EFFECT OF A POLYUNSATURATED FATTY ACID MIMETIC ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN THE APOE DEFICIENT MOUSE

Fatemeh Moheimani

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Department of Immunopathology

Women's and Children's Hospital

Faculty of Health Sciences

Department of Paediatrics

The University of Adelaide

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CHAPTER 4

KINETICS OF DEVELOPMENT OF ATHEROSCLEROTIC LESIONS IN THE APOE DEFICIENT MOUSE MODEL

4.1 Introduction

ApoE$^{-/-}$ mice created by gene targeting in ES cells develop severe hypercholesterolemia and atherosclerosis (Plump and Breslow, 1995). Animals develop atherosclerotic lesions in the aortic roots, at the lesser curvature of aortic arch, the principal branches of the aorta, and in the pulmonary and carotid arteries (Nakashima *et al.*, 1994). Although the advanced lesions of atherosclerosis occur at the same site in apoE$^{-/-}$ mice on both a normal chow diet and a Western-type diet, the lesions in the apoE$^{-/-}$ mice on a Western-type diet are more advanced and occur much earlier (Plump *et al.*, 1992).

Although the apoE$^{-/-}$ mice have been widely used as an experimental model for atherosclerosis, there are no comprehensive reports on the time related development of the lesions in either apoE$^{-/-}$ mice with normal diet and high fat diet (Western type diet). It would therefore be highly desirable to conduct studies of quantitation and kinetics of lesion formation. This was examined both by measuring the area that the lesion occupies as well as grading the type of lesion (as described in chapter 3). It was also our objective to try to develop any potential mathematical relationship which governs the time related development of atherosclerotic lesions.

4.2 Kinetics of development of atherosclerotic lesions

4.2.1 Selection of the most appropriate site of proximal aorta for measurement of plaque area

To measure the plaque area for each mouse at a consistent site, the section of proximal aorta at the level close to the aortic valve cusps and coronary arteries was selected for

measurement (Figure 2.2). This area, which is a lesion prone site, correlated with the area of maximum atheroma in the majority of animals examined (Plump *et al.*, 1992). Aortic area and plaque area were measured digitally using Measure Master (Leading Edge, SA, Australia) and the percentage of lumen occupied by plaque was calculated for each animal.

4.2.2 Measurement variability

To assess repeatability (measuring the same section by the same observer) and reproducibility (measuring the same section by different observer) of the techniques used in this study, six cases were selected randomly in order to determine intraobserver and interobserver variability. In this analysis, each section was measured three times by each observer (Table 4.1). We used analysis of variance to assess the consistency of measurement for observers over cases and between observers. Variance ratio or F ratio was calculated for observers and observers×cases. F ratio was 0.1110 for observers; $P>0.05$, showing that there is no significant difference between the two observers and each had almost the same accuracy level. F ratio was 0.5477 for observers × cases; $P>0.05$, showing that there was no interaction between observers and cases or observers were consistent over the cases.

Table 4.1. Intraobserver and interobserver variability.

Case Number	Observer 1				Observer 2			
	Measurement repeat				Measurement repeat			
	1	2	3	Mean±SEM	1	2	3	Mean±SEM
98	12	12	11	11.67±0.33	11	13	12	12.00±0.58
101	10	11	11	10.67±0.33	12	11	11	11.33±0.33
107	15	17	17	16.33±0.67	17	18	17	17.33±0.33
136	13	13	12	12.67±0.33	13	14	11	12.67±0.88
135	23	22	23	22.67±0.33	18	17	17	17.33±0.33
140	6	6	6	6.00±0.00	7	7	6	6.67±0.33

Values represent % of lumen occupied by the lesion. The results are presented as measurement repeats and mean±sem from a single section from each case.

4.2.3 Time related development of atherosclerotic plaque in apoE$^{-/-}$ mice subjected to a Western diet

To assess the pattern of development of atherosclerotic lesions in the experimental model, apoE$^{-/-}$ male mice subjected to a high fat diet containing 21% fat and 0.15% cholesterol that resembles the Western diet in humans. The animals were sacrificed at various times thereafter and the heart and aorta removed for sectioning, staining and examination.

In the high fat diet group, there were 3 animals providing the baseline value with 0 weeks of high fat diet. Two animals were sacrificed after 1 and 2 weeks of high fat diet, 2 animals after 3 weeks of high fat diet, 1 animal after 4 weeks of high fat diet, 14 animals after 5 weeks of high fat diet, 2 animals after 7 weeks of high fat diet, 1 animal after 12, 15, 18 and 19 weeks of high fat diet, and 6 animals after 30 weeks of high fat diet.

The results showed that the percentage of the aortic lumen occupied by plaque dramatically increased in an exponential manner when the animals were subjected to a high fat diet. By week 19 of a high fat diet the rate of increase in the percentage of the aortic lumen occupied by plaque appeared to slow down, levelling out between 20 and 30 weeks of high fat diet (Figure 4.1). In order to achieve a mathematical model to predict lesion development in apoE$^{-/-}$ mice on high fat diet, the data was subjected to analysis using a SPSS package (version 12.0.1). The data were best fitted to an exponential mathematical model, $y=57.4-e^{(-0.1x+4.2)}$. The upper limit of plaque size or

asymptote is 57.4%. To calculate this equation the results of plaque size in animals which had received 2 weeks or more of high fat diet were used. This was because plaque size was too small at 1 week of high fat diet to give a reliable measurement. Based on the exponential model, the time for a 25% lesion development (LD_{25}) was about 7.9 or 8 weeks. The values of LD_{25} and maximal lesion attainable enable the data to be subjected to statistical analyses, for example for comparing drug evaluation on atherosclerosis. With the aid of this model information can be obtained with a reduced animal requirement in a predictive manner.

In addition, linear regression was used to assess the association of plaque area to aortic cross sectional area. There was a significant Pearson's correlation between aortic cross sectional area and plaque area ($r=0.99$, $p<0.001$). As animals received more weeks of atherogenic diet (high fat diet) the plaque area increased leading to enlargement in proximal aorta size as an adaptive response (Figure 4.2). This might be the reason that the kinetics of development of plaque in this model level off to become asymptotic to 57.4% as an upper limit.

At the start of the experiment, animals had mainly type I lesions (n=2) except one animal with type II. Mice developed mostly type II lesions (n=3) after 1-3 weeks of high fat diet although one showed type VIa. Animals receiving 4-7 weeks of high fat diet showed atherosclerotic lesions ranging from type II, III, IV, Va and Vb. The most common were type II in 8 cases, III in 4 cases, IV in 3 cases, Va in 1 case and Vb in another one. After 12-19 weeks of high fat diet, 2 animals showed type Vc, one developed type Vb and one had type Va. By 30 weeks the animals developed a range of

very advanced lesion, mainly VIb, VIa and Vc in 3, 2 and 1 animal, respectively (Figure 4.3 and Figure 4.4).

Although each mouse is represented by a point, some points are superimposed in the figures.

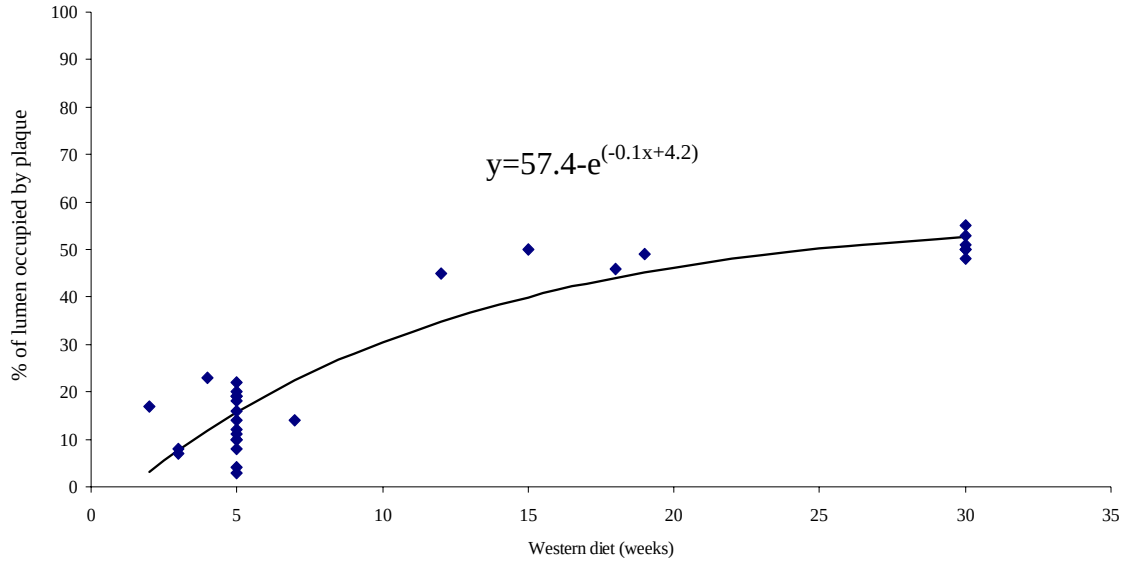

Figure 4.1. Time related development of atherosclerotic lesions in apoE$^{-/-}$ mice on Western diet as assessed by lumen occupation. Each mouse is represented by a point, involving a total of 29 mice. The best model that the data were fitted in was an exponential equation of the type $y=c-e^{(-ax+b)}$ (c=asymptote).

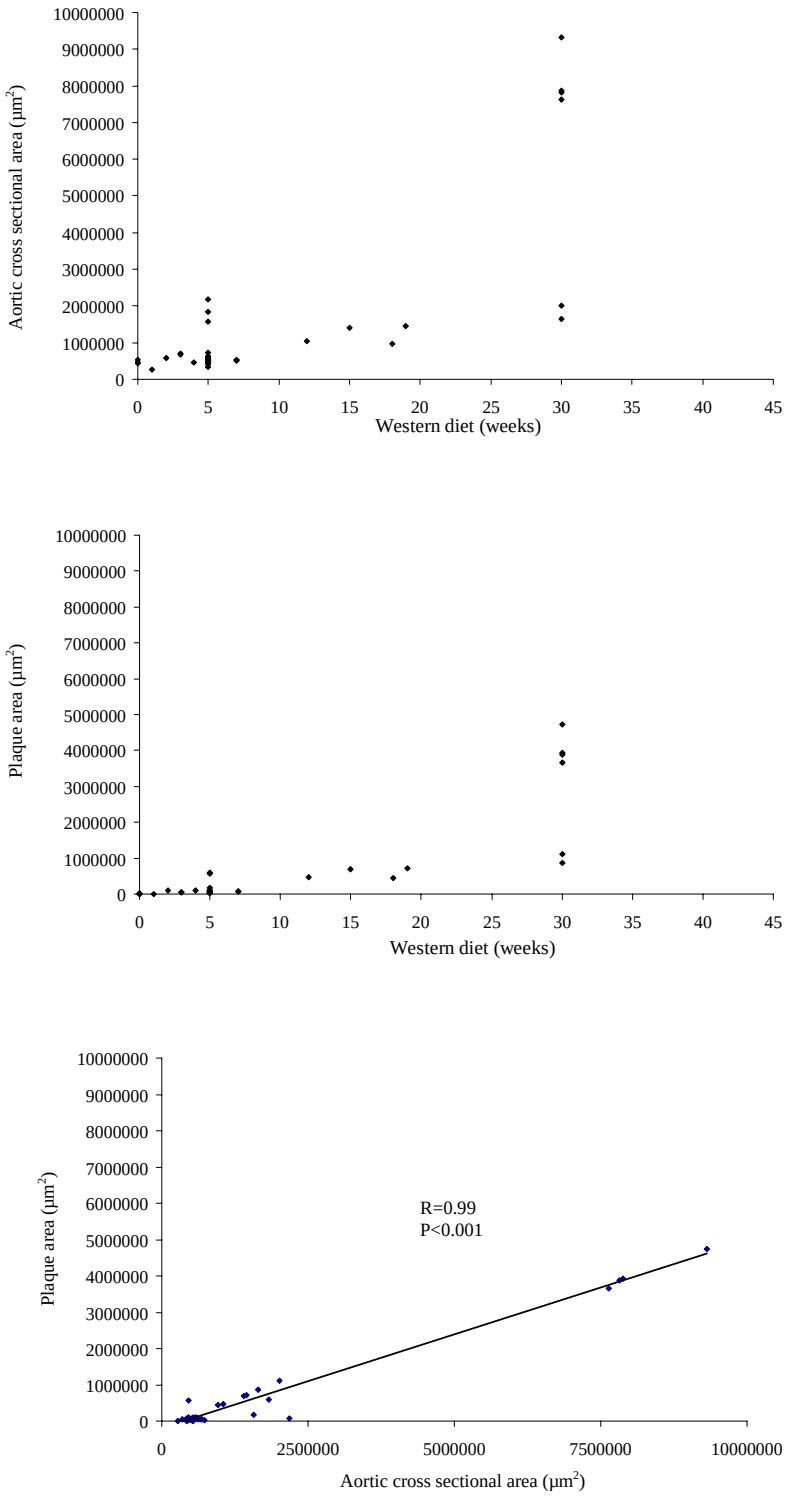

Figure 4.2. Compensatory aortic enlargement in animals fed a high fat diet. Each mouse was indicated by a point on the scatter plot.

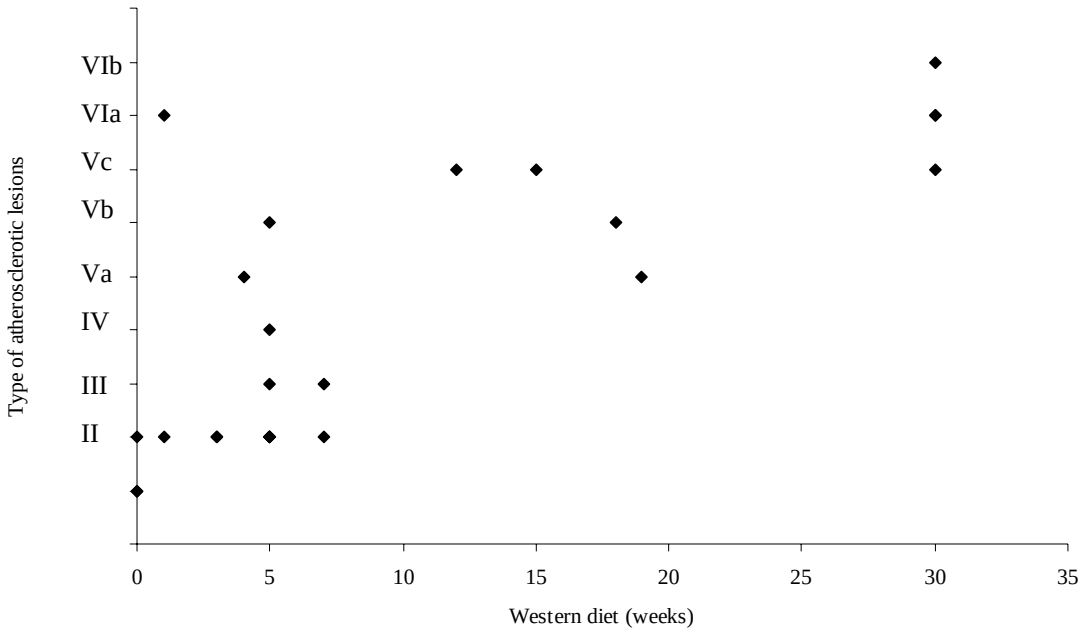

Figure 4.3. Time related atherosclerotic lesion in apoE$^{-/-}$ mice on Western diet as a function of lesion types. Lesions were classified as type I, II, III, IV, Va, Vb, Vc, VIa, VIb. Each mouse is represented by a diamond.

