (chapter 10). Presently there is no evidence that the enzymes involved in the breakdown of phospholipids are influenced by dietary fish oil (chapter 3 and 7).

Progressive Ca^{2+} overload appears also to be an important latent precursor of overt arteriosclerosis [19]. Whether modification of Ca^{2+} overload plays a role in the prevention of atherosclerosis by fish oil consumption needs to be investigated.

From the preceding paragraphs it is clear that dietary n-3 fatty acids may be beneficial in the prevention of development of ischemic heart disease. The mechanisms by which this can occur are schematically presented in *figure 2.*

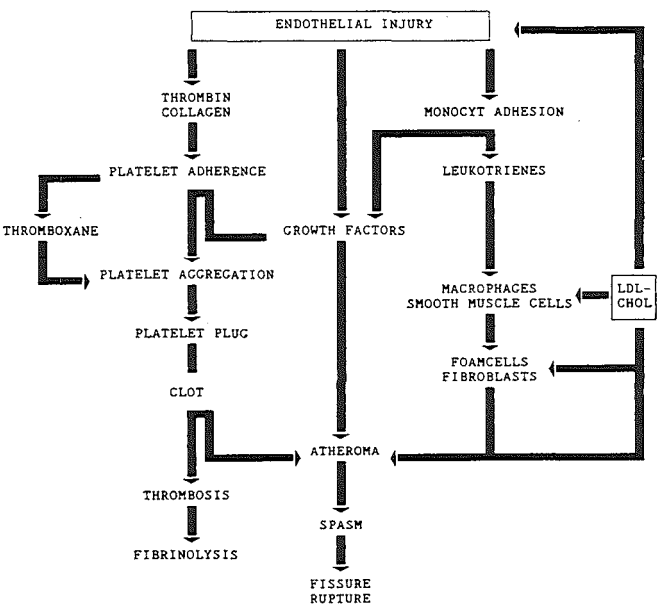

Figure 2 The atherosclerotic proces

Endothelial injury and LDL-cholesterol are according to current views the primary factors which initiate the development of an atherosclerotic lesion (chapter 12). Endothelial injury can be caused by mechanical stress (e.g. hypertension, high blood viscosity), or by viral, immunological, chemical (e.g. smoking, lipid peroxidation), or radiation origin. In this context it is interesting that immunological responses can also be modified by fish oil consumption [20, 21]. Lipid peroxidation could be increased after high n-3 PUFA intake, but this can be prevented, at least in the pig, by dietary supplementation of natural antioxidants (chapter 10). Dietary fish oil

definitely affects the prostaglandin metabolism and thereby the responses to endothelial injury, but this issue needs further investigation. The shift in TXA/PGI ratio by fish oil may also play a role in the prevention of coronary spasm. The hypocholesterolemic effect of the high doses of n-3 PUFA's found in all our studies can not be disregarded, but its importance is still controversial. Other possible effects of n-3 fatty acids on the process of atherosclerosis have recently been discussed by Leaf and Weber [22].

The extent of atherosclerosis in man is difficult to assess. Intervention studies on the arrest of atherosclerosis in man deal with the difficulties of long follow up and less sensitive methods on quantification [23-25]. At present angiography is the only clinical tool to evaluate atherosclerotic lesions. In the future doppler-, echographic- and magnetic resonance phase shift techniques may offer possibilities to quantify the extent of atherosclerosis more precisely. A newly developped technique to study arteriolar flow is the retinal fluorotachometry [26], which may offer possibilities to quantify arteriosclerosis.

In conclusion dietary fish oil produces several biochemical effects which potentially modify the development and regression of atherosclerotic lesions. Whether these effects found in experimental studies can be extrapolated to the human situation requires clinical trials. Fish oil supplements could then become a valuable part of the therapeutic arsenal against ischemic heart disease. Until now only mixtures of EPA and DHA were tested. However, recently pure concentrates of EPA and DHA have become available which enables investigators to study seperately the effects of these fatty acids. As n-6 PUFA's share many but not all beneficial effects with n-3 PUFA's, comparison with a n-6 PUFA's rich diet is relevant too. The provided evidence in this thesis of the effects of dietary fish oil on plasmalipid levels, prostaglandin synthesis, platelet aggregation and atherosclerosis warrants further clinical trials. However, combination of fish oil supplements with anticoagulants will lead to increased bleeding tendency and, therefore, must be dissuaded. Another important message of this thesis is that n-3 PUFA's are susceptible to peroxidation, but this seems to be prevented by addition of natural antioxidants to the diet. It is premature to give a dietary advice on the base of this thesis, as the effects described in this thesis were found after rather high doses of fish oil. The inclusion of several fish dishes per week will supply only a modest amount of n-3 PUFA's and it is unpredictable if such a diet will exert an antiatherogenic effect.

