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

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Cardiovascular disease is the most common cause of death in the developed world and about one million fatalities in the United Sates yearly have been reported (Ridker *et al.*, 2001). In a report from Australian Bureau of Statistics in 2004, cardiovascular disease remains one of the leading causes of death in Australia in 2002, accounting for 50294 or 38% of all deaths (ABS, 2004).

Atherosclerosis is a chronic inflammatory vascular disease, characterised by a thickening of the vascular wall due to lipid accumulation, infiltration by circulating monocytes and T cells and proliferation of smooth muscle cells (Ross, 1999a; Ross, 1999b; Libby *et al.*, 2002). Despite the common belief that atherosclerosis is a disease of old age, the initial atherosclerotic lesion may form from infancy and develop to advanced lesions during life (Stary *et al.*, 1994). It is a multifactorial disease with both genetic and environmental predisposing factors. Among several risk factors, inherited defects in lipid metabolism and diet-induced hypercholesterolemia have received most attention (Reddick *et al.*, 1994).

One of the risk factors for atherosclerosis is apoE deficiency. ApoE is a 34 kD glycoprotein that is synthesised in the liver, brain and other tissue in both humans and mice. It is a structural component of all lipoproteins except low density lipoprotein (LDL) and mediates clearance of chylomicron remnants and very low density lipoproteins (VLDL) (Zhang *et al.*, 1992). ApoE genetic variation in humans has a significant effect on lipoprotein patterns and atherosclerosis susceptibility. Humans

with apoE deficiency have high levels of cholesterol resembling type III hyperlipoproteinemia, early development of atherosclerosis and xanthomas (Plump *et al.*, 1992).

A high fat diet is another major risk factor for atherosclerosis. The majority of polyunsaturated fatty acids (PUFA) received in the Western diet, are *n*-6 fatty acids and saturated fatty acids. These promote inflammation and the community is well aware that this diet needs supplementation with *n-3* fatty acids to balance the levels (a ratio of *n-6/n-3* of 1-4/1 in diet rather than the ratio of 20-16/1 in the current Western diet) and prevent the effects of *n-6* fatty acids (Simopoulos, 1999; Simopoulos, 2002). The protection by *n-3* fatty acids occurs through the production of eicosanoids that have several fold less pro-inflammatory activity than those produced through the oxidation of *n-6* fatty acids, through their anti-thrombotic effects (Simopoulos, 1999) and by their ability to inhibit expression of cell adhesion molecules (CAM) on blood vessel walls (De Caterina *et al.*, 1999). Recently we have developed a novel group of PUFA which were more selective in targeting pro-inflammatory pathways than the natural *n-3* fatty acids. One of these, β-oxa 23:4*n-6* was found to selectively inhibit the up-regulation of CAM by inhibiting the activation of the transcriptional factor NFκB (Ferrante *et al.*, 2005b). Since these inflammatory variables are required in the development of atherosclerosis, it was the objective of this thesis to examine whether this novel fatty acid could protect against this disease in the apoE$^{-/-}$ experimental mouse model.

New approaches to target the inflammatory pathways involved in development of atherosclerosis and to improve treatment of atherosclerosis are essential to improve the long term outcome and quality of life for individuals with this disease.

1.2 Structure of arteries

Arteries consist of three major layers; the intima, media and adventitia respectively from inside to outside (Libby, 2001). The tuinca intima, the innermost layer of the arterial wall, consists of endothelium and a subendothelial layer (Ilveskoski, 2001). The major cells in human arterial intima are endothelial cells and smooth muscle cells with isolated macrophages (Stary *et al.*, 1992). The subendothelial layer of tuinca intima consists of two layers. The inner layer, a proteoglycan layer mainly contains nonfibrous connective tissue. There are small amounts of elastic fibers, smooth muscle cells and macrophages in this layer. The musculoelastic layer is a thicker layer which underlies the proteoglycan layer contains a large number of smooth muscle cells and elastic fibers (Stary *et al.*, 1992; Ilveskoski, 2001; Libby, 2001).