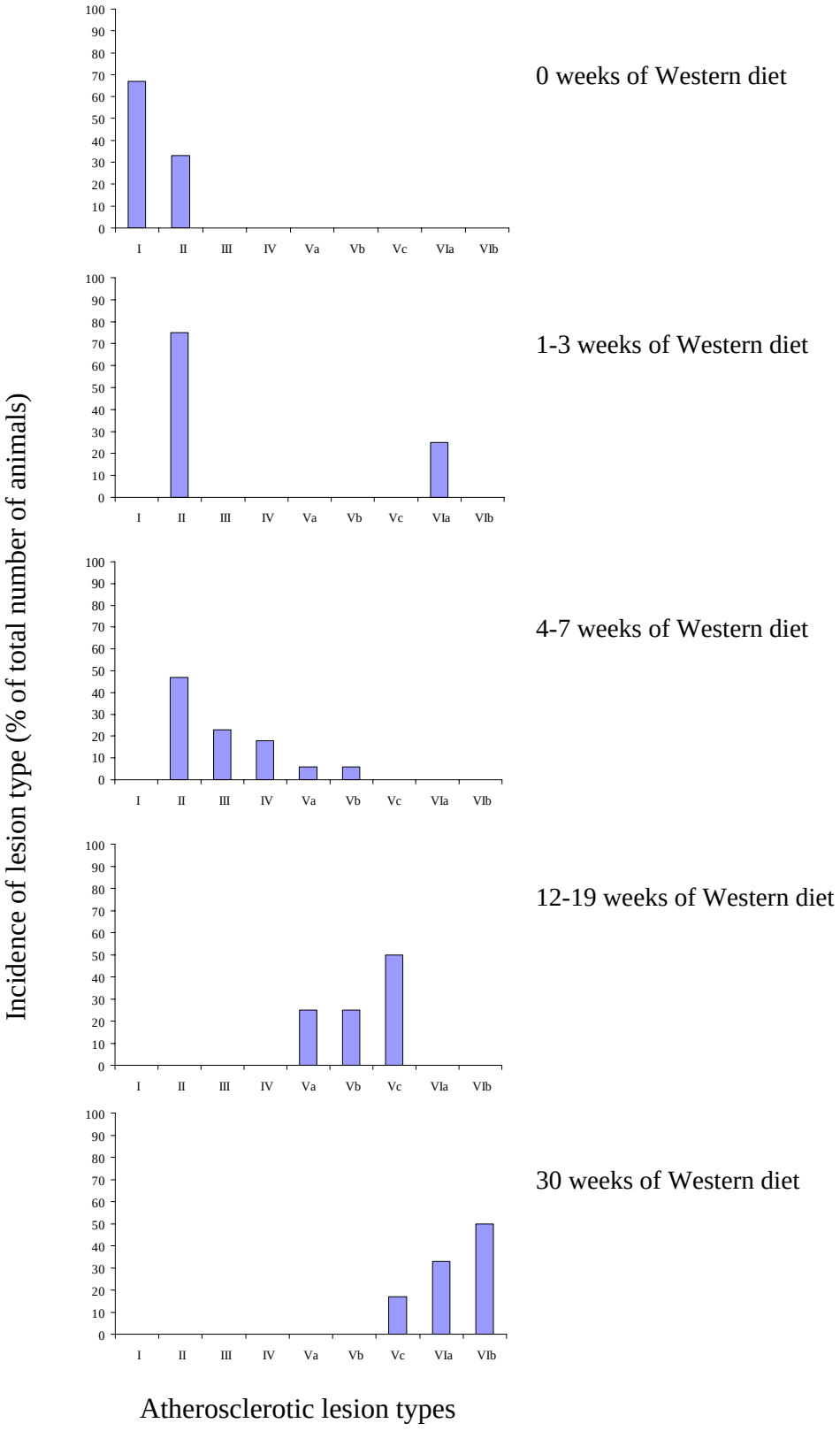

Figure 4.4. Relation between duration of Western diet and atherosclerotic lesion type in apoE$^{-/-}$ mice. Animals developed type I and II at 0 week of diet, mainly type II after 1-3 weeks of diet, mainly types II-IV after 4-7 weeks of diet, type V after 12-19 weeks of diet and mainly type VI after 30 weeks of diet.

4.2.4 Development of atherosclerotic lesions in apoE$^{-/-}$ mice on a normal diet

To assess the pattern of development of atherosclerotic lesions in apoE$^{-/-}$ mice that have not been exposed to a high fat diet, another group of animals were fed a normal diet. Animals were sacrificed at different times and the heart and aorta removed for sectioning, staining and examination.

Four animals were sacrificed at 6 weeks of age, 2 animals at 7 weeks of age, 3 animals at 8 weeks of age, 1 animal at 10 weeks of age, 4 animals at 11 weeks of age, 2 animals at 12 weeks of age, and 3 animals at each time point of 21, 29, 34 and 39 weeks of age were sacrificed. As animals in this group received a normal diet from birth, their age is equal to the weeks of normal diet.

ApoE$^{-/-}$ mice on a normal diet did not show any detectable (visible) atherosclerotic plaque before 7 weeks of age. The results showed that the percent of the aortic lumen occupied by the plaque dramatically increased in an exponential manner in mice from 10 weeks of age. Animals developed very small plaques by 12 weeks. The plaque size increased significantly by 21 weeks of age and then levelled off and became asymptotic to the upper limit of 39.8% (Figure 4.5). The mathematical model, $y=39.8-e^{(-0.2x+5.8)}$, can also be applied to predict the lesion development in apoE$^{-/-}$ mice on normal diet, with the reservation that the number of mice were limited. The LD_{25} is about 15.3 weeks of age. In order to calculate this equation animals at 10 weeks of age or older were used as the measurement technique was not accurate enough to pick up very small lesions in younger animals.

Linear regression was also used to assess the relationship between of plaque area and aortic cross sectional area. There was a significant Pearson's correlation between aortic cross sectional area and plaque area ($r=0.50$, $p<0.05$). As the animal's age increased the plaque area increased leading to compensatory dilation of the proximal aorta and an increase in aortic cross sectional area (Figure 4.6). This might be the reason why the kinetics of development of plaque in this model levelled off to become asymptotic with 39.8% as an upper limit (Figure 4.6).

There was no sign of atherosclerotic plaque by 6 weeks of age in apoE$^{-/-}$ mice fed a normal diet (n=4), although focal aortic calcification was found in one of the animals. Mice showed very early lesions at 7-12 weeks of age (n=12); type I (n=7), type II (n=4) and one animal did not develop any lesion. By 21 weeks of age, animals developed type VIa (n=1) and type VIb (n=2) lesions. At 29 weeks, mice developed type VIa (n=3). They developed advanced type; type Vc (n=1), type VIa (n=2) at 34 weeks. By 39 weeks of age the animals had developed type IV (n=1) and type Vc (n=2) lesions (Figure 4.7 and Figure 4.8). Because there were limited animal numbers in this group, especially between 12 and 21 weeks, we may have missed some types of atherosclerotic lesions.

Although each mouse is represented by a point, some points are superimposed in the figures.

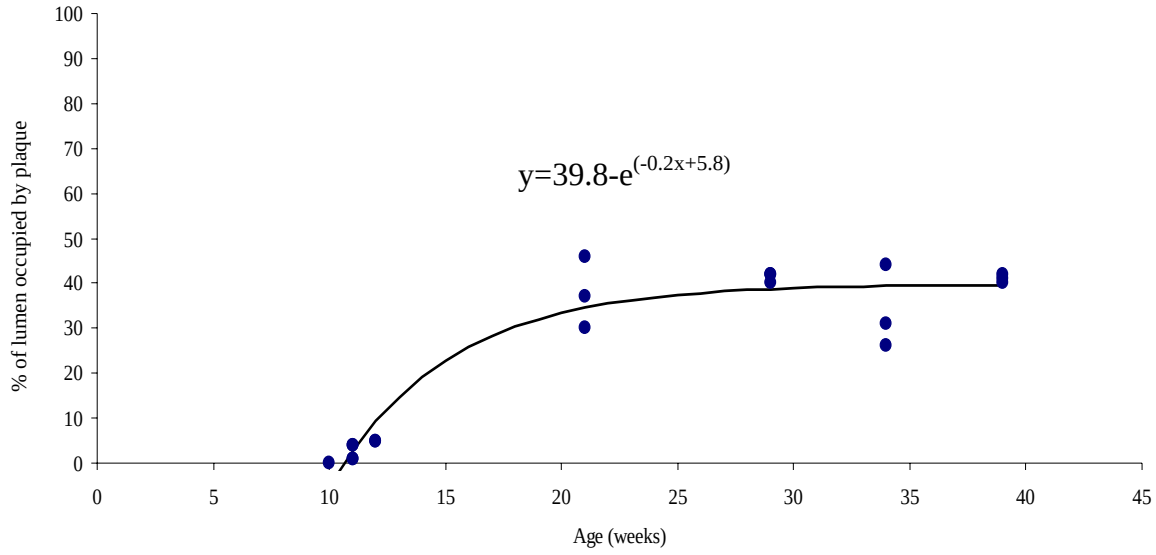

Figure 4.5. Age-related development of atherosclerotic lesion in apoE deficient mice fed a normal diet. Each mouse is represented by a point, involving a total of 19 mice. The best-fit model for the data was again an exponential relationship as described in Fig 4.1: $y=c\text{-}e^{(-ax+b)}$.

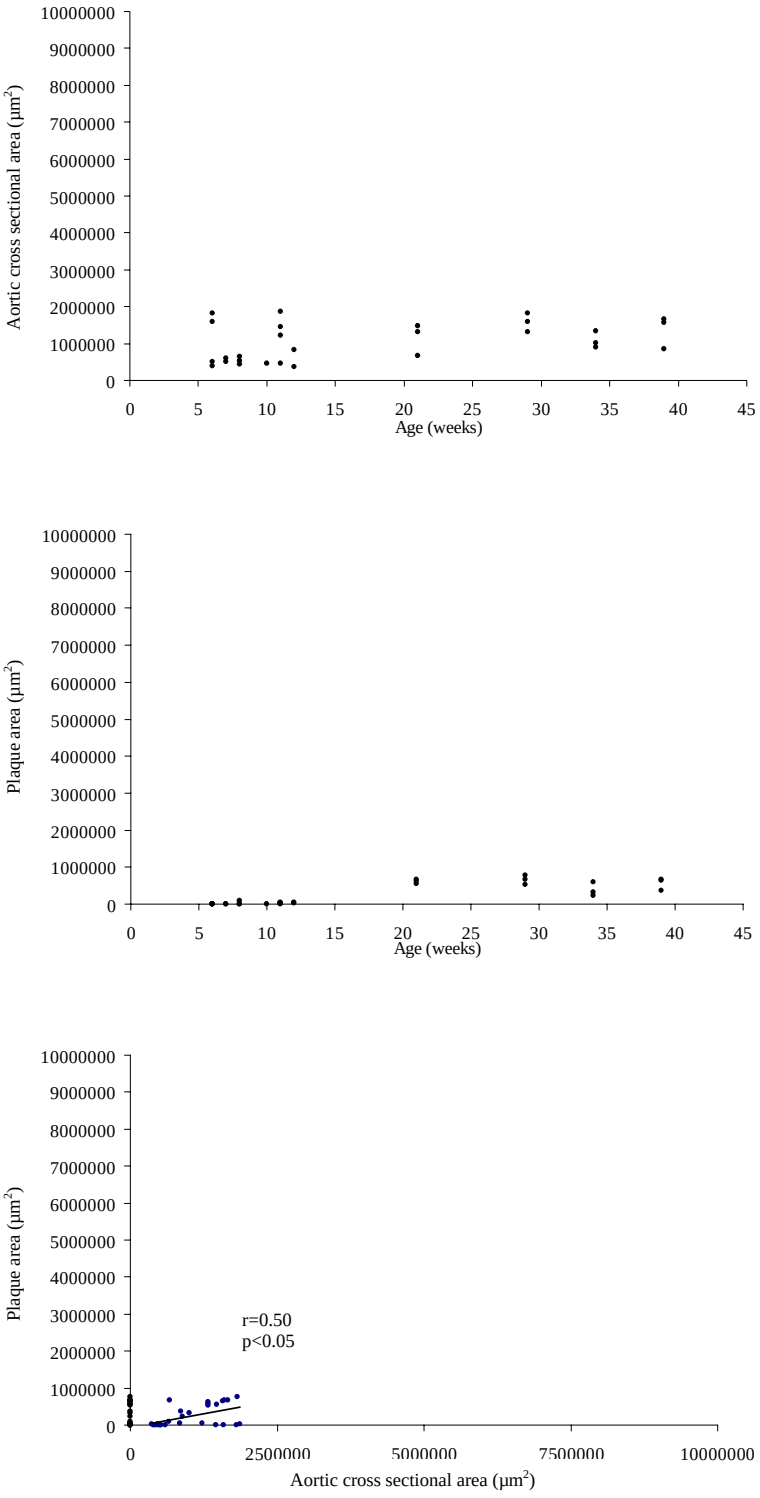

Figure 4.6. Compensatory aortic enlargement in animals fed a normal diet. Each animal was represented by a point.

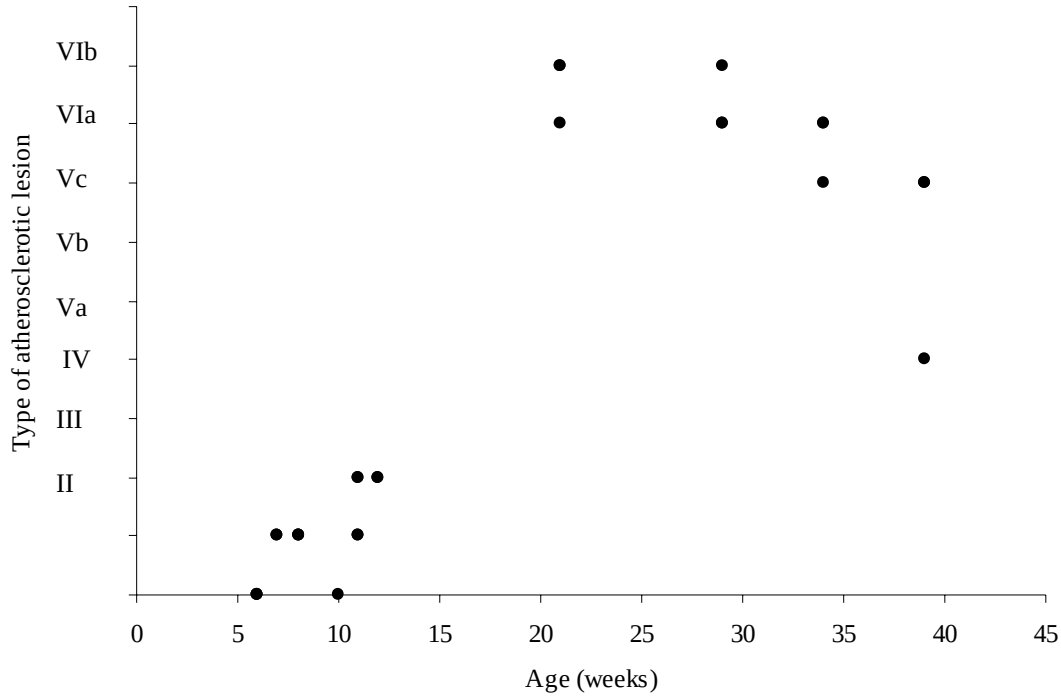

Figure 4.7. Lesion types seen at different ages in apoE$^{-/-}$ mice fed a normal diet. Each mouse is represented by a point. Animals developed type I, II, IV, Vc, VIa and VIb or did not develop any lesion.