REFERENCES

1. Ruiter A, Jongbloed AW, van Gent CM, Danse LHJC, Metz SHM: The influence of dietary mackerel oil on the condition of organs and on blood lipid composition in the young growing pig. Am J Clin Nutr 1978; 31: 2159-66.
2. Shimokawa A, van Houtte PM: Dietary cod-liver oil improves endothelium-dependent responses in hypercholesterolemic and atherosclerotic porcine coronary arteries. Circulation 1988; 78: 1421-30.
3. Bang HO: More on fish oil. New Engl J Med 1987; 316: 624-5.
4. Blackburn H, Heyden S, Berenson GS, Jacobs D, Christakis G, Joosens JV, Christian JC, Kagan A, Epstein F, Kannel WB, Feinleib M, Morrison JA, Havas S, Roberts NJ, Heiss G, Wijnder EL. Conference on the health effects of blood lipids: Optimal distributions for populations. Prev Med 1979; 8: 612-78.
5. Verschoor L: Bij wie moeten metingen van serumlipiden worden verricht? Hart Bull 1987; suppl 1: 41-3.
6. Knuiman JT, Katan MB: Cholesterol niveaus in serum in Nederland in vergelijking met die in de Verenigde Staten. Ned Tijdschr Geneesk 1985; 129: 2500-05.
7. Thorogood M, Carter R, Benfield L, Mc Pherson, Mann JI: Plasma lipids and lipoprotein cholesterol concentrations in people with different diets in Brittain. Brit Med J 1987; 295: 351-3.
8. Gudbjarnason S, Oskarsdottir G, Doell B, Hallgrimsson J: Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 1978; 25: 130-44.
9. Gudbjarnason S, Benediktsdottir E: Role of arachidonic acid metabolism in development of fatal ventricular fibrillation in rats. J Mol Cell Cardiol 1985; 17: 112A.
10. Black KL, Culp B, Madison D, Randall OS, Lands WEM: The protective effect of dietary fish oil on cerebral infarction. Prostaglan Med 1979; 5: 257-68.
11. Culp BR, Lands WEM, Lucchesi BR, Pitt R, Romson J: The effects of dietary supplementation of fish oil on experimental myocardial infarction. Prostaglandins 1980; 20: 1021-31.
12. Hock CE, Holahan MA, Reibel DK: Effect of dietary fish oil on myocardial phospholipids and myocardial ischemic damage. Am J Physiol 1987; 21: H554-60.
13. Furster V, Adams PC, Badimon JJ, Chesebro JH. Platelet inhibitor drugs role in coronary artery disease. Prog Cardiovasc Dis 1987; 29: 325-467.
14. Essed CE, Van den Brand M, Becker AE. Transluminal coronary angioplasty and early restenosis. Br Heat J 1983; 49: 393-6.
15. Ross R. The pathogenesis of atherosclerosis - an update. N Engl J Med 1986; 314: 488-500.
16. Slack JD, Pinkerton CA, van Tassel J: Can oral fish oil supplement minimize restenosis after percutaneous transluminal coronary angioplasty? J Am Coll Card 1987; 9: 64A.
17. Dehmer GJ, Popma JJ, van den Berg EK, Eichhorn EJ, Prewitt JB, Campbell WB, Jennings L, Willerson JT, Schmitz JM: Reduction in the rate of early restenosis after angioplasty by a diet supplemented with n-3 fatty acids. N Engl J Med 1988; 319: 733-40.