The internal elastic lamina is part of the media that separates the intima from the media. However, in some parts of arteries with geometric transitions, like bifurcations, branches and curvatures, the internal elastic lamina is not well defined. Thus, it is difficult to distinguish intima and media in these regions and they appear as a single unit (Stary *et al.*, 1992). The tunica media contains smooth muscle cells and an elastic-rich extracellular matrix. The external elastic lamina separates media form adventitia (Libby, 2001).

The tunica adventitia, the outermost layer of the arterial wall, contains sparse collagen fibrils, vasa vasorum and nerve endings. The major cell types in the adventitia are sparse fibroblasts and mast cells (Libby, 2001).

1.3 Atherosclerosis

1.3.1 Definition of atherosclerosis

Atherosclerosis comes from the Greek words meaning: athero (gruel or paste) and sclerosis (hardness). It is a chronic disease of elastic arteries and large and medium size muscular arteries characterised by thickening of the vascular wall. Atherosclerotic plaques are intimal lesions which protrude into and obstruct the vascular lumen and weaken the underlying media (Gotlieb and Havenith, 1991; Schoen, 2005).

1.3.2 Main hypotheses for development of atherosclerosis

Several theories have been put forward to explain the development of atherosclerosis. These are now considered collectively to explain atherosclerosis development with inflammation of vessel wall as the main primary event. Several of these hypotheses are discussed briefly below (Weissberg and Rudd, 2002).

Monoclonal theory. This theory proposes that a dysfunction in smooth muscle cells as the basis for the disease. The initiating factor is a mutagen that mainly affects smooth muscle cells. For example Marek's disease virus, a herpes-type DNA virus, induces

fatty lesions in chickens and promotes the accumulation of cholesterol and cholesteryl ester in cultured arterial smooth muscle cells. The possibility is raised that a form of selection occurs that promotes monoclonality. Therefore, some smooth muscle cells in the intima preferentially proliferate in the microenvironment of early lesions, like hypercholesterolemia (Gotlieb and Havenith, 1991).

Lipid hypothesis. The lipid hypothesis, proposed first in 1913 by Anitschkow claims that the gradual accumulation of lipid in the arterial wall is responsible for the development of atherosclerosis. This is supported by findings that link elevated serum lipid levels with the risk of development of atherosclerotic lesions in humans (Weissberg and Rudd, 2002).

Thrombogenic hypothesis. In the thrombogenic hypothesis, atherosclerotic lesions grow by the gradual incorporation of luminal thrombus into the arterial wall. The presence of fibrin and platelet-derived protein in both developing and advanced lesions as well as the presence of a large amount of platelet-derived growth factor support this theory. However, this theory is difficult to prove because plaque infiltration by immature blood vessels is common in advanced lesions, which also leads to haemorrhage and thrombosis (Weissberg and Rudd, 2002).

Response/modified response to injury hypothesis. Virchow in 1856 proposed that the endothelial damage or dysfunction is a basic stage and atherosclerosis is a healing response of the arterial intima to a prior mechanical injury (Gotlieb and Havenith, 1991;

Weissberg and Rudd, 2002). Ross and Glomset (1973) initially published a modified version of the response to injury hypothesis and made subsequent changes later (Weissberg and Rudd, 2002). Based on the modified response to injury hypothesis, atherosclerosis begins as a protective or healing process to endothelial cell injury. Injurious agents, such as oxidised-LDL and toxic factors or radicals originating from cigarette smoke, induce some changes in both endothelial cells and circulating lymphocytes. Endothelial dysfunction makes endothelial surfaces more adhesive therefore lymphocyte adherence to endothelium increases. Adherent leukocytes enter the intima resulting in chronic inflammation within the intima of the artery. During this inflammatory process, different cells become active and release growth regulatory molecules such as cytokines that lead to migration and replication of macrophages and smooth muscle cells, resulting in an inflammatory fibroproliferative response (Ross, 1999a; Ross, 1999b).