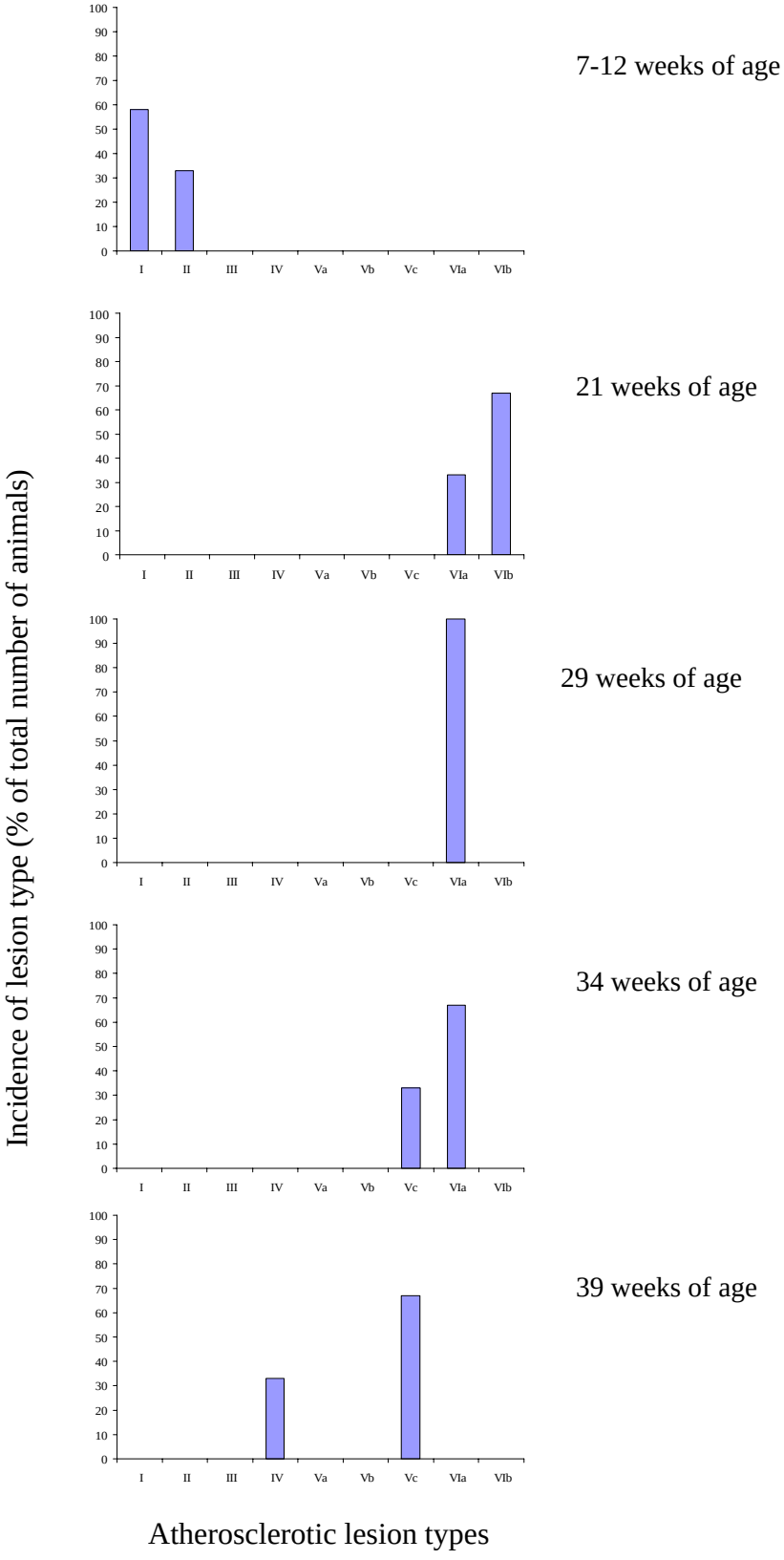

Figure 4.8. Relation between duration of normal diet and atherosclerotic lesion type in apoE$^{-/-}$ mice. Animals developed types I and II at 7-12 weeks of age, types VI at 21 and 29 weeks of age, types V and VI at 34, and types IV and V at 39 weeks of age.

4.2.5 Comparison between high fat diet and normal diet on size and type of atherosclerotic lesions in apoE$^{-/-}$ mice

ApoE$^{-/-}$ mice (n=6), 6 weeks of age, were fed a high fat diet for 5 weeks. They developed atherosclerotic plaque occupying on average 11.8±2.2% of the aortic lumen. This was significantly higher than animals (n=4) of the same age, fed a normal diet which developed plaque, occupying on average only 2.5±0.8% of the lumen (Figure 4.9 (A)). On the other hand animals (n=6), at 9 weeks of age, were fed a high fat diet for 30 weeks had plaques occupying 51.2±1.0% of the aortic lumen compared with animals (n=3) at the same age fed a normal diet that had plaque about 41.0±0.6% of the lumen (Figure 4.9 (B)).

These results show that a high fat diet accelerated the development of atherosclerosis in young apoE$^{-/-}$ mice. Animals fed 5 weeks of high fat diet, from 6 weeks of age, developed plaques about 4.8 times larger than animals, at the same age, fed a normal diet. This compares with a 1.2 fold difference between mice which had been fed a high fat diet for 30 weeks and their age-matched on a normal diet. This small difference between them is likely due to the fact that over this period, the degree of atherosclerosis in normal diet group had caught up with the high fat diet.

The results from examination of atherosclerotic plaques showed apoE$^{-/-}$ mice fed a normal diet developed mainly type I (n=2) and type II (n=2) at 11 weeks of age. At 39 weeks of age, they developed advanced lesions type IV (n=1), type Vc (n=2). Animals at 11 weeks of age following a high fat diet for 5 weeks, developed plaques mainly of

type II (n=5) and type III (n=1) and after 30 weeks of high fat diet, animals had very advanced lesions including; type Vc (n=1), VIa (n=2) and type VIb (n=3) (Table 4.2).

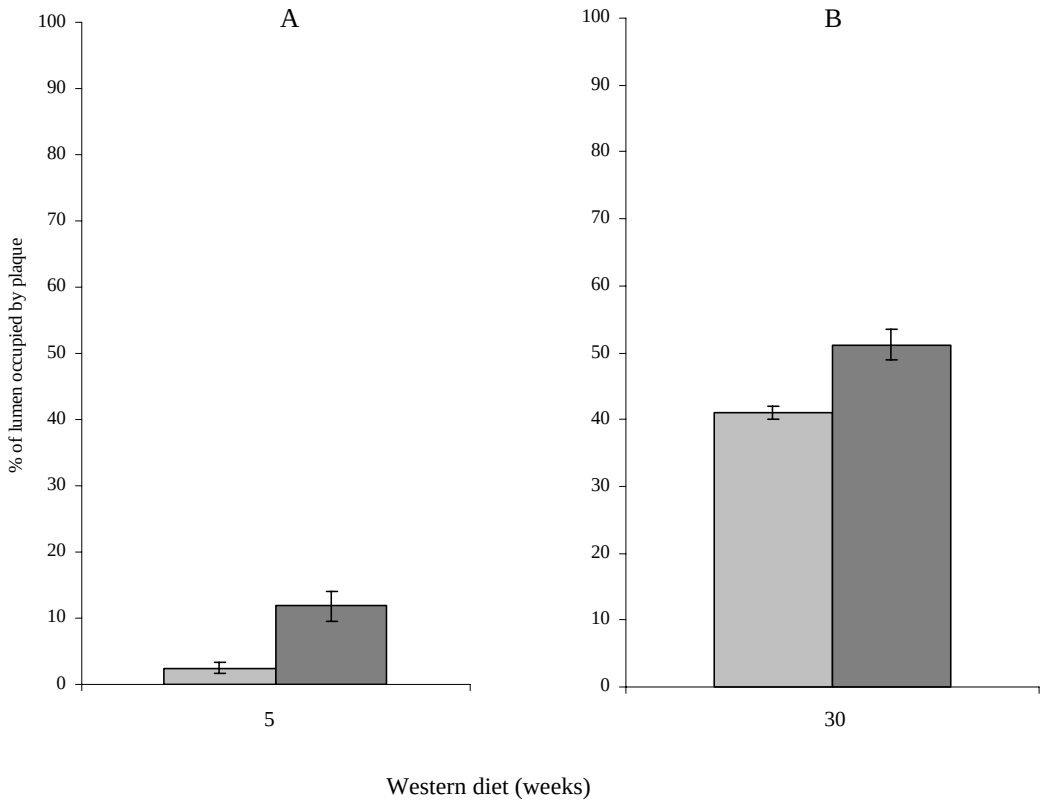

Figure 4.9. Comparison between high fat diet and normal diet for different periods on the size of atherosclerotic lesion in apoE$^{-/-}$ mice. Light grey bars represent animals fed a normal diet and dark grey bars represent animals fed a high fat diet. (A) At 6 weeks of age, animals were fed a high fat diet for 5 weeks and they developed plaque (expressed as percentage of lumen occupied) 4.8 times bigger than animals on a normal diet. (B) At 9 weeks of age, animals were fed a high fat diet for 30 weeks and they developed plaque just 1.2 times bigger than animals on a normal diet.

Table 4.2. Incidence of difference lesion types in mice fed normal or high fat diet.

Weeks	Types of diet	Lesion type								
		I	**II**	**III**	**IV**	**Va**	**Vb**	**Vc**	**VIa**	**VIb**
5	**ND**	50%	50%							
	HFD		83%	17%						
30	**ND**				33%			67%		
	HFD							17%	33%	50%

Numbers indicate occurrence out of the total number of animals studied in each age group. ND indicates a normal diet and HFD indicates a high fat diet.

4.2.6 Relationship between the size of the atherosclerotic lesion and the type of lesion

As the atherosclerotic lesions progress from a very early type to an advanced type, the lesion size increases. Linear regression was used to assess the association of percentage of lumen occupied by lesion and different types of atherosclerotic lesions. There was a significant Pearson's correlation between plaque size and atherosclerotic lesion types in animals that were fed a high fat diet ($r=0.87$, $p<0.0001$), and also animals that were fed a normal diet ($r=0.93$, $p<0.0001$) (Figure 4.10). Therefore is a linear relationship between the size of the lesion (percentage) and the type of lesion however the components of the atherosclerotic lesion are the most important determination for in grading the lesion (described in details in chapter 3).

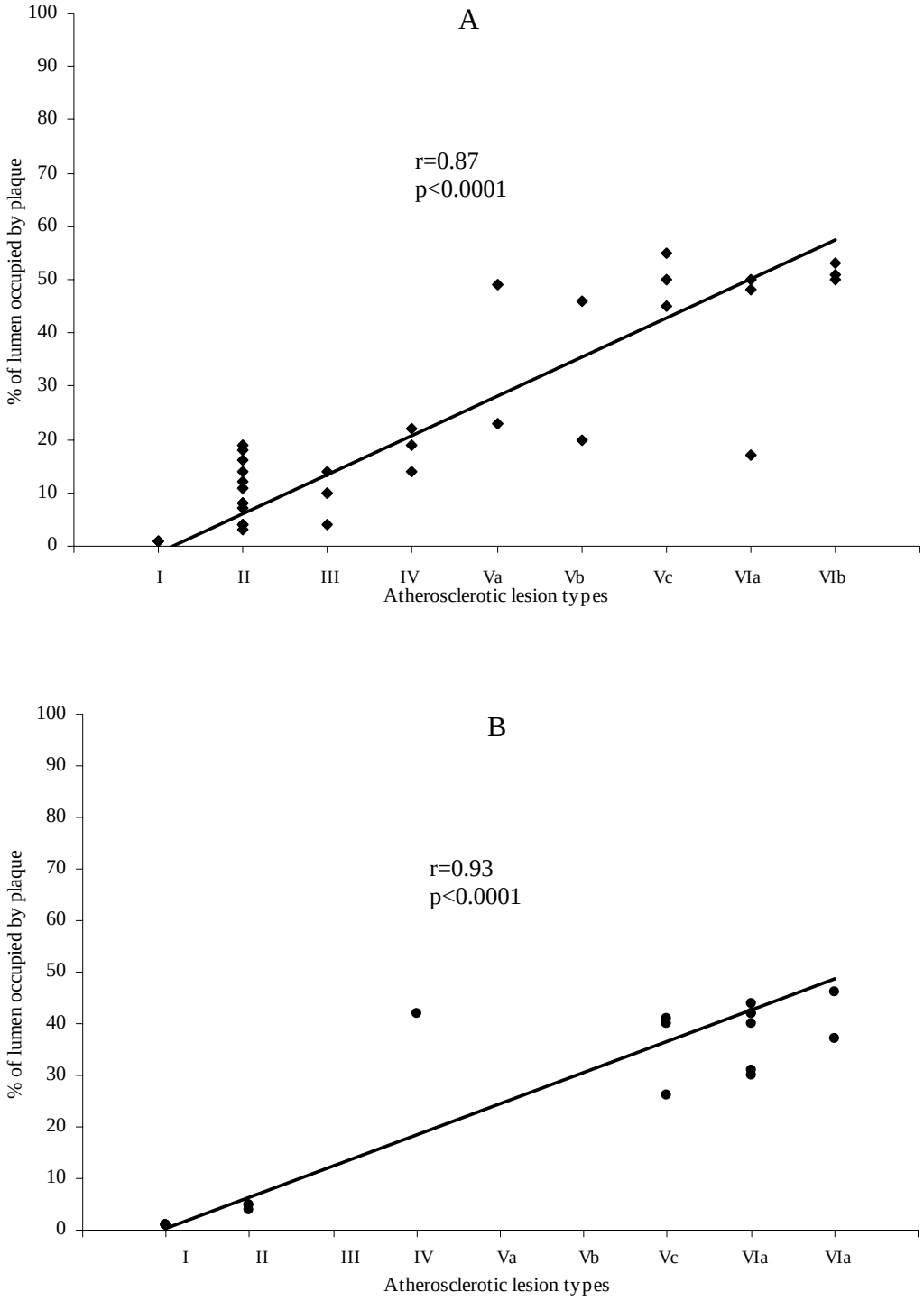

Figure 4.10. Relationship between the size of the atherosclerotic lesion (percentage) and the type of lesion. There is a linear relationship between percentage of lumen occupied by plaque and lesion types in animals fed a high fat diet (A) and in animals fed a normal diet (B). Each mouse is represented by a point.