18. Dehmer GJ, Schmitz JM, Willerson JT. Reduction in coronary restenosis with n-3 fatty acids. N Engl J Med 1989; 320: 466-7.
19. Fleckenstein A, Fleckenstein-Grün G. Cardiovascular protection by calcium antagonists. Eur Heart J 1980; 1 (Suppl B): 15-21.
20. Lee TH, Hoover RL, Williams JD, Sperling RI, Ravalese J, Spur BW, Robinson DR, Gorey EJ, Lewis RA, Austen KF. Effects of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. N Engl J Med 1985; 312: 1217-24.
21. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JWM, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaefer EJ, Wolff SM, Dinarello CA: The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989; 320: 265-71.
22. Leaf A, Weber PC. Cardiovascular effects of n-3 fatty acids. N Engl J Med 1988; 318: 549-57.
23. Manilow MR, Blaton V. Regression of atherosclerotic lesions. Arteriosclerosis 1984; 4: 292-5.
24. Arntzenius AC, Kromhout D, Barth JD, Reiber JHC, Bruschke AVG, Buis B, Van Gent CM, Kempen-Voogd N, Strikwerda S, Van der Velde EA. Diet, lipoproteins and the progression coronary atherosclerosis. N Engl J Med 1985; 312: 805-11.
25. Blankenhorn DH, Nessim SA, Johnson RL, Sanmarco ME, Azen SP, Cashin-Hempill L. Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass graft. JAMA 1987; 257: 3233-40.
26. Schulte AVMCL. Retinal fluorotachometry. Academic thesis: Erasmus University Rotterdam, The Netherlands, Rotterdam, 1986.

SAMENVATTING

Uit epidemiologische onderzoeken onder veel vis etende Eskimo's en Japanners is gepostuleerd dat de meervoudige onverzadigde lange keten vetzuren van de n-3 familie in de vis beschermen tegen ischemische hartziekten.

De laatste decennia is veel onderzoek gedaan naar de effecten van deze n-3 vetzuren. Echter de resultaten van deze studies waren vaak controversieel. Dit kan voor een deel verklaard worden door een verschil in dosering of de keuze van het controle dieet, zoals besproken in het overzichts verhaal (hoofdstuk 1). Vele studies waren echter ongecontroleerd.

De opzet van dit proefschrift was om via goed gecontroleerde studies meer duidelijkheid te verschaffen over de effecten van n-3 vetzuren op de verschillende onderdelen van het atherosclerotisch proces. Voor deze studies werd een biggen model gekozen. Het varken vertoont namelijk grote overeenkomsten met de mens in anatomie, fysiologie en vet stofwisseling. Een studie naar catecholaminen gevoeligheid in levertraan gevoede ratten was gebaseerd op eerder onderzoek van Gubjarnason et al. (hoofdstuk 7).

In de in dit proefschrift beschreven studies werd een cholesterol en vooral triglyceriden verlagend effect van visolie aangetoond (hoofdstuk 4, 5 en 6). De effecten worden verklaard door een verlaging van de VLDL synthese (hoofdstuk 1 en 6). De plasmacholesterol verlagende effecten worden pas waargenomen bij hoge doseringen n-3 meervoudig onverzadigde vetzuren. De geconsumeerde n-3 vetzuren worden ingebouwd in de membranen van hart, bloedplaatjes en andere cellen. De inbouw van n-3 meervoudig onverzadigde vetzuren (eicosapentaeenzuur , 20:5 n-3, en docosahexaeenzuur 22:6 n-3) gaat ten kosten van de hoeveelheid arachidonzuur (20:4 n-6) in de membraan fosfolipiden (hoofdstuk 4, 5, 7, 8, 9 en 11). Hierdoor worden de eigenschappen van deze membranen veranderd (hoofdstuk 1, 3 en 10). De prostaglandinen synthese ondergaat grote veranderingen na visolie consumptie. De thromboxaan synthese wordt fors verlaagd terwijl de prostacycline productie minder wordt verlaagd (hoofdstuk 8 en 9). Ook worden er prostaglandinen uit n-3 vetzuren gesynthetiseerd, maar dit is echter slechts een fractie (<15%) van de totale productie (hoofdstuk 13). De bloedplaatjes aggregatie is verminderd na visolie consumptie. In een biggenmodel met een lichte vernauwing van de kransslagader bleek visolie de intima proliferatie te remmen, vermoedelijk via een remming van de interactie tussen bloedplaatjes en de beschadigde vaatwand (hoofdstuk 11). In atherosclerotische biggen gaf visolie een regressie van de kransslagader vernauwingen, waarschijnlijk via een krachtige verlaging van het plasmacholesterol (hoofdstuk 13). In de hier beschreven studies werden geen effecten van visolie op bloeddruk gevonden. Wel gaf visolie een versterkte doorbloeding van de kransslagader tijdens reperfusie (hoofdstuk 8 en 9) en een verlaging van het aantal ritmestoornissen tijdens reperfusie in een van deze studies (hoofdstuk 9). De versterkte bloeddoor-

stroming van de kransslagader na een kortdurende afsluiting van dit vat wordt waarschijnlijk veroorzaakt door de veranderingen in prostaglandinen synthese.