Inflammation theory. Ross, in his recent reviews of the pathogenesis of atherosclerosis highlighted the important role of inflammation at every step of disease development together with the importance of endothelial dysfunction (Weissberg and Rudd, 2002). Substantial biological data implicate inflammatory pathways in early atherogenesis, in the progression of the lesions and finally in the thrombotic complications of the disease. Also major risk factors for atherosclerosis such as oxidised LDL, dyslipidemia, hypertension, diabetes, obesity and infection are counted as triggers for inflammation in atherogenesis (Libby *et al.*, 2002).

Atherosclerosis preferentially occurs in sites which are referred to as lesion prone sites.

1.3.3 Definition of adaptive intimal thickening

There is variation in the thickness of the arterial intima in different regions because of variation in mechanical stresses resulting from variations in blood flow and/or wall tension. The intima, as a living and active tissue, responds to reduced shear stress and increased tensile stress by an increase in thickness, a process known as adaptive intimal thickening. Adaptive intimal thickening is an adjustment to changes in pulse rate, blood pressure, arterial geometry, flow rate, and resistance to flow in distal vascular segments in supplied organs. However, adaptive intimal thickening does not mean an abnormal and diseased artery. Adaptive intimal thickening has two patterns, diffuse intimal thickening that usually occurs in relatively straight arterial segments with a uniform distribution of mechanical stresses and secondly, eccentric intimal thickening occurring in regions of bifurcations and branches where both shear and tensile stresses are not uniform (Stary *et al.*, 1992).

The turnover of endothelial cells and smooth muscle cells and the concentrations of low density lipoproteins and other plasma components increase in adaptive intimal thickening compared with an adjacent region without thickening. However, these increases do not mean abnormality unless they cause tissue damage (Stary *et al.*, 1992).

During the process of atherogenesis, advanced lesions develop first in the regions with adaptive intimal thickening (Stary *et al.*, 1992). Physiologic fluid shear stress exerts on atheroprotective effect in vivo as atherosclerosis preferentially occurs in areas of

disturbed flow or low shear stress, whereas regions with steady laminar flow and physiologic shear stress are protected (Yamawaki *et al.*, 2005). These susceptible regions of arteries, including branches, bifurcations and curvatures, are known as the atherosclerotic lesion prone sites (Ross, 1999b). In locations of the arterial tree with adaptive intimal thickening, specific mechanical stresses exist whether high concentrations of atherogenic lipoproteins (e.g. LDL) are present or not. When atherogenic lipoproteins increase, the same mechanical forces may increase lipoprotein deposition in the same regions, leading to transformation into atheromatous lesions. Although the initial signs of development of advanced lesions occur at the site of adaptive intimal thickening, the advanced lesions are not confined to these regions and nearly all regions in the aorta and many arteries may become the sites of advanced lesions (Stary *et al.*, 1992).

At the lesion prone sites the expression of adhesion molecules on endothelium, such as E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), increases upon exposure to risk factors and results in monocyte and T cell adherence, migration and accumulation at these sites (Ross, 1999b).

There is significant evidence that CAMs play key roles in atherogenesis. Many atherogenic factors, such as hypercholesterolemia, lysophosphatidylcholine and advanced glycation end products (AGE) have been reported to increase ICAM-1 and VCAM-1 expression on endothelial cells (Huo and Ley, 2001). Oxidised LDL enhances VCAM-1 expression only in endothelial cells stimulated with cytokines such