4.3 Summary

Attempts to quantify the atherosclerotic plaques in apoE$^{-/-}$ mice established several important issues. One approach was to express the lesion size as a ratio or % of aortic lumen area. Using this index, we found that the development of lesions under the influence of a high fat diet followed an exponential relationship during this supplementation. When the data was analysed the equation governing this relationship was:

$$y = c - e^{(-ax + b)}$$

The asymptotic nature of this relationship was due to the changes in the size of the aorta. Thus with increases in plaque size as the period of high cholesterol supplementation increased, the size of the blood vessel dramatically increased. Atherosclerotic plaques were also evident in mice which received the normal diet. The kinetics of plaque development under a normal diet condition was also exponential. However it was evident that the establishment and progression of lesion development were promoted by the high fat diet.

The data suggest that gauging atherosclerotic lesion size as a % of aortic lumen size will not give an accurate estimation of lesion size in the later stages as the curve tends towards an asymptotic character. Thus pathogenesis may be best assessed as the absolute size of the plaque as well as the change in size of the aorta. Since during the early stage of plaque development there is insignificant change in aortic size, this period may be ideal to gauge as % of plaque to aortic size. From this the number of days it takes to develop 25% of the final lesion size (LD_{25}) can be deduced and such values are amenable to statistical analyses.

When the size of the atherosclerotic lesion was examined against the lesion types there was a significant correlation between them therefore as the size increased there was a progression from type I/II to type V/VI.

CHAPTER 5

EFFECT OF β-oxa 23: 4*n*-*6* ON THE DEVELOPMENT OF ATHEROSCLEROSIS

5.1 Introduction

There is significant evidence that cell adhesion molecules (CAMs) play key roles in atherogenesis. *In vivo*, increases in CAM expression is localised to human arteries with atherosclerotic lesions and in lesion-prone sites on the aorta of mice and rabbits (Johnson-Tidey *et al.*, 1994; Ross, 1999b). Studies in animal models have also demonstrated that preventing the expression of CAMs through inactivating mutations caused by homologous recombination (Nageh *et al.*, 1997; Ramos *et al.*, 1999; Collins *et al.*, 2000; Huo and Ley, 2001), and antibody neutralisation of CAMs, reduces the recruitment of monocytes to atherosclerotic plaques and reduces lesion size (Patel *et al.*, 1998; Huo and Ley, 2001). Consequently, strategies to reduce CAM expression are attractive approaches to reduce or impede the development of atherosclerosis. One of the essential factors that is required for the up-regulation of CAM expression is the transcription factor, NFκB. Therefore, Inhibition of NFκB signalling pathway can be an attractive target for the development of drugs to suppress atherosclerosis (May *et al.*, 2000; Valen *et al.*, 2001).

The n-3 fatty acids and fish oil are currently believed to possess cardioprotective actions and one well-studied action is the suppression of CAM expression (De Caterina *et al.*, 1999). Recently we have demonstrated that a novel PUFA, β-oxa 23: 4*n-6* (MP3) suppresses the expression of CAM and hence leukocyte-endothelium interaction (Robinson *et al.*, 1999; Ferrante *et al.*, 2005b). This molecule, containing an oxygen atom in the β position of the carbon backbone, is better than docosahexaenoic acid (DHA; 22:6n-3) at suppressing tumour necrosis factor (TNF)-, lipopolysaccharide (LPS)- or phorbol 12-myristate 13-acetate (PMA)-induced expression of E-selectin,

ICAM-1 and VCAM-1 in vitro. However, unlike DHA which is a strong stimulator of the phagocyte respiratory burst and hence is a promoter of neutrophil-mediated tissue damage, MP3 is relatively poor at stimulating this response. Preliminary studies have found MP3 to be effective in vivo at suppressing LPS-stimulated upregulation of E-selectin expression in the aortae of mice and prevent the infiltration of leukocytes, including monocytes, into sites of inflammation (Ferrante *et al.*, 2005b). Preliminary data from our laboratory had also demonstrated that MP3 inhibits the ability of TNF to activate the IκB kinase-NFκB signalling pathway. DHA was less effective than MP3 at antagonising the action of TNF on this pathway, consistent with its weaker ability than MP3 at suppressing CAM expression. It would be thus expected that MP3 would be effective in reducing/preventing the development of atherosclerosis. The focus of this chapter is therefore to investigate the effects of MP3 on development of atherosclerosis, using the apoE$^{-/-}$ mouse experimental model.

5.2 Effect of MP3 on the development of atherosclerosis

The first set of experiment was conducted to find out the effect of short-term treatment with MP3 in preventing the development of atherosclerotic lesions in apoE$^{-/-}$ mice on a high fat diet. Nine 11 week old male apoE$^{-/-}$ mice were divided into two groups of 4, test and control groups. The remaining mouse was sacrificed to establish a baseline. Mice received IP injection of 40mg/kg body weight of MP3 or vehicle once daily for 4 weeks duration. Previous studies in our laboratory have demonstrated that this dose of MP3 was effective at suppressing LPS-induced E-selectin expression in the murine aorta (Ferrante *et al.*, 2005b). The mice were placed on a high fat diet one day after the first injection of MP3 or vehicle. One mouse from each group was sacrificed every

week thereafter. The heart and approximately 5mm attached aorta was isolated, fixed in 10% buffered formalin. The fixed heart was cut transversely caudal to the atria and embedded in paraffin. The tissue then sectioned serially, at 5µ thickness beginning from the transverse cut. The sections were stained with Haematoxylin and Eosin (see chapter 2).

Measurement of plaque size in sections of aortic roots revealed that MP3 treated mice had smaller lesions than age matched controls for the first 3 weeks of treatment (Figure 5.1) owing to limited number of mice a statistical analysis was not possible. Both plaque area and the percentage of the lumen occupied by plaque showed the same pattern (Figure 5.1). The test group also had less advanced lesions than the control group. The baseline animal developed a type II lesion at the start of the experiment on normal diet and the other animals in the control group developed type II, IV, II and Va lesions over the next four weeks of high fat diet. In comparison the animals in the test group showed no lesion, type II, I and Va lesions respectively over the same period (Table 5.1).

In the second experiment six male apoE$^{-/-}$ mice at 5 weeks of age were divided into two groups and placed on a high fat diet. The test group received IP injections of MP3 (40mg/kg) once daily on the day that the diet was changed, and the control group received IP injections of vehicle. One animal from each group was sacrificed after 3, 5 and 7 weeks of treatment. Animals' hearts were fixed in 10% formalin and underwent sectioning. Sections were stained and lesions were examined.

The results showed that MP3 treated mice appeared to have smaller atherosclerotic lesions than controls for the first 2 weeks of treatment (Figure 5.2). Owing to limited number of mice, the statistical analysis was not possible. There was no difference in the grade of atherosclerotic lesions between the test group and control group (Table 5.2).

In the third experiment seventeen 8 week old male apoE$^{-/-}$ mice were placed on a high fat diet. Two mice were immediately sacrificed to establish a baseline and the others were divided into two groups. The test group of 8 mice received IP injections of MP3 (40mg/kg) daily starting on the day that diet was changed, and the control group of 7 mice received an appropriate amount of vehicle. Treatment continued for five weeks at which stage the animals were sacrificed, hearts were fixed and sectioned. The sections were stained and examined for lesion size and type.

At 8 weeks of age the mice showed negligible plaque formation; 1.0±0.0%. The average percentage of lumen occupied by plaque in the test group after 5 weeks of MP3 treatment was 13.9±1.5% which was similar to the control group, 13.6±2.5% (Figure 5.3). This lack of effect of MP3 was evident when the results were also expressed as aortic cross sectional area and plaque area. The aortic cross sectional area after 5 weeks of high fat diet in the test group was 604,132±37,017μm^2 and in the control group was 560,458±39,399μm^2, and the absolute plaque area in test group was 85,107±11,985μm^2 and in control group was 71,816±10,394μm^2, showing no difference between test and control groups(Figure 5.3).

The test group developed a wide range of lesion types (early to advanced lesions); type II (n=1), III (n=1), IV (n=1), Vb (n=4), whereas control group showed mainly type III (n=4), type IV (n=3) and type II (n=1) (Table 5.3). Therefore, MP3 did not affect lesion types in the mice.

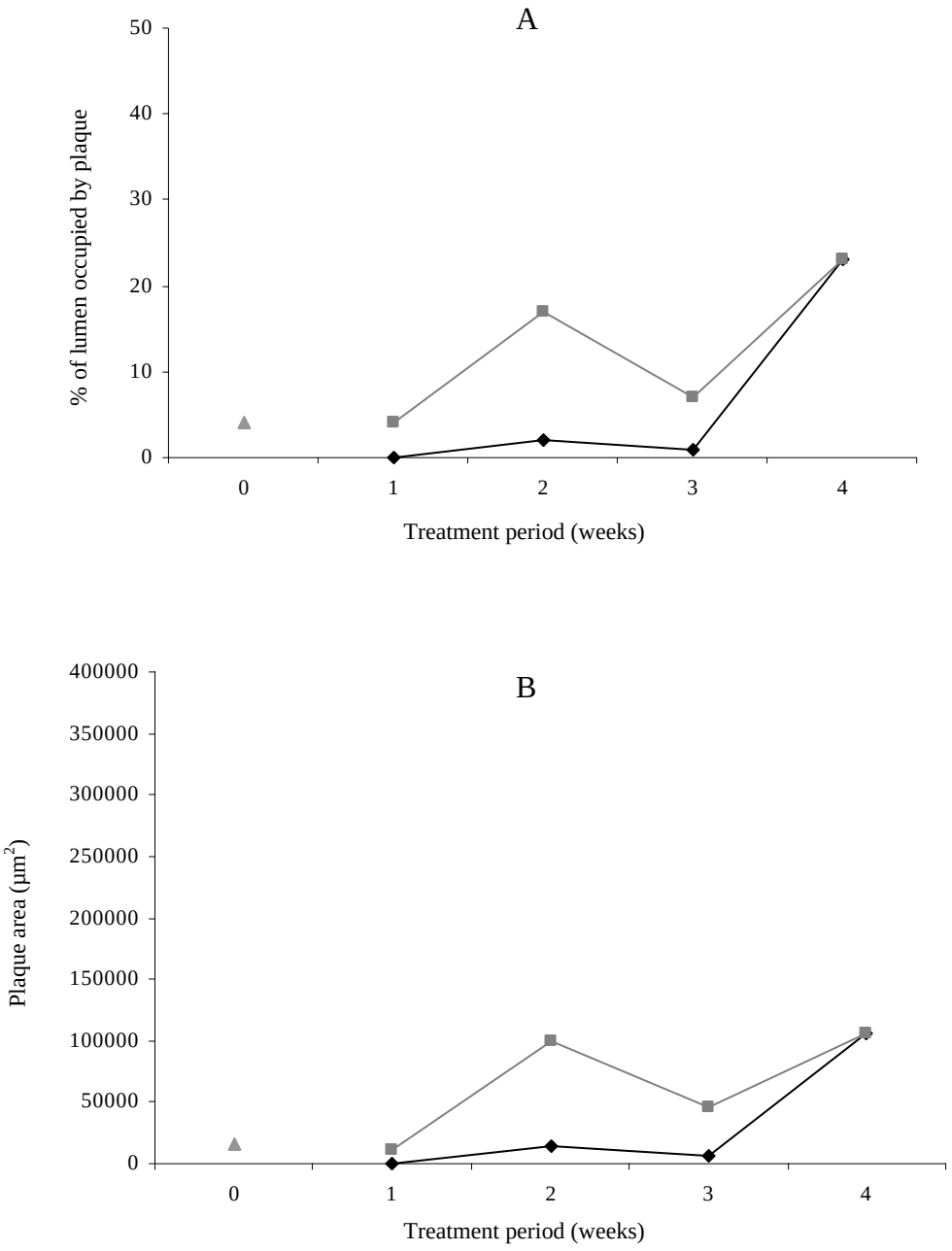

Figure 5.1. Effect of MP3 on the development of atherosclerosis. Mice were placed on a high fat diet at 11 weeks of age. They were injected IP with 40mg/kg of MP3 (◆) or vehicle (■) once daily, starting a day before being put on the high fat diet. (▲) Shows vehicle for non treated mouse (baseline). Each point represents one mouse. (A) The results are expressed as the percentage of lumen occupied by plaque. (B) The results are expressed as the plaque area.

Table 5.1. Effect of MP3 (40mg/kg) on the development of different types of atherosclerotic lesion in apoE$^{-/-}$ mice fed a high fat diet. The hearts from the mice in figure 5.1 were graded for the severity of atherosclerosis.

	Lesion types				
	Weeks of treatment				
Group	0	1	2	3	4
Baseline	II				
Test		*-	II	I	Va
Control		II	IV	II	Va

* This animal did not develop any plaque.

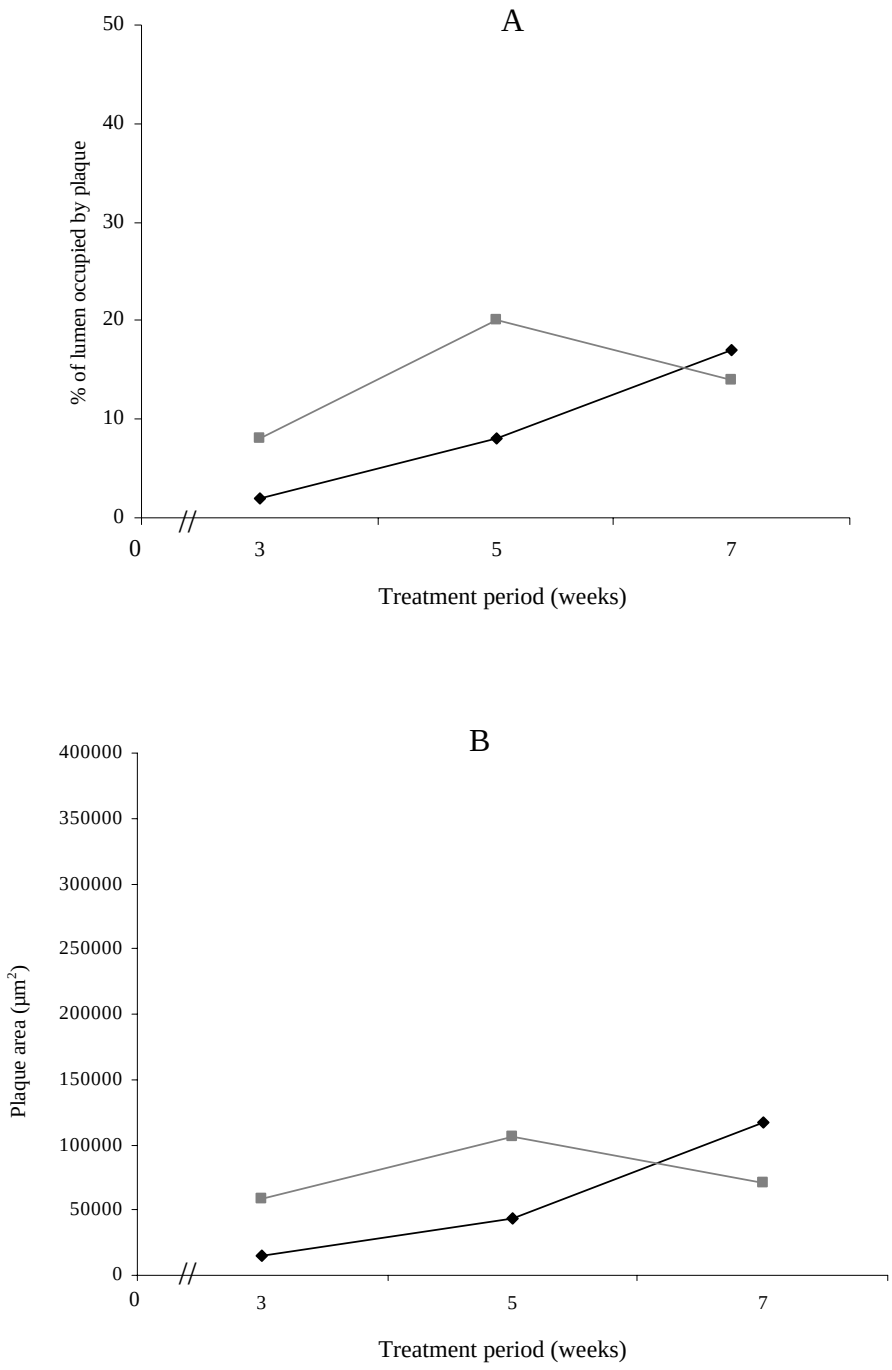

Figure 5.2. Effect of MP3 (40mg/kg) on development of atherosclerosis in apoE$^{-/-}$ mice fed a high fat diet from 5 weeks of age. Mice were injected IP with MP3 (◆) or vehicle (■) once daily. Each point represents a mouse. (A) The results are expressed as the percentage of lumen occupied by plaque. (B) The results are expressed as the plaque area.