Concluderend, visolie heeft effecten op verschillende onderdelen van het atherosclerotisch proces. Echter de effecten op bloedplaatjes - vaatwand interactie moeten nog nader worden onderzocht, want juist dit onderdeel lijkt een van de belangrijkste aangrijpingspunten te zijn. Het is waarschijnlijk dat visolie supplementen een waardevolle rol kunnen spelen in de therapie van ischemische hartziekten. Verdere klinische evaluatie zal moeten aantonen of de in dit proefschrift beschreven effecten van hoge doseringen visolieconcentraten ook bij de mens haalbaar zijn. Het gebruik van visolie preparaten in combinatie met antistolling moet worden afgeraden in verband met een verhoogde bloedingsneiging. Het is echter niet mogelijk om op basis van dit proefschrift een algeheel voedingsvoorschrift te geven. Het nuttigen van enkele vismaaltijden per week zal slechts een geringe hoeveelheid n-3 meervoudig onverzadigde vetzuren opleveren. Het is tot op heden niet duidelijk of dergelijke hoeveelheden beschermen tegen hart en vaatziekten.

DANKWOORD

Bij het tot stand komen van dit proefschrift ben ik velen dank verschuldigd voor de geboden hulp.

Ik dank mijn promotoren Prof. P.G. Hugenholtz en Prof.Dr. W.C. Hülsmann voor de geboden mogelijkheden om dit onderzoek te kunnen verrichten en hun begeleiding bij de vervaardiging van dit proefschrift.

Ik dank Dr. P.D. Verdouw hoofd van de afdeling experimentele cardiologie die mij introduceerde in het wetenschappelijk onderzoek en voor zijn vertrouwen in mij en steun. Dr. J.M.J. Lamers dank ik voor de stimulerende samenwerking en uitgebreide adviezen. Ik weet dat zij de jaren van samenwerking graag bekroond hadden gezien met voltooien van de Rotterdam marathon, maar hierbij heb ik wegens blessures helaas verstek moeten laten gaan.

Grote dank gaat uit naar mijn naaste medewerkers Dhr. R.H. van Bremen, Dhr. R.J. Rensen, Mw. A.M. Rutteman, Dhr. W.B. Vletter, Dhr. J.P.C. Heiligers, Mw. L. van der Werf, Mw. M. Groh-Hoogenboom, Mw. J.T. de Jonge-Stinis, Mw. M. Dubelaar, Dhr. L.M. Scheek, Drs. L.M. Sassen, Drs. L.J. van Woerkens, Dr. P.W. Achterberg en Drs. W.J. van der Giessen.

Ik dank Dhr. H. Morse en Dr. M.C. Blok voor hun adviezen bij de samenstelling van de dieten.

Dr. M. Klompe, Dr. A. Montfoort, Drs. C.E. Essed, Dr. P.H.E. Groot, Prof.Dr. J.F. Koster, Dr. A. van Tol, Dr. F.J. ten Cate, Ir. C.J. Slager, Dr. G. Hornstra, Dr. L.H.J.C. Danse, Dr. W.C. de Bruin, Dr. D. van Heuven-Nolsen, Dr. F.P. Nijkamp, Dr. F.J. Zijlstra, Prof.Dr. J.R.T.C. Roelandt, Prof.Dr. J.F. Jongkind, Prof.Dr. A.E. Becker en Prof.Dr. A.J. Vergroesen dank ik voor hun hulp of adviezen.

Dhr. E.W.M. Lansbergen, Dhr. R. Bunk, Dhr. W.J. Kruidenier en Dhr. M.E.L. van Wassenaer van het Centraal Proefdieren Bedrijf dank ik voor hun medewerking.