as tumour necrosis factor α (TNF) and interleukin 1β (Huo and Ley, 2001), which are produced at sites of inflammation. *In vivo*, increase in CAM expression is localised to human arteries with atherosclerotic lesions and in lesion-prone sites on the aortae of mice and rabbits (Johnson-Tidey *et al.*, 1994; Ross, 1999b). One of the essential intracellular factors that is required for the up-regulation of CAM expression on the endothelium is the transcription factor, NFκB. The activity of NFκB is tightly regulated by cytokines and other stimuli. In the resting cells, NFκB dimers are sequestered in the cytoplasm by IκB proteins. Upon activation, IκB is phosphorylated by a signalosome complex of IκB kinases. The phosphorylated IκB dissociates from NFκB and undergoes proteosome-mediated degradation, permitting the nuclear translocation of NFκB and increase in CAM expression (May *et al.*, 2000; Valen *et al.*, 2001; Lentsch and Ward, 1999) (Figure 1.1).

The process of atherosclerosis begins by tethering and rolling, or saltatory, of leukocytes (monocytes and T cells) along the endothelial surface mediated by endothelial E and P-selectins and leukocyte L-selectin (Ross, 1999b; Libby, 2002). This is followed by firm adhesion of leukocytes onto the endothelium resulting from binding of β1 and β2 integrins on leukocytes and ICAM-1 and VCAM-1 on endothelial layers. Migration of leukocytes into the intima is then mediated by oxidised-LDL, monocyte chemotactic protein 1 (MCP-1), interleukin-8 (IL-8), platelet-derived growth factor, macrophage colony-stimulating factor (M-CSF) and osteopontin. The monocytes differentiate into macrophages and ingest a modified form of LDL to become foam cells (Ross, 1999b; Libby, 2002). Foam cell formation is mediated by oxidised-LDL, M-CSF, tumour necrosis factor α (TNF α) and interleukin 1(IL-1). Then fatty streaks form which consist of lipid laden monocytes and macrophages (foam

cells), T cells and smooth muscle cells. Activated macrophages release inflammatory cytokines and growth factors that may be responsible for recruitment of additional monocytes in the developing lesion and stimulation of smooth muscle cell migration and proliferation (Ross, 1999b). These processes progress to form intermediate and advanced lesions, which consist of a fibrofatty matrix of connective tissue, smooth muscle and foam cells. Intermediate and advanced lesions at this stage tend to form a fibrous cap on the lesion that walls off the lesion from the lumen and represents a type of healing or fibrous response to the injury. Continuing influx and activation of macrophages, which release metalloproteinases and other proteolytic enzymes capable of degrading the collagen, can lead to thinning of the fibrous cap, haemorrhage and thrombus formation and occlusion of the artery at end stages of atherosclerosis (Figure 1.2) (Ross, 1999b).