Table 5.2. Effect of MP3 (40mg/kg) on the grade of atherosclerotic lesion in apoE$^{-/-}$ mice fed a high fat diet from 5 weeks of age. The hearts from the mice in figure 5.2 were graded for the severity of atherosclerosis.

	Lesion types		
	Weeks of treatment		
Group	3	5	7
Test	II	Vb	II
Control	II	Vb	III

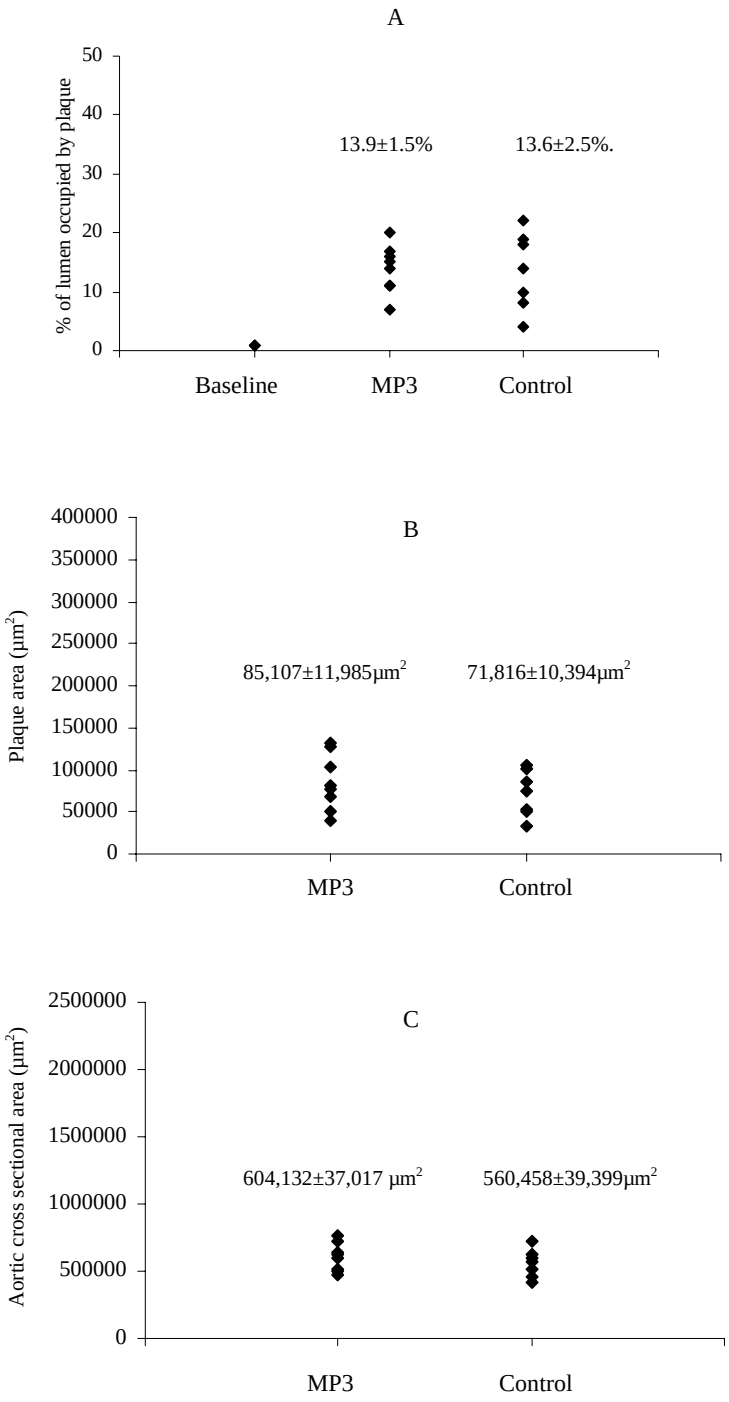

Figure 5.3. Effect of MP3 on the development of atherosclerosis in apoE$^{-/-}$ mice maintained on a high fat diet. Mice, at 8 weeks of age, were treated daily with either 40mg/kg of MP3 or vehicle for 5 weeks and then killed for tissue sectioning. The results are presented as (A) the percentage of lumen occupied by plaque, (B) plaque area and (C) aortic cross sectional area. The mean±sem is shown above the points.

Table 5.3. Effect of 40 mg/kg MP3 for 5 weeks on atherosclerotic lesion types in apoE$^{-/-}$ mice fed a high fat diet. The hearts from the mice in figure 5.3 were graded for the severity of atherosclerosis.

	Lesion type								
Group	**I**	**II**	**III**	**IV**	**Va**	**Vb**	**Vc**	**VIa**	**VIb**
Test		14%	14%	14%		58%			
Control		13%	50%	37%					

The % represented occurrence among the population in each group

5.3 The ability of MP3 to reverse atherosclerosis

The aims of these tests were to determine whether or not MP3 treatment in apoE$^{-/-}$ mice had a curative action in animals that already had developed advanced atherosclerotic lesions.

Concurrent with the experiments in figures 5.1-5.3, an investigation was also conducted to determine whether MP3 was able to reverse atherosclerosis. Nine 11 week old male apoE$^{-/-}$ mice were placed on a high fat diet and at 22 weeks of age were divided into two groups. The test group of 5 were injected IP with MP3 (40mg/kg) once daily and the control group of 4 were injected with vehicle. These mice continued to be on a high fat diet during the treatment and treatment was continued for 8 weeks. Two animals from the test group and one animal from the control group were sacrificed at 1 week of treatment and then one animal from each group was sacrificed at 4, 7 and 8 weeks of treatment. The hearts were isolated and underwent the process of fixation, sectioning and staining.

It is evident from the results in figure 5.4 that the size of the lesions in the control group had reached almost the maximum size over the study period based on the model in chapter 4. There was no consistent effect of MP3 on the degree of atherosclerosis expressed as either percentage of lumen occupied by plaque or plaque area (Figure 5.4). It was noted the atherosclerotic lesions were of an advanced type in both groups (Table 5.4).

The failure of MP3 to prevent or reverse the development of atherosclerosis could be due to an insufficient amount of MP3 being administered. We therefore investigated whether a dose of 70mg/kg would reduce atherosclerosis. To achieve a high degree of atherosclerosis, the mice (9×8 weeks of age) were given a high fat diet for 5 weeks and then returned to normal diet for 1 week. The rational for this latter manipulation was to reduce the level of serum lipids prior to MP3 treatment to facilitate the uptake of MP3 by the tissue. At the end of the week of normal diet, the animals were divided into 2 groups, a test group of 5 animals which received five injections (IP) of 70 mg/kg of MP3; given on days 1, 2, 4, 6 and 8, and a control group of 4 received an equivalent amount of vehicle. The animals were then sacrificed, hearts were isolated, fixed and sectioned for staining and examination.

The mean percentage of lumen occupied by lesion in the test group was 20.2±4.3% compared to 22.0±3.3% in the control group, which showed there was no significant difference between test and control groups (Figure 5.5). The plaque area, which was 126,699±32,934μm^2 in the test group and 139269±14017μm^2 in the control, was also not significantly different (Figure 5.5). The aortic cross sectional area, 595,634±35,757μm^2 in the test group and 657,044±65,004μm^2 in controls, also showed there was no difference between test and control groups (Figure 5.5). Table 5.5 shows the distribution of different atherosclerotic lesion types in both test and control groups. In the test group animals developed type IV (n=2) and type Vb (n=3) lesions, and in the control group animals developed a similar range of lesion types with type IV (n=2), type Vb (n=1) and type VIb (n=1) lesions. The data show that MP3 did not affect the grade of atherosclerotic lesions.

These findings showed that although the animals tolerated 70mg/kg MP3, the fatty acid was not found to effectively suppress the plaque size in highly developed lesions.

The lack of the expected protective effects of MP3 may have been because 1 week wash out period with normal diet prior to MP3 treatment was insufficient and/or the duration of MP3 (70mg/kg) treatment was not adequate. To overcome these limitations fourteen 5 week old mice received a high fat diet for 5 weeks to accelerate development of atherosclerotic lesions. Then they were placed back on a normal diet. The treatment with MP3 was started 4 weeks after removing the high fat diet. The animals were divided in two groups of 7. The test group received 70 mg/kg MP3, IP, once daily and the control group received the appropriate amount of vehicle. Treatment was continued for 4 weeks and then the animals were sacrificed for isolation of hearts, tissue sectioning, staining and examination.

The results showed that the percentage of the aortic lumen occupied by the plaque in the test group was 26.6±1.1% compared to 29.9±3.3% in control (Figure 5.6). This difference was not statistically significant. Measurement of the plaque areas in test group; 157,194±6,905.1μm, compared to the control group, 204,099±32,257 (Figure 5.6) showed that the difference remained not statistically significant. The mean of the aortic cross sectional area was 596521±30147μm in the test group and 657991±38147μm in the control group, showing there is no difference and a similar distribution (Figure 5.6).

The data presented in table 5.6 show that the test group mainly developed type IV (n=5) and type Vc (n=2) atherosclerotic lesions, and the control group developed type III (n=2), type IV (n=3) and type VIb (n=2) lesions.

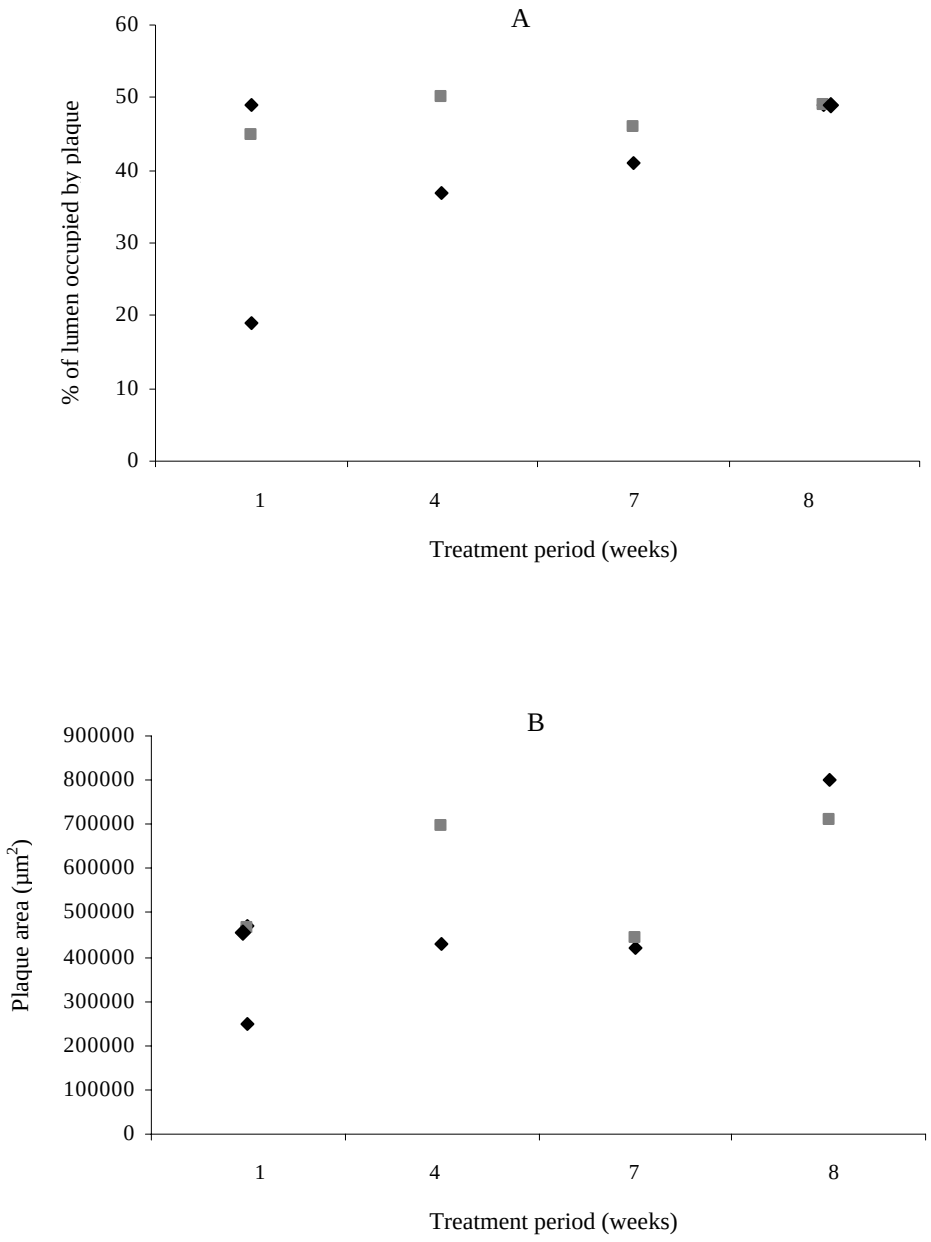

Figure 5.4. The curative effect of 40mg/kg MP3 on atherosclerosis in apoE$^{-/-}$ mice with advanced lesions. The test group and control group are represented by (◆) and (■), respectively. Each mouse is represented by one point. As there were 2 animals at 1st week of treatment, there are two points for test group. (A) The data are expressed as the percentage of lumen occupied by plaque and (B) represents the data as plaque area.

Table 5.4. The effect of MP3 (40mg/kg) on plaque type in apoE$^{-/-}$ mice on a long term high fat diet. The hearts from the mice in figure 5.4 were graded for the severity of atherosclerosis. As there were 2 animals at first week of treatment, there are two types of lesion at this time point.

	Lesion types			
	Weeks of treatment			
Group	1	4	7	8
Test	IV&Va	Va	Vc	Vc
Control	Vc	Vc	Vb	Va

Note. All animals show advanced plaque type.