De medewerkers van het Laboratorium voor Experimentele Chirurgie ben ik grote dank verschuldigd voor hun assistentie: Dhr. J. Kasbergen, Mw. J. de Kam, Dhr. E. Ridderhof, Dhr. E.C.C. Collij, Dhr. R.C. Spruyt en Dhr. W.P.Schalkwijk.

Mijn opleiders tijdens mijn stage Inwendige Geneeskunde Drs. C. Verdoorn en Dr. B.P. Hazenberg wil ik danken voor hun steun bij de tot standkoming van dit proefschrift.

Merck Sharp & Dohme, Hastar Enterprises, Wyeth, Sanofi, Squibb, Eli Lilly en Bristol-Myers worden bedankt voor de verleende steun.

Grote dank ben ik verschuldigd aan Mw. P.H. Vegter, Mw. M.A. van Ee, Dhr. B. de Haan, Mw. T. Jansen, Mw. M.J. van Bergen en Mw. T.R. Spruyt-Kramer voor hun hulp bij de vervaardiging van dit proefschrift.

Bovenal echter wil ik mijn vrouw danken voor haar steun en verdraagzaamheid gedurende de afgelopen jaren.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 16 juni 1957 te Rotterdam geboren. In 1976 behaalde hij het eindexamen Atheneum B aan de Scholengemeenschap Casimir te Vlaardingen. Tijdens de studie geneeskunde aan de Erasmus Universiteit te Rotterdam was hij werkzaam op het laboratorium voor Experimentele Cardiologie onder begeleiding van Dr. P.D. Verdouw. Na het artsexamen in februari 1984 was hij werkzaam als wetenschappelijk medewerker op deze afdeling. Van mei 1987 tot mei 1989 volgde hij de stage Inwendige Geneeskunde bij Drs. C. Verdoorn en Dr. B.P. Hazenberg in het Diakonessenhuis Refaja te Dordrecht. Vanaf juni 1989 volgt hij de opleiding tot cardioloog in het Thoraxcentrum van het Academisch Ziekenhuis Rotterdam "Dijkzigt" onder leiding van Professor Dr. J.R.T.C. Roelandt.

LIST OF PUBLICATIONS

Verdouw, P.D., Hartog, J.M., Ten Cate, F.J. & Hugenholtz, P.G. Beschermende werking van nifedipine tijdens reperfusie van het ischemische hart. Hart Bulletin suppl. 1: 15-18, 1980.

Verdouw, P.D., Hartog, J.M. & Rutteman, A.M. Systemic and regional myocardial responses to AR-L 115 BS, a positive inotropic imidazo-pyridine, in the absence or in the presence of the bradycardiac action of alinidine. Basic Res. Cardiol. 76: 328-343, 1981.

Verdouw, P.D., Hartog, J.M., Ten Cate, F.J., Schamhardt, H.C., Bastiaans, O.L., van Bremen, R.H., Serruys, P.W. & Hugenholtz, P.G. Effects of nifedipine on the recovery of regional myocardial performance during reperfusion of ischemic myocardium. Progr. Pharmacol. 4/2: 91-100, 1981.

Verdouw, P.D., Ten Cate, F.J., Hartog, J.M., Scheffer, M.G. & Stam, H. Intracoronary infusion of small doses of nifedipine lowers regional myocardial O_2-consumption without altering regional myocardial function. Basic Res. Cardiol. 77, 26-33, 1982.

Verdouw, P.D., Hartog, J.M., Scheffer, M.G., van Bremen, R.H. & Dufour, A. The effects of Cibenzoline, an imidazoline derivative with antiarrhythmic properties, on systemic hemodynamics and regional myocardial performance. Drug Dev. Res. 2: 519-532, 1982.

Verdouw, P.D., Hartog, J.M. & Roelandt, J.R.T.C. Het hart blijft op de goede plaats. Loopsport 10: 25-27, 1984.

Verdouw, P.D., Hartog, J.M., Rijsterborgh, H., Vletter, W.B., McGhie, J., Gorissen, W., Ten Cate, F.J. & Roelandt, J.R.T.C. Sporthart bij lange afstandlopers. Arts in Beweging 3: 8-9, 1985.