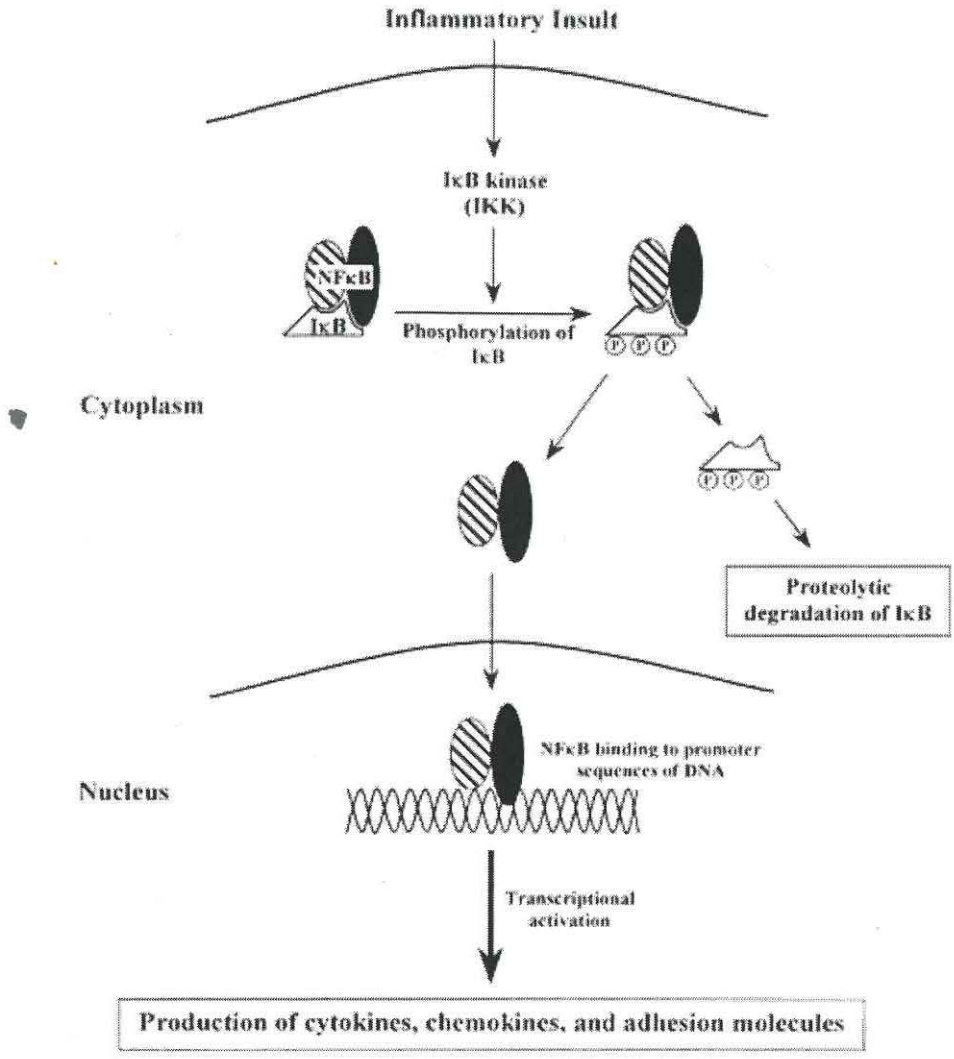


Figure 1.1. Mechanism of activation of NFκB dimers which up regulate CAM expression and play important role in the atherogenesis (Lentsch and Ward, 1999).