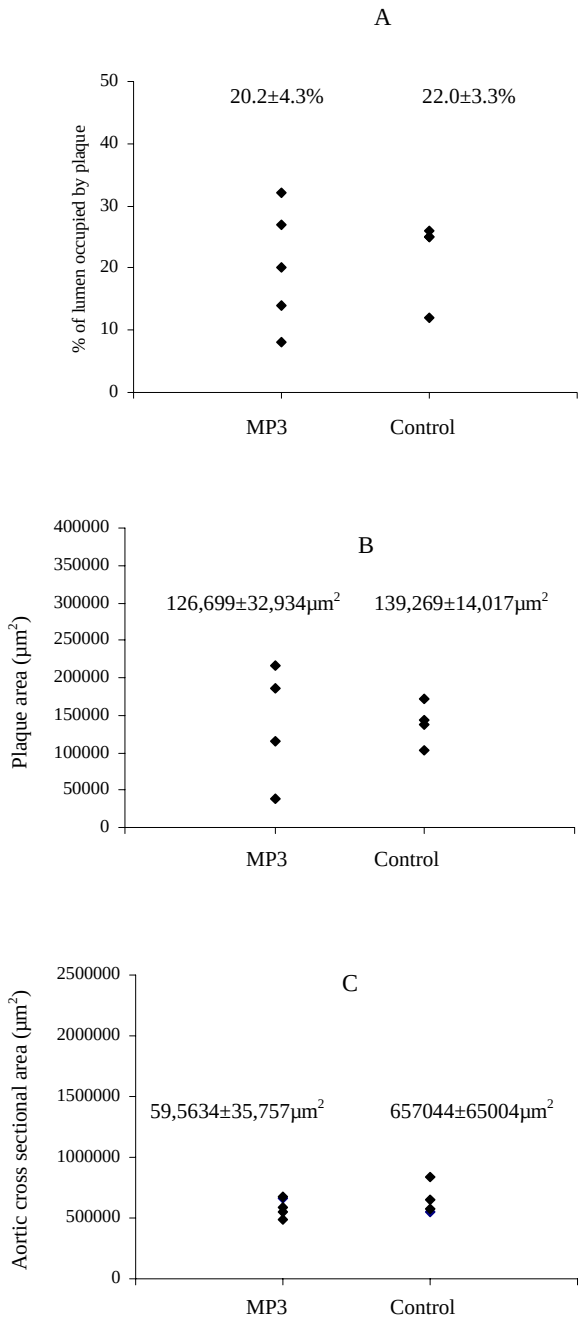

Figure 5.5. Curative effect of 70mg/kg MP3 for short term on development of atherosclerosis in apoE$^{-/-}$ mice. The results are expressed as (A) percentage of lumen occupied by plaque, (B) plaque area, (C) aortic cross sectional area. Each point represents a mouse. The mean±sem of each group is given above each group. There were no differences between test and control groups.

Table 5.5. Curative effect of 70mg/kg MP3 for short term on grade of atherosclerotic lesion types in apoE$^{-/-}$ mice. The hearts from the mice in figure 5.5 were graded for the severity of atherosclerosis.

	Lesion type								
Group	**I**	**II**	**III**	**IV**	**Va**	**Vb**	**Vc**	**VIa**	**VIb**
Test				40%		60%			
Control				50%		25%			25%

The % represents the occurrence among the population for each group.

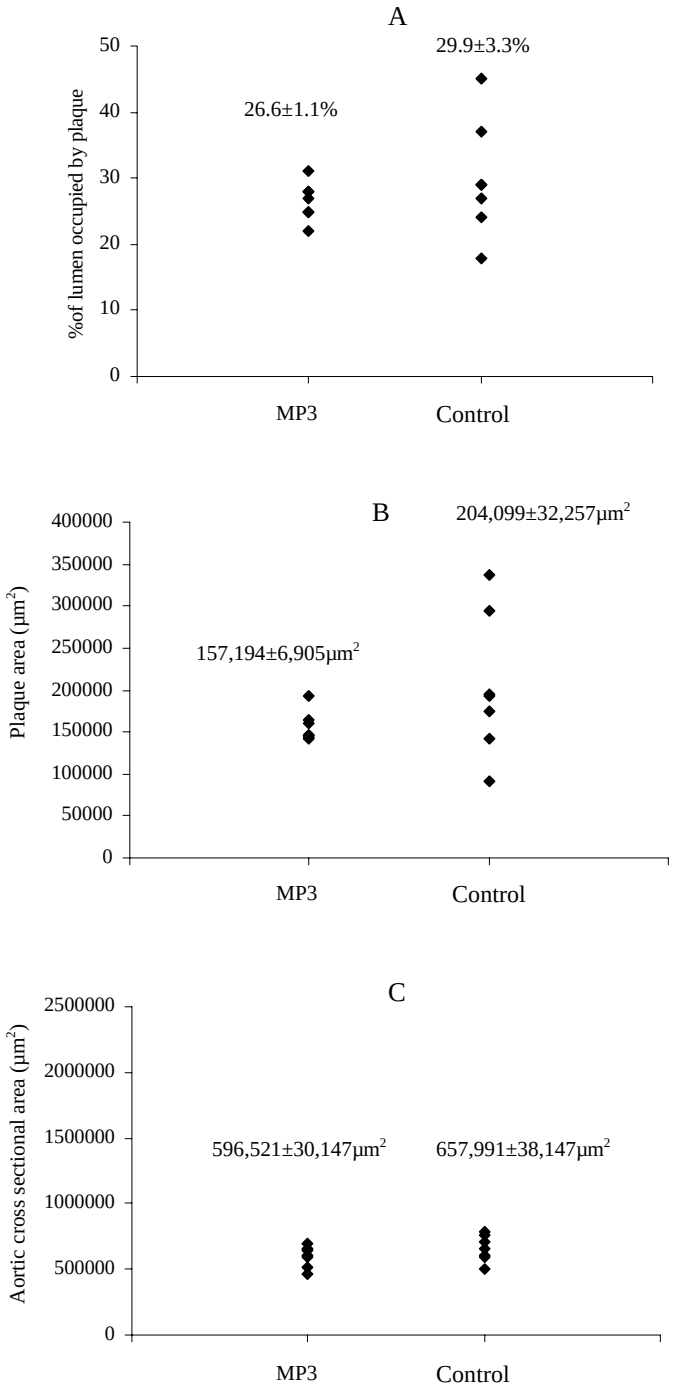

Figure 5.6. Effect of 70mg/kg MP3 for 4 weeks on development of atherosclerosis in apoE$^{-/-}$ mice fed a normal diet. Animals received daily treatment for 4 weeks while their high fat diet had been removed 4 weeks before starting treatment. The results are expressed as (A) percentage of lumen occupied by plaque, (B) plaque area, (C) aortic cross sectional area. The mean±sem of each group is given above each group. Each point represents a mouse.

Table 5.6. Effect of 70mg/kg MP3 for 4 weeks on grade of atherosclerotic lesion types in apoE$^{-/-}$ mice fed a normal diet. The hearts from the mice in figure 5.6 were graded for the severity of atherosclerosis.

	Lesion type								
Group	**I**	**II**	**III**	**IV**	**Va**	**Vb**	**Vc**	**VIa**	**VIb**
Test				71%			29%		
Control			29%	42%					29%

The % represents occurrence among the population in each group.

5.4 Effect of treatment with MP3 prior to initiation atherosclerosis development with high fat diet

Although the above experiments were conducted to increase the level of MP3 in the mice under normal diet conditions, it seems that these manipulations were still inadequate to show statistically reduction in lesion development. Another possibility for this failure of MP3 to suppress lesion size despite being given at 70mg/kg is that the fatty acid might not be able to affect the development of atherosclerosis when lesions had already developed.

To ensure that a greater amount of MP3 was present in the animals at the start of atherosclerotic diet ten 4-5 week old mice were treated with MP3 (70mg/kg) daily for 2 weeks prior to being placed on a high fat diet. The control group of nine 4-5 week old mice received adequate amount of vehicle. Three animals from each group were then sacrificed after 2 weeks. The remaining mice were placed on the high fat diet and they continued to receive the MP3 or vehicle every other day for 5 weeks. The mice were then sacrificed, hearts were isolated, sectioned and stained for examination.

The results showed that after 2 weeks of MP3 treatment on normal diet, the mice did not show any visible signs of atherosclerotic plaque except for one mouse in the control group which showed 1% plaque. After 5 weeks of further treatment with MP3 and in presence of a high fat diet, the results showed the mean percentage of lumen occupied by plaque was 9.3±2.6% for the test group compared to 11.7±2.3% for the control group (Figure 5.7). This represented a 20.5% reduction in plaque size caused by MP3

however it did not reach a statistically significant level ($p>0.05$). The results of direct measurement of plaque area; 36,135±7,503.9μm the test group and 13,8551±45,913μm in the control group however, showed a significant difference in the plaque area between test and control ($p<0.05$). The measurement of the aortic cross sectional area, 434,115±46,882μm in the test group and 1,146,953±326,877μm in control group, also showed a preventive effect of treatment on compensatory aortic enlargement in the test group ($p<0.05$) compared to control group (Figure 5.7). Therefore, this dose and treatment regime prevent both plaque formation and compensatory aortic dilation. This also explains why the results expressed as the percentage of lumen occupied by plaque did not revealed a significant difference between test and control groups.

The test group developed type II (n=4), type III (n=2), type Vb (n=1) lesions and the control group developed type II (n=5) and type Vb (n=1) lesions (Table 5.7). Therefore, treatment did not decrease the score of atherosclerotic lesions despite reducing lesion area.

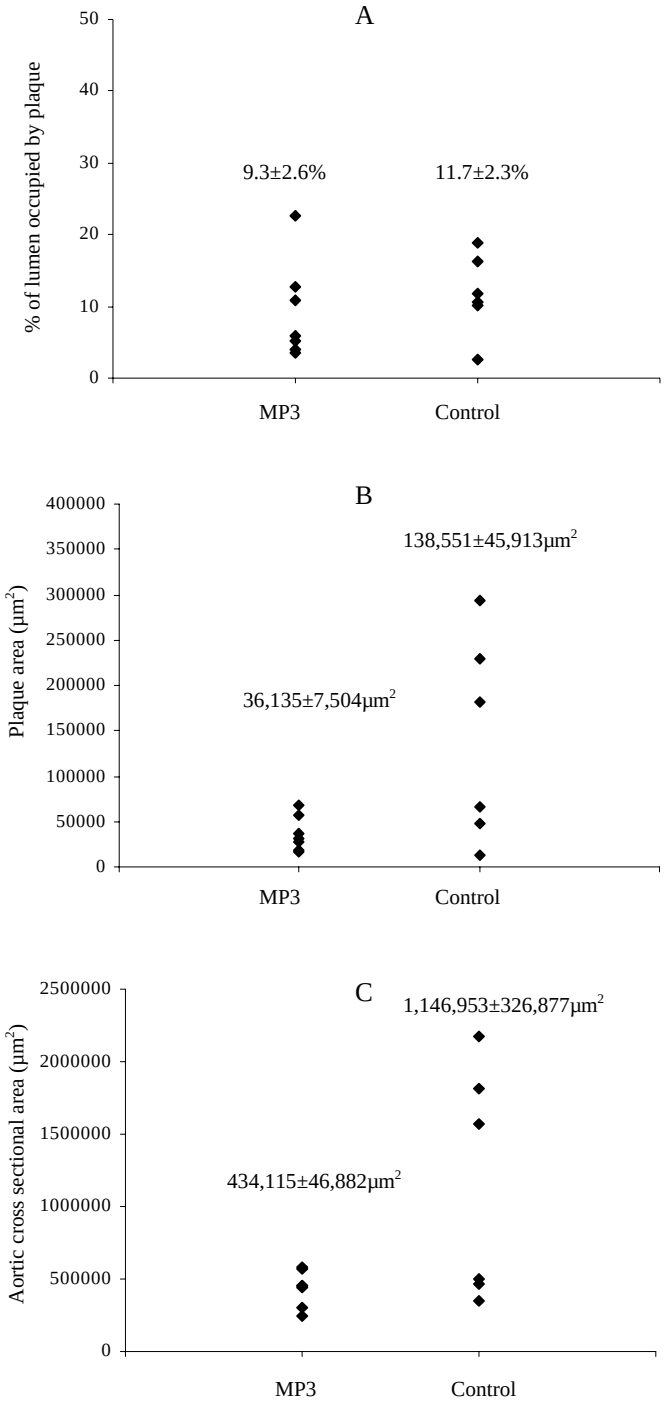

Figure 5.7. Effect of 70mg/kg MP3 on development of atherosclerosis in apoE$^{-/-}$ mice fed a normal diet for two weeks and a high fat diet for remaining of the experimental period. Mice were treated as described in the section 5.4 and the results are expressed as (A) percentage of lumen occupied by plaque, (B) plaque area, (C) aortic cross sectional area. Each mouse is represented by a point. The mean±sem of each group is given above each group. There were significant differences between test and control in B and C: $p<0.05$.

Table 5.7. Effect of 70mg/kg MP3 on the grade of atherosclerotic lesions in apoE$^{-/-}$ mice fed a normal diet during for two weeks and a high fat diet for rest of the experiment. The hearts from the mice in figure 5.7 were graded for the severity of atherosclerosis.

	Lesion type								
Group	**I**	**II**	**III**	**IV**	**Va**	**Vb**	**Vc**	**VIa**	**VIb**
Test		57%	29%			14%			
Control		83%				17%			

The % represented occurrence among the population in each group.

5.5 Summary

The results showed that β-oxa 23:4*n-6* (MP3) could protect against atherosclerosis and this protection was dose dependent. Thus irrespective of the treatment schedule used, doses of 40mg/kg body weight did not reach statistical differences. Protection was seen at a dose of 70mg/kg but this was highly dependant on the treatment schedule. The protection was seen when MP3 treatment was started at 4-5 weeks of age when there were no visible plaques and prior to the animals being placed on a high fat diet. Protection was evident by examination of both plaque area and aortic area as MP3 treatment reduced these values. However expressing the results as the percentage of lumen occupied by plaque did not show any difference which is most likely due to compensatory aortic enlargement described in chapter 4. However treatment did not affect the type of atherosclerotic lesions, suggesting that although the lesions are smaller in size after MP3 treatment the type of the lesions remain similar.