Hartog, J.M. & Verdouw, P.D. Alleviation of myocardial ischaemia after administration of the cardioselective beta- adrenoceptor antagonist bevantolol. Cardiovasc. Res. 20: 264- 268, 1986.

Verdouw, P.D. & Hartog J.M. Provocation and suppression of ventricular arrhythmias in domestic swine. In: Swine in Cardiovascular Research. Eds: Stanton H.C. and Mersmann H.J.. CRC Press Inc., Boca Raton, Florida, Vol II, 121-156, 1986.

Verdouw, P.D., Hartog, J.M., Wolffenbuttel, B.H.R., Berk, L., Duncker, D.J., Schmeets, I.O.L., Sassen, L.M.A., Rensen, R.J., Bremen, R.H. van & Hugenholtz, P.G. Intracoronary and intravenous effects of calcium antagonists on cardiac efficiency after and without beta-adrenoceptor blockade. In: New Therapy of Ischaemic Heart Disease and Hypertension. Ed: Lichtlen P.R., 347-356, 1986.

Duncker, D.J., Hartog, J.M., Hugenholtz, P.G., Saxena, P.R. & Verdouw, P.D. The effects of nisoldipine (BAY K 5552) on cardiovascular performance and regional blood flows in pentobarbital - anaesthetized pigs with or without beta-adrenoceptor blockade. Br. J. Pharmacol. 88: 9-18, 1986.

Verdouw, P.D., Hartog, J.M., Saxena, P.R. & Hugenholtz, P.G. Systemic and regional hemodynamic, antiarrhythmic and antiischemic effects of bevantolol in anesthetized pigs. Am. J. Cardiol. 58: 8E-16E, 1986.

Hartog, J.M., Bremen, R.H. van & Verdouw, P.D. On the cardiovascular and antiarrhythmic actions of the cardioselective beta-adrenoceptor antagonist

bevantolol in the pig. Drug Dev. Res. 7: 23-33, 1986.
Roelandt, J., Verdouw, P.D., Rijsterborgh, H. & Hartog, J.M. Aerobic excercise and cardiac size: an echocardiographic study of Rotterdam marathon runners. In: Sports Cardiology. Exercise in health and cardiovascular disease. Eds: Fagard, R.H., Bekaert I.E.. Martinus Nijhoff Publishers, Dordrecht/Boston/Lancaster, 85-91, 1986.
Montfoort, A., Werf, L. van der, Hartog, J.M., Hugenholtz, P.G., Verdouw, P.D., Hülsmann, W.C. & Lamers, J.M.J. The influence of fish-oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidyl ethanolamine. Basic Res. Cardiol. 81: 289-302, 1986.
Verdouw, P.D., Hartog, J.M., Duncker, D.J., Roth, W & Saxena, P.R. Cardiovascular profile of pimobendan, a benzimidazole pyridazinone derrivative with vasodilating and inotropic properties. Eur. J. Pharmacol. 126: 21-30, 1986.
Hartog, J.M., Lamers, J.M.J. & Verdouw, P.D. The effects of dietary mackerel oil on plasma and cell membrane lipids, on hemodynamics and cardiac arrhythmias during recurrent acute ischemia in the pig. Basic Res. Cardiol. 81: 567-580, 1986.
Berk, L., Schmeets, I.O.L., Sassen, L.M.A., Rensen, R.J., Bremen, R.H. van, Hartog, J.M., Serruys, P.W. & Verdouw, P.D. On the time course of systolic myocardial wall thickening during coronary artery occlusion and reperfusion in the absence and presence of synchronized diastolic coronary venous retroperfusion in anesthetized pigs. Clinics of CSI, Ed. Mohl, W. Steinkopff Verlag Darmstadt, 277-280, 1986.
Hartog, J.M., Lamers, J.M.J. & Montfoort, A. Diëten met visolie. Effect op plasmalipidenspiegels, membraan- vetzuurcompositie en aggregatieneiging van trombocyten, en bloedviscositeit bij biggen. Hart Bulletin 17: 103-107, 1986.
Hartog, J.M., Saxena, P.R. & Verdouw, P.D. Inotropic and vasodilating properties of amrinone depend on the mode of administration. Eur. Heart J. 7: 1067-1076, 1986.
Duncker, D.J., Dalen, F.J. van, Hartog, J.M., Lamers, J.M.J., Rensen, R.J., Saxena, P.R. & Verdouw, P.D. Usefulness of pimobendan in the treatment of heart failure. Arzneim. Forsch./Drug Res. 36: 1740-1744, 1986.
Lamers, J.M.J., Hartog, J.M., Verdouw, P.D. & Hülsmann, W.C. Dietary fatty acids and myocardial function. Bas. Res. Cardiol. 82: 169-181, 1987.
Hartog, J.M., Lamers, J.M.J., Achterberg, P.W., Heuven-Nolsen, D. van, Nykamp, F.P. & Verdouw, P.D. The effects of dietary mackerel oil on the recovery of cardiac function after acute ischemic events in the pig. Bas. Res. Cardiol. 82: 183-194, 1987.
Hartog, J.M., Saxena, P.R. & Verdouw, P.D. Cardiovascular actions of amrinone in anesthetized open-chest pigs with an acute mild stenosis in the left anterior descending coronary artery. Can. J. Cardiol. 3 (1), 33-38, 1987.
Hartog, J.M., Lamers, J.M.J., Montfoort, A., Becker, A.E., Klompe, M., Morse, H., Cate, F.J. ten, Werf, F. van der, Hülsmann, W.C., Hugenholtz, P.G. & Verdouw, P.D. Comparison of mackerel-oil and a lard-fat enriched diets on plasma lipids, cardiac membrane phospholipids, cardiovascular performance and morphology in young pigs. Am. J. Clin. Nutr. 46, 258-266, 1987.
Verdouw, P.D. & Hartog, J.M. Doelmatigheid van calciumantagonisten bij ischemie