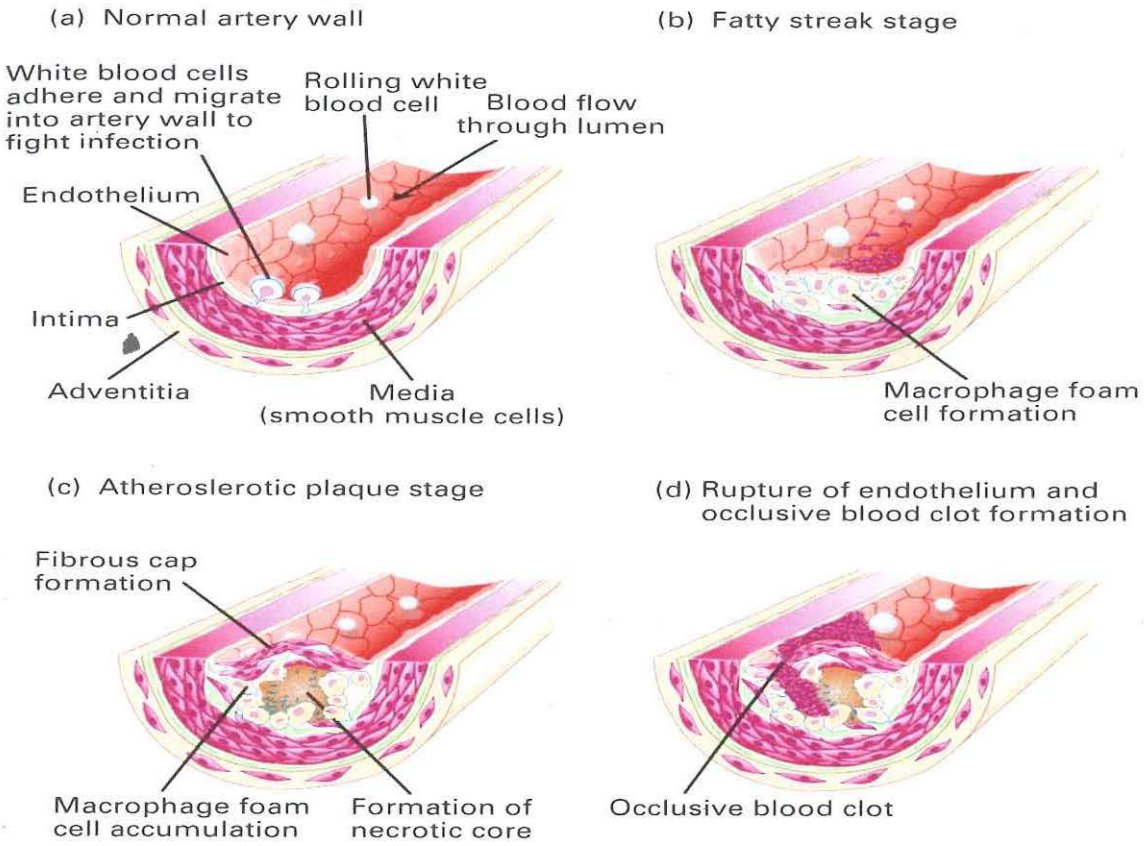


Figure 1.2. Features of atherogenesis. Endothelial dysfunction (a) is the first stage of atherosclerosis followed by formation of fatty streaks (b), advanced and complicated lesions (c), and unstable fibrous plaque formation (d) in atherosclerosis (Ross, 1999b).

1.3.4 Classification of atherosclerosis

It is essential to classify the different types of atherosclerotic lesion in order to facilitate distinction between the different stages of atherogenesis by morphological examination.

The American Heart Association's (AHA) committee on vascular lesions has described different types of atherosclerotic lesions and provided a histological classification of all human atherosclerotic lesion types. In the histological classification, lesions are designated by Roman numerals, which indicate the usual sequence of lesion progression (Stary *et al.*, 1994; Stary *et al.*, 1995; Stary, 2000b).

1.3.4.1 AHA classification

In the 1990s, a classification of atherosclerosis was presented by Herbert C. Stary and colleagues in four reports, by the Committee on Vascular Lesions of the Council on atherosclerosis, American Hearth Association (AHA). This classification is based on consistent morphological characteristics of the lesion. The lesions are divided into early, intermediate and advanced forms. Early lesions (Type I and Type II) usually occur in infants and children. Those considered intermediate lesions (Type III) develop gradually after puberty and are a transitional stage between early and advanced lesions. Advanced lesions (Type IV-VIII), are characterised as those where components disorganise the intimal structure and deform the arterial wall and usually begin in the third decade of life (Table 1.1, Figure 1.3 and Figure 1.4) (Stary *et al.*, 1994; Stary *et al.*, 1995; Stary, 2000b).

The susceptibility of locations for development of atherosclerotic lesions can be divided into three degrees; (1) locations at which lipid does not accumulate and foam cells do

not form except in the presence of a very high level of atherogenic lipoproteins (as in homozygous familial hypercholesterolemia); (2) moderately susceptible sites at which foam cell formation occurs (lesion types I and II) but progression to atheroma is slow and late; and (3) highly susceptible sites at which there is early-onset foam cell accumulation and progression to advanced types happen earlier than elsewhere (Stary, 2001).

Type I lesion. This early change appearing in the blood vessels frequently develops in infants and children. The lesion consists of isolated macrophages and foam cells accumulating at adaptive intimal thickenings or lesion prone sites (Stary *et al.*, 1994).

Type II lesion. The type II lesion consists of multiple layers of foam cells which appear as yellow-coloured streaks, patches or spots on the intimal surface. Fatty streaks can be recognised by their red colour after staining with Sudan III or Sudan IV. The lesion components can include intimal smooth muscle cells with lipid droplets in addition to foam cells. There are more macrophages in type II lesions than in type I lesions and a normal intima. A few T lymphocytes and isolated mast cells might be present in the lesion (Stary *et al.*, 1994).