CHAPTER 6

DISCUSSION

6.1 Criteria for grading atherosclerosis in apoE$^{-/-}$ mice

The data demonstrated that by examination the aortic roots of apoE$^{-/-}$ mice at the level that aortic valve cusps are visible, a lesion prone site at which consistently develops plaque (Nakashima *et al.*, 1994), atherosclerotic lesions could be classified into six categories (Table 6.1). While type I lesions were characterised by a sparse infiltrate of macrophages and foam cells, type II were composed of a greater number of macrophages, including foam cells and characterised by two or more cell layers, surrounded by a thin cap that may be a thickened endothelial layer. Cholesterol clefts surrounded by foam cells and macrophages were a major component of type III lesions. A confluent lipid core, a thin fibrous cap and penetration of plaque into the media were the characteristics of type IV lesions. The atherosclerotic lesions progressed to more advanced types including type V, characterised by a well-developed fibrous cap and/or connective tissue (Va) or calcification (Vb) or ossification (Vc). The most advanced lesions or type VI were characterised with incipient aneurysm (VIa) or actual aneurysm formation (VIb) and/or inflammation including neutrophils (Figure 6.1). Plaque rupture, which could lead to haemorrhage and thrombus, was not observed in the aortic roots, possibly due to the healing process and fibrous cap formation which provides protection from rupture. Whether it is a case of area under study or the duration of study (up to a maximum of 39 weeks of age) remains to be evaluated. Interestingly, plaque rupture has been reported in the brachiocephalic arteries of this model after 8 weeks of high fat diet. These early acute plaque ruptures may heal over and become incorporated into the lesion as buried fibrous caps, which can be counted to give another estimate of plaque stability (Johnson *et al.*, 2005). In addition, a highly consistent rate of progression including vascular narrowing characterised by atrophy of the media and perivascular inflammation in the innominate artery in apoE$^{-/-}$ mice has been reported. In

that study animals aged 42 to 57 weeks showed intraplaque haemorrhage and a fibrotic conversion of necrotic zones accompanied by loss of the fibrous cap, and by 60 weeks of age showed collagen-rich fibrofatty nodules lesion with xanthomas at the edges (Rosenfeld *et al.*, 2000). As these findings have been reported in the literature, lesions with plaque rupture leading to haemorrhage and thrombosis were designated as type VIc even though we were unable to detect them.

The data showed that animals had mainly type I and a few of them type II lesions at a young age. When the animals were fed a high fat diet, they developed an almost consistent pattern of very early (type I) to very advanced types (type VIb) types of lesions over time (Figure 4.4). By 3 weeks of diet mainly type II was observed. A range of types II to IV of lesions were evident during the fourth to seventh weeks of diet and they progressed to type V from 12 to 19 weeks. The most advanced types (VI) were observed at 30 weeks of diet. By comparing of the sequence of progress in humans (Figure 6.2) with apoE$^{-/-}$ mice, plaque formation at infancy, puberty, late adolescence, early middle age and late middle age in humans can be addressed to 0, 1-3, 4-7, 12-19 and 30 weeks of high fat diet in apoE$^{-/-}$ mice, respectively. However due to the limited numbers of animals in the normal diet group, development of atherosclerotic lesions could not be shown to follow in the same consistent manner and not all lesion types were identified (Figure 4.8).

As the lesions progress to advanced type the size increases and there is a linear relationship between the percentage of lumen occupied by plaque and lesion types. However, variation in components of the plaque play an important role in grading the atherosclerotic lesions in apoE$^{-/-}$ mice.

There are many similarities in lesion components at different stages of disease between humans described by Stary (Stary *et al.*, 1994; Stary *et al.*, 1995; Stary, 2000b) (Figure 6.2) and apoE$^{-/-}$ mice described in this thesis, however there are a number of differences as well. In humans, the main layer involved in atherosclerotic lesions is the intima. The media and adventitia adjacent to the lesions are not diseased, except in very advanced stages when extracellular lipid deposits may modify media and adventitia (Stary *et al.*, 1994). Moe (2004) reported that arteriosclerosis affects the medial layer of elastic arteries leading to thickening and stiffness. In apoE$^{-/-}$ mice however medial involvement can be seen from type IV. In these mice, type I lesions were characterised by a slight increase in intimal thickness whereas type II lesions tended to disorganise the normal structure of the intima and narrow the lumen. Type IV lesions penetrate into the media and causes medial elastolysis. The elastin degradation may be due to a high amount of extracellular lipid deposits and/or large numbers of macrophages that release matrix metalloproteinases (MMPs). However, in humans; structural disorganisation and thickening of the intima occur from type IV and only types V, VI, VII and VIII show a narrowing of the lumen (Stary *et al.*, 1995). In apoE$^{-/-}$ mice, the cap in type II and III is the pre-existing intima at lesion prone sites, and a fibrous cap including smooth muscle cells and collagen, usually forms from type IV, while in humans a fibrous cap forms from types V and VI lesions (Stary, 2000b).

One interesting observation made during this study, was that in addition to calcification and ossification within the atherosclerotic plaque in the proximal aorta in apoE$^{-/-}$ mice with advanced disease, calcification was sometimes observed caudal to the site of maximal plaque formation in the aortic valve ring in animals with only very early disease. Calcium deposits in smooth muscle cells were likely to be due to arterial

osteoblast and osteoclast participation and this has been previously described (Stary, 2001; Doherty *et al.*, 2003). Intracellular calcification becomes extracellular and ossifies after disintegration of dead cells as lipidic remnants of macrophage foam cells and smooth muscle cells calcify. In addition, cartilaginous metaplasia, localised mainly in aortic valve attachments as a response to changes in mechanical stresses, is hypothesised as the pathway of artery wall calcification (Qiao *et al.*, 1995) providing a pathway for ossification at this level, even at early stages of the disease. In addition, recent advances in medical imaging including intravascular ultrasound, have demonstrated that in humans calcification can also occur early in the course of atherosclerotic disease (Moe, 2004).

Another important finding of this study was the presence of neutrophils in the atherosclerotic lesions in highly advanced stages of the disease. The presence of neutrophils was usually coincident with incipient or actual aneurysm formation and/or severe chronic inflammation external to the aorta especially around the coronary arteries. Neutrophils are probably attracted to the plaque in response to advanced inflammation and release proteolytic and elastolytic enzymes such as neutrophil elastase (NE) which leads to digestion of elastin, collagen, fibronectin, laminin and proteoglycans (Dollery *et al.*, 2003). This tissue destruction can lead to aneurysm, haemorrhage and plaque instability. In a small number of cases, neutrophils were also observed around cholesterol clefts (extracellular lipid pools). Formation of these extracellular lipid pools among the layers of cells may lead to more damage and attract neutrophils to the sites or matrix breakdown as a result of NE activity leading to the formation of cholesterol clefts.

Activated neutrophils may also play a proatherogenic role by releasing oxygen derived species leading to the formation more oxidised-LDL. Unfortunately, the role of neutrophils in atherogenesis has been neglected and future research is needed to determine their role and contribution to this disease. In humans, clinical studies have demonstrated the presence of activated neutrophils in patients with unstable angina pectoris (UAP) and acute myocardial infarction (AMI) (Naruko *et al.*, 2002). The postulated functions of neutrophils include endocytosis of foreign material or secretion of enzymes such as elastase and myeloperoxidase which may cause destabilisation of atherosclerotic plaques (Naruko *et al.*, 2002). A proatherogenic role for NE has been implicated because of its ability to break down HDL, increase LDL uptake and induce foam cell formation in humans (Dollery *et al.*, 2003).

Although qualitative systems can be subjective, the grading criteria for atherosclerotic lesions in apoE$^{-/-}$ mice developed in this study describes different lesion types in terms of cellular and non-cellular constituents. By using inbred animals with the same genetic background and the same environmental factors, the accuracy of lesion characterisation is increased compared to humans with all different genetic background and environmental factors. Although there are some differences between the atherosclerotic lesions in apoE$^{-/-}$ mice and humans, there are significant similarities in the morphology of lesions formed in this model compared with specific stages of the human disease. We did not observed any sudden deaths as a result of myocardial infarction or stroke even after progression of atherosclerotic lesions to very advanced lesions however Johnson and colleagues reported 73.8% sudden death during 40 weeks of high fat feeding (Johnson *et al.*, 2005). Therefore apoE$^{-/-}$ is a suitable model for assessing atherosclerosis.

Table 6.1. Criteria for atherosclerotic lesions in apoE$^{-/-}$ mice.

Lesion type	Lesion component	Cap formation	Media involvement
Type I	Isolated monocytes, macrophages and foam cells	No cap	Intact
Type II	Greater intensity of macrophages and foam cells, two or more cell layers	Thin cap	
Type III	Cholesterol clefts (extracellular lipid pools) surrounded by foam cells and macrophages		
Type IV	Confluent lipid cores surrounded by macrophages, foam cells and/or connective tissue	Fibrous cap	Partial disruption
Type V	Lipid core consisting collagen and smooth muscle cells (Va), calcification (Vb) and ossification (Vc)	Well developed fibrous cap	
Type VI	Incipient aneurysm (VIa) or actual aneurysm (VIb) with inflammation (including neutrophils)		Complete disruption

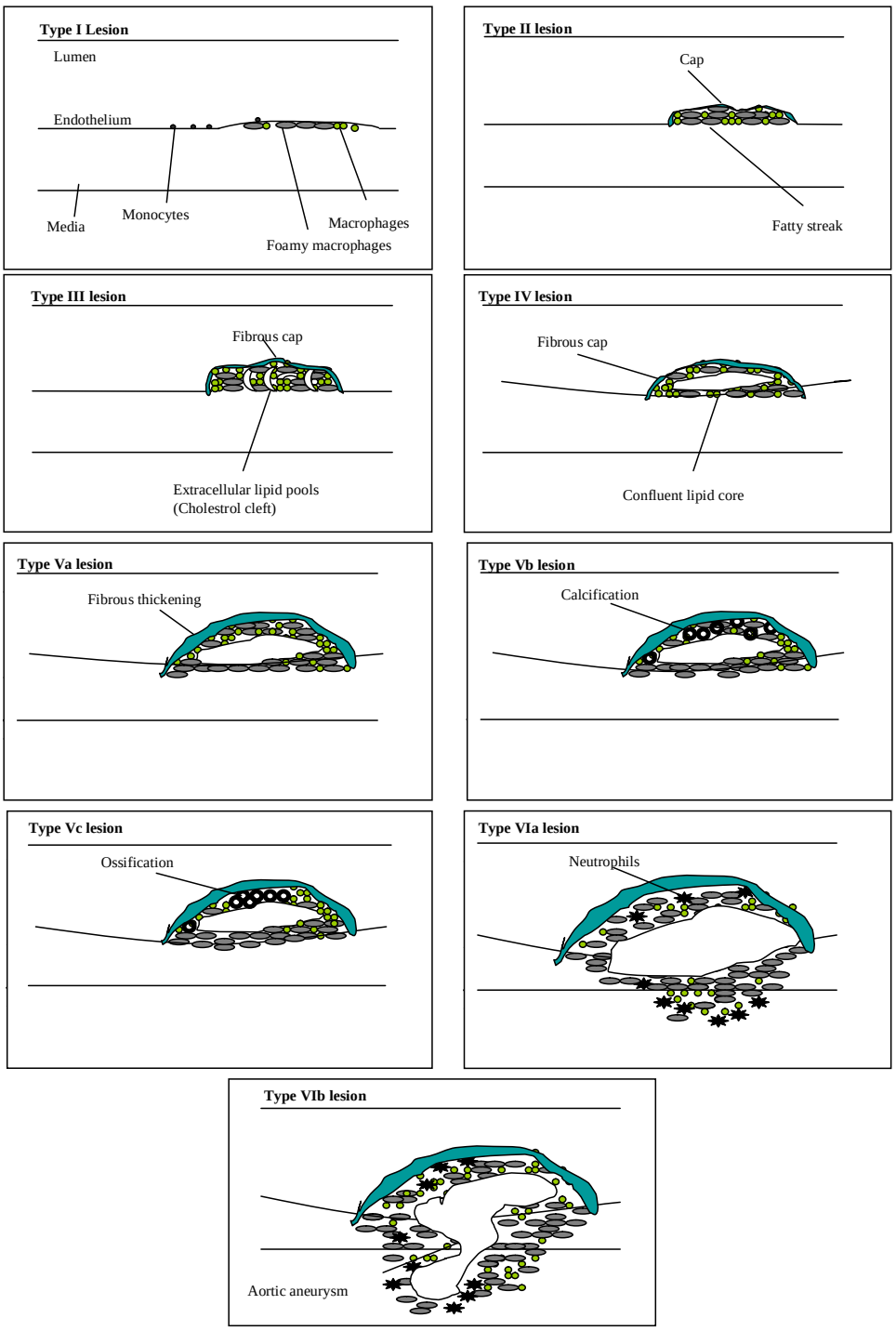

Figure 6.1. Diagrammatic representation of grading criteria in apoE$^{-/-}$ mice. Atherosclerotic lesions in this model were classified into six types based on histological characterisations described in details in chapter 3.

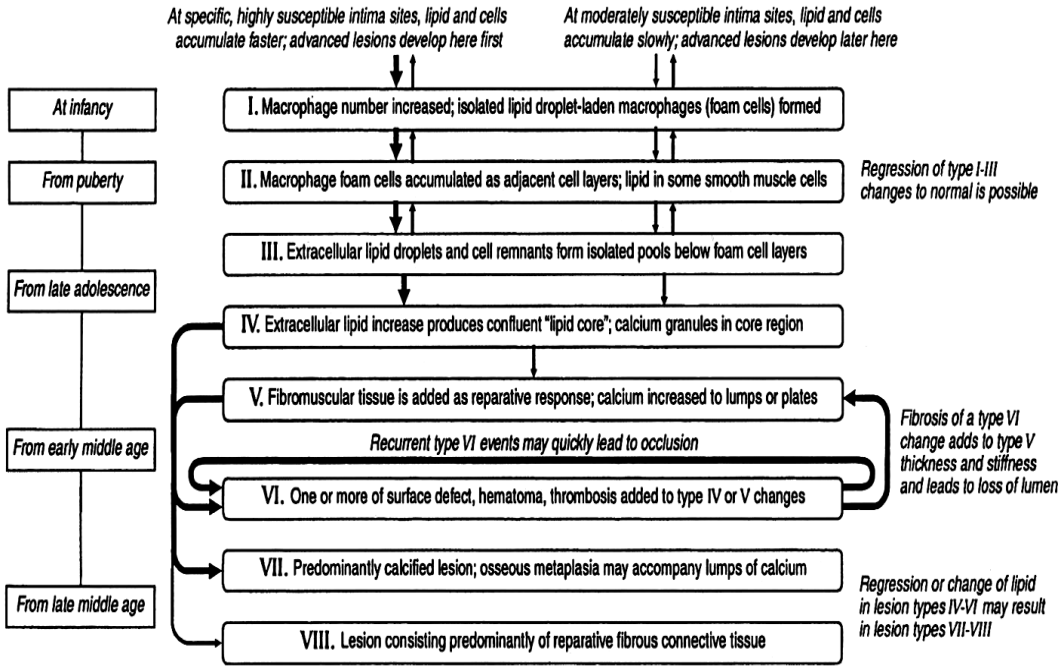

Figure 6.2. Outline of the sequence in the progress of atherosclerotic lesions in humans. Thick and thin arrows differentiate between the relative ease with which lesions develop at specific sites, or they indicate the relative frequency and importance of pathway section. Adapted from Stary 2001 (Stary, 2001).

6.2 Kinetics of development of atherosclerosis in apoE$^{-/-}$ mice

Because of the subjective and tedious nature of quantitative assessments, we used a computer-assisted method to measure plaque size which showed good intraobserver and interobserver agreement (Table 4.1). It was simple and readily applied to H&E stained slides. As well-defined areas were chosen for quantitation of lesion area for all animals, comparisons between the results are objective.

Plaque size development occurred in an exponential manner represented by the mathematical relationship $y=c-e^{(-ax+b)}$. If a is positive then as x becomes very large y will approach the value c which is an upper limit or asymptote. This model increases very rapidly at first and then levels off to become asymptotic to an upper limit. The mathematical method enables us to calculate the time for a 25% lesion development (LD_{25}) and estimate the upper limit of plaque size. The equation will also allow future experiments to be conducted on only a small number of animals as it can predict the development of the plaque.