en de mogelijke invloed op de ontwikkeling van atherosclerose van de big. In: Calciumantagonisme en myocardischemie. Eds: Hugenholtz, P.G. & Van Zwieten, P.A. 59-69, 1987.

Hartog, J.M., Verdouw, P.D., Klompe, M. & Lamers, J.M.J. Dietary mackerel oil in pigs: effect on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. J. Nutr. 117, 1371-1378, 1987.

Duncker, D.J., Hartog, J.M., Levinsky', L. & Verdouw, P.D. Systemic haemodynamic actions of pimobendan (UD-CG 115 BS) and its O-demethylmetabolite UD-CG 212 Cl in the conscious pig. Br. J. Pharmacol. 91, 609-615, 1987.

Lamers J.M.J., Hartog J.M. & Verdouw P.D. Dietary fish oil reduces intimal proliferation of the coronary artery caused by implantation of a constrictor in pigs. Biomed. Biochim. Acta 47: S83-85, 1988.

Hartog J.M., Lamers J.M.J., Essed C.E., Schalkwijk W.P. & Verdouw P.D. Does platelet aggregation play a role in the reduction in localized intimal proliferation in normolipidemic pigs with fixed coronary artery stenosis fed dietary fish oil? Atherosclerosis 76: 79-88, 1989.

Hartog, J.M., Lamers, J.M.J., Hülsmann, W.C., Verdouw, P.D. & Hugenholtz, P.G. Dietary fish oil and the prevention of ischemic heart disease (in press).

Groot, P.H.E., Hartog, J.M., Dubelaar, M.L., Scheek, L.M., Verdouw, P.D. & Lamers J.M.J. The effects of diets supplemented with lard fat or mackerel oil on plasma lipoprotein lipid concentrations in domestic swine. Atherosclerosis (in press).

Verdouw, P.D., Sassen, L.M.A., Hartog, J.M., Woerkens, L.J. van & Lamers, J.M.J. Intimal proliferation in coronary arteries of normolipidemic pigs with a fixed stenosis. Eur. Heart J. (in press).

Sassen, L.M.A., Hartog, J.M., Lamers, J.M.J., Klompe, M., Woerkens, L.J. van & Verdouw, P.D. Mackerel oil and atherosclerosis in pigs. Eur. Heart J. (in press).

Lamers, J.M.J., Sassen, L.M.A., Hartog, J.M. & Verdouw, P.D. Dietary n-3 fatty acids and ischemic heart disease (in press).

Sassen, L.M.A., Lamers, J.M.J., Hartog, J.M. & Verdouw, P.D. Failure of diltiazem to suppress atherogenesis in pigs (submitted).