In animals fed a high fat diet (21% fat and 0.15% cholesterol) resembling a Western diet, the equation is $y=57.4-e^{(-0.1x+4.2)}$, the upper limit of plaque size is 57.4% and LD_{25} is 7.9 weeks of high fat diet. Development of atherosclerotic lesions in apoE$^{-/-}$ mice which were fed a normal diet also followed a similar pattern $y=39.8-e^{(-0.2x+5.8)}$ with an upper limit of plaque size equal to 39.8% and LD_{25} 15.3 weeks of age. The results also show that a high fat diet accelerates plaque formation in younger animals more than in older animals. Five weeks of a high fat diet leads to formation of atherosclerotic plaques 4.8 times larger than those seen in animals fed a normal diet at 11 weeks of age.

On the other hand 30 weeks of high fat diet appeared to increase plaque size only 1.2 times compared to animals on a normal diet at 39 weeks of age.

Compensatory cardiac hypertrophy (data not shown) and compensatory dilation of the proximal aorta were observed mainly in high fat diet group. An increase in artery size in the relatively early phase of advanced human atherosclerotic lesion formation as compensatory enlargement or vascular remodelling was reported first by Glagov (Stary, 2001). Although the exact mechanisms of vascular remodelling in humans are not clear, proposed mechanisms include alterations in shear stress, shear stress-induced NO production, endothelial dysfunction, hypertension and the activation of matrix degradation proteins (Lutgens *et al.*, 2001). Lutgens and colleagues reported compensatory enlargement in the carotid arteries, the thoracic aorta and abdominal aorta in apoE$^{-/-}$ mice on an atherogenic diet, however constrictive remodelling and ischemic organ damage did not occur (Lutgens *et al.*, 2001). This vascular remodelling is an adaptive response to chronic changes in hemodynamic conditions. As plaque size increases, proximal aortic enlargement occurs as a compensatory response to extensive atheroma, showing a linear relationship between the aortic cross sectional area and plaque size in animals fed a high fat diet. In addition, the presence of large numbers of macrophages, which release matrix metalloproteinases (MMPs) (Lutgens *et al.*, 2001), and neutrophils, which release NE, in advanced lesions may also play important roles in remodelling of the proximal aorta as a result of elastic fibre degradation within the media and aneurysm formation. This compensatory enlargement is the reason why more extensive occlusion of the aorta was not observed and why the kinetics of development levelled off and reached a plateau level with ongoing survival of affected animals. It also points out the importance of direct measurement of plaque size in

addition to expressing results as a percentage of lumen area during the assessment of inhibitory effects of different medications. If treatment is able inhibit compensatory aortic enlargement in addition to suppressing plaque size, a change may not be appreciated when results are expressed as the percentage of plaque area to lumen area.

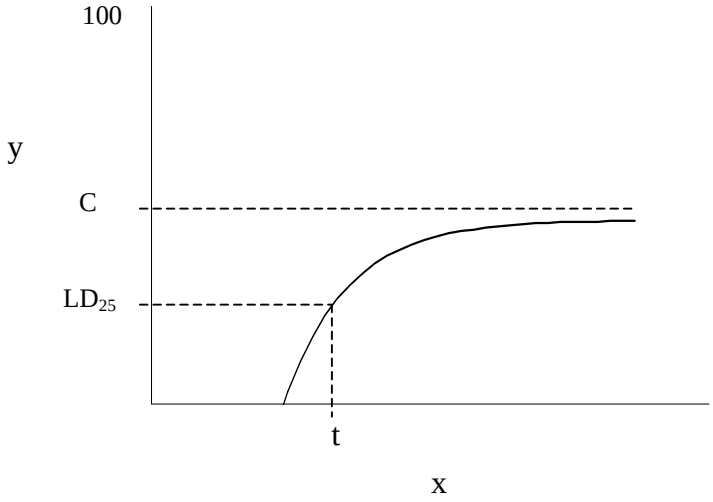

Figure 6.3. Exponential model: $y=c-e^{(-ax+b)}$. This mathematical model enables us to calculate the time (t) required for 25% lesion formation and estimate the upper limit of plaque size or asymptote (c).

6.3 MP3 protects against atherosclerosis

The data demonstrated that mice receiving the novel polyunsaturated fatty acid β-oxa-23:4*n-6* (MP3) were protected against atherosclerosis. This fatty acid protected against experimental atherosclerosis in a dose dependent manner and under condition that favoured increased uptake of MP3 in tissues. Of the two doses examined, protection was clearly evident at 70mg/kg under conditions that the level of fat supplementation was restricted to favour the uptake of MP3 into the tissue. It is well accepted that in trials involving *n-3* supplementation, greater efficacy is seen if the level of *n-6* fatty acids in the diet are simultaneously reduced (Simopoulos, 1999; Simopoulos, 2002). Interestingly, MP3 acted most effectively when the animals were treated with 70mg/kg from an early age. Using a different schedule in which the treatment was delayed until plaque development had been established, even 70mg/kg failed to give significant level of protection. Thus apart from protection being a function of amount of MP3 incorporated into the tissue, our data would be consistent with the view that MP3 is most effective in preventing the development of atherosclerotic plaque at early stages. Further experiments would be required to determine whether increasing the dose of MP3 above 70mg/kg can cure the disease in the advanced stages. Nevertheless our findings would support the potential use of MP3 during childhood since it is now evident that atherosclerosis begins at the young age (Stary *et al.*, 1994; Ross, 1999a). Consistent with this, the data in figures 5.1 and 5.2 show that MP3 (40mg/kg) appeared to afford some protection for the first few weeks of treatment. However, this protective effect was lost after a longer period of high fat diet and this was most likely to be due to the low dose of the fatty acid being administered and the unrestricted intake of fat and cholesterol.

It was evident from our results that there were limitations in the expression of level of plaque development based on a ratio of plaque area to aortic cross area (the percentage of the lumen occupied by plaque) because plaque development can be associated with changes in size of the aorta. Consequently, examination of the results obtained with the effective dose of MP3 failed to show significant protection when the data were expressed as a ratio of these two parameters. In comparison, analysis of these results as plaque size *per se* demonstrated that there was a 70% decrease in plaque area. Importantly, this protection was also evident in a parallel reduction in the size of the aorta. Clearly, these findings point to the fact that it is not accurate to express the results as percentage (ratio) when assessing the effect of anti-atherogenic agents.

In contrast to these results from the quantitative analyses, examination of plaque characteristics by criteria established in this thesis did not revealed the expected effect of MP3. It is not clear to us as to why this was so. However, it would indicate that decreasing the lesion size can occur without a corresponding change in the characteristics of the lesions (type). It would be interesting to do similar comparisons between these two types of assessment criteria with respect to other anti-atherogenic agents. Since the above finding seems to be inconsistent with the data in chapter 4 showing there is a significant correlation between the plaque size and lesion type, the findings suggest this relationship may not hold when the body is subjected to anti-atherogenic stimuli.

While the exact mechanisms by which MP3 protects against atherosclerosis remain to be elucidated, several pieces of evidence point to an effect of MP3 on intracellular signalling molecules that induce the expression of cell adhesion molecules (CAM) on

blood vessels walls. These molecules are responsible for leukocyte adhesion and infiltration into the blood vessel wall. Recently, Ferrante *et al* 2005b have reported that MP3 inhibits the activation of transcription factor, NFκB and thereby prevented the increase in expression of CAM in and leukocyte adhesion to blood vessels. It is well established that inhibition of CAM expression protects against atherosclerosis, including in animal models such as apoE$^{-/-}$ mice (Nageh *et al.*, 1997; Ramos *et al.*, 1999; Collins *et al.*, 2000; Huo and Ley, 2001).

Some light can be shed on the basis by which MP3 inhibits CAM expression. This has been in part described in figure 6.4. MP3, when added to tissues/cells, becomes incorporated into the *sn*-2 position of the membrane phospholipids such as phosphatidylinositol 4,5 bisphosphate (PIP2) (Ferrante *et al.*, 2005b) in competition with other polyunsaturated fatty acids such as AA, and in this manner the ability of phosphatidylinositol 3 kinase (PI3K) to interact with PIP2 may be compromised leading to poor production of PIP3 and in this manner prevent phosphoinositide-dependent kinase (PDK) from activating protein kinase B (Akt). This then results in an inability of atherogenic substances such as TNF to activate the IKK-NFκB pathway. Alternatively, MP3 may block Akt activation by stimulating the transcriptional activation of peroxisomal proliferator-activated receptor (PPARα) (Figure 6.4). It has been established that oxidised products of PUFA inhibit NFκB activation and that such lipid metabolised do this by activating PPARα (Sethi *et al.*, 2002). Such a mechanism will explain how MP3 can inhibit NFκB activation with the associated inhibition of CAM expression on blood vessel walls in the presence of atherogenic factors.

Whether or not MP3 will on its own right become a therapeutic for cardiovascular diseases remains to be identified.

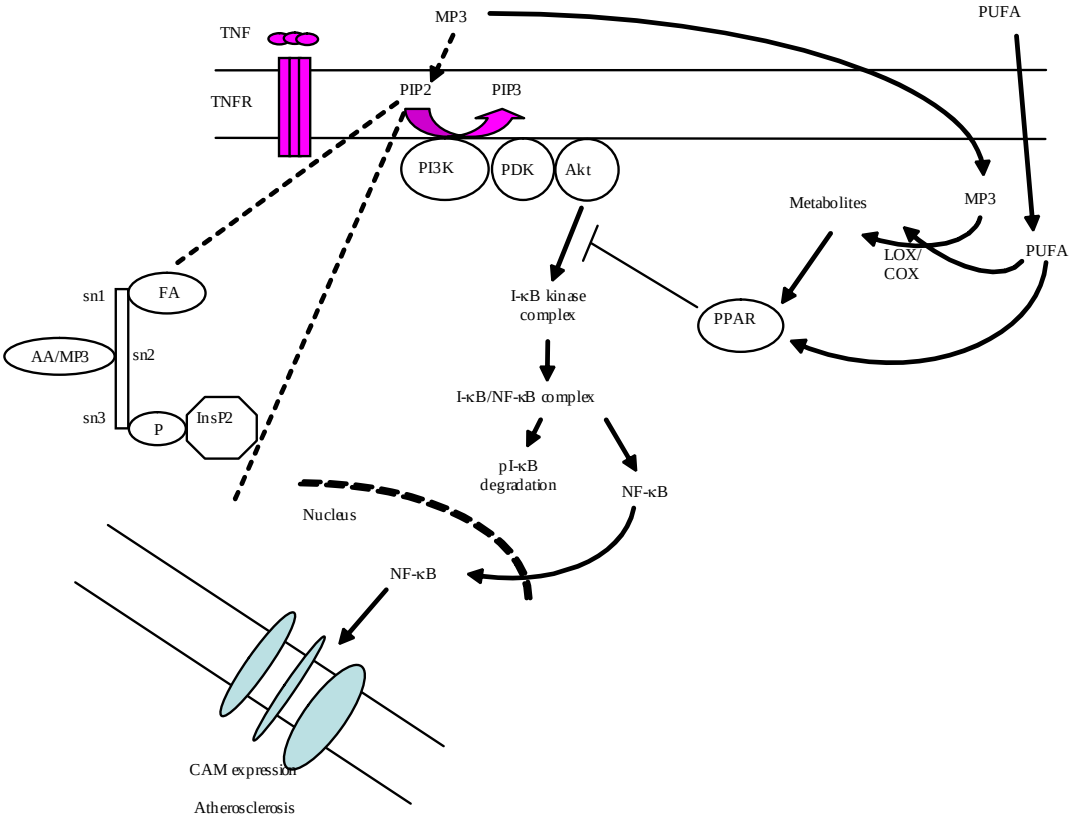

Figure 6.4. Diagrammatic representation of possible mechanism by which MP3 prevents against atherosclerosis. The diagram shows that atherogenic agents up-regulate CAM via the AKT-NFκB pathway. MP3 inhibits AKT activation either by altering the structure of PIP2 or stimulating PPARα. PIP2: phosphatidylinositol 4,5 bisphosphate, PI3K: phosphatidylinositol 3 kinase, PPAR: Peroxisome proliferator activated receptor, PDK: phosphoinositide-dependent kinase, Akt: PKB/protein kinase B, CAM: cell adhesion molecules (E-selectin, ICAM-1, VCAM-1), LOX: lipoxygenases, COX: cyclooxygenases, FA: Fatty acid.

6.4 Concluding remarks

This thesis has established criteria for grading different stages of atherosclerotic lesion development in apoE$^{-/-}$ mice. These were graded from types I to VI based on histological characteristics of lesion components including macrophages density, foam cell formation, cholesterol clefts, confluent lipid core, fibrous cap formation, calcification, ossification and aneurysm. Interestingly our work also highlighted the presence of neutrophils in the atherosclerotic lesions which has not been previously appreciated. Nevertheless, this may contribute to pathogenesis through the release of oxygen radicals and myeloperoxidase, thereby causing lipid peroxidation and activation of blood vessel walls. This classification showed both similarities and differences when compared to the classification of human plaques by Stary *et al* (1994, 1995 and 2000b).

Examination of lesion development quantitatively, using the size of the plaque as a proportion to lumen size showed that plaque development occurred in an exponential manner, governed by the general relationship of $y=-e^{-x}$. It was evident that the asymptotic nature of this development was the result of a corresponding increase in blood vessel size which accompanied the increase in plaque size. This shows that one should not rely solely on this type of expression. Clearly, emphasis needs to be placed on the actual size of the lesion. This is important when examining anti-atherogenic agents since reduction in plaque size is likely to lead to normalisation of blood vessel size. If comparison is going to be made using percentage of lesion size to lumen size, then the comparison should be made during the earlier stages of lesion formation.

Our findings showed that the novel polyunsaturated fatty acid, MP3, could protect against the development of atherosclerosis which is consistent with our stated

hypothesis and we have thus achieved the overall objective of the thesis. This anti-atherogenic action was evident in plaque area (70% reduction) and aortic cross sectional area (60% reduction). Although it was not possible to conduct the required number of experiments to establish efficacy doses and appropriate treatment schedules within the time frame of this thesis, the data suggested that the protection may also be a function of the level of MP3 incorporated into tissue. This work has therefore revealed that novel PUFA could be explored as potential anti-atherogenic factors

6.5 Future research

There is a need to conduct more experiments to examine the efficacy of MP3 in protecting against atherosclerosis under several different variables, which are conducive to attaining tissue levels that cause a significant reduction in cell adhesion molecules on blood vessel walls. This may be achieved through pharmacokinetic studies.

While we have already identified targets for MP3, it remains important to examine these targets directly in this disease. This could include the composition of cellular incorporate, expression of CAM and activation of transcription factors.

The infiltration of plaque lesions by neutrophils has not been well appreciated. Directions should be initiated in not only confirming our findings but also to aim to understand whether these cells play a significant role in pathogenesis of atherosclerosis.

Although we have substantially increased level of knowledge about characteristics of the plaque at the histological level it is evident that this can be extended in terms of

development of calcification and ossification, particularly as we saw these very early in the disease.

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