In the original classification in 1994 by Stary, type II lesions were divided into two subgroups; *type IIa* or progression-prone lesion which developed at specific adaptive intimal thickenings that are prone to progress to advanced lesions and *type IIb* or progression-resistant lesion which are found in locations with a thin intima and do not progress or progress slowly to advanced lesions. However in an up-dated report in 2000, this distinction was not drawn and both *type IIa* and *typeIIb* were categorised as

type II. Instead, the effect of highly versus moderately susceptible arterial sites was emphasised (Stary *et al.*, 1994; Stary, 2000b).

Type III lesion. The type III lesion, known as the intermediate lesion, the transitional lesion and preatheroma, represents a morphological and chemical bridge between the early and advanced lesions. The lesion consists of extracellular lipid pools among the layers of smooth muscle cells, generally localised at lesion prone sites, a few lymphocytes, plasma cells and mast cells. The presence of the type III lesion can confirm the development of atherosclerosis, through early lesions to advanced lesions (Stary *et al.*, 1994).

Type IV lesion. Type IV or atheroma is the first lesion considered as an advanced type due to severe intimal disorganisation by the confluent lipid core and initially is an eccentric lesion. The confluent lipid core may form by combination of isolated lipid pools from the type III lesion. The intima above the confluent lipid core contains macrophages and smooth muscle cells with or without lipid droplets, lymphocytes and mast cells. The organelles of some smooth muscle cells may be calcified and calcium particles may be found within the lipid core. The lesion is large enough to be visible the unaided eye. Although atheroma usually does not narrow the vascular lumen, this lesion is clinically important. The layer above the confluent core consists of mainly macrophages and a small amount of collagen so the lesion is prone to rupture (Stary *et al.*, 1995).

Type V lesion. The type V lesions cause a narrowing of the lumen and may develop fissures, haematoma and thrombus. In 1995 Stary classified type V into three

subgroups: *Type Va lesion (fibroatheroma)* with fibrous connective tissue as the major component, which may be a multilayered lesion consisting of several lipid cores separated by thick layer of fibrous connective tissue: *Type Vb (VII) lesion (calcific lesion)* with calcification as the major component in addition to fibrous connective tissue: and *Type Vc (VIII) lesion (fibrotic lesion)* with no lipid core and containing a minimal amount of lipid (Stary *et al.*, 1995). However, in the AHA-recommended classification in 2000, types Vb and Vc are classified as types VII and VIII (Stary, 2000b).

Type VI lesion. The type VI lesion or complicated lesion is the result of development of type IV and type V and leads to morbidity and mortality. The type VI lesion may be subdivided into; *Type VIa* with surface disruption, *Type VIb* with haematoma or haemorrhage, and *Type VIc* with thrombosis. Type VIabc includes all three features (Stary *et al.*, 1995).

Table 1.1. Classification of atherosclerotic lesions used in pathology (Stary, 2000a).

Histologic classification and terms used for atherosclerotic lesions		Additional terms used and often based on appearance with the unaided eye	
Type I lesion	Initial change	Fatty dot, fatty streak	Early lesion, minimal lesion
Type II lesion	IIa: Progression-prone IIb: Progression-resistant		
Type III lesion	Preatheroma	Intermediate lesion, transitional lesion	
Type IV lesion	Atheroma	Fibrolipid plaque, fibrous plaque, plaque	Advanced lesion, raised lesion
Type V lesion	Fibroatheroma		
Type VI lesion	Fissured lesion, ulcerated lesion, hemorrhagic lesion, thrombotic lesion	Complicated lesion	
Type VII lesion	Calcific lesion	Calcified plaque	
Type VIII lesion	Fibrotic lesion	Fibrous plaque, plaque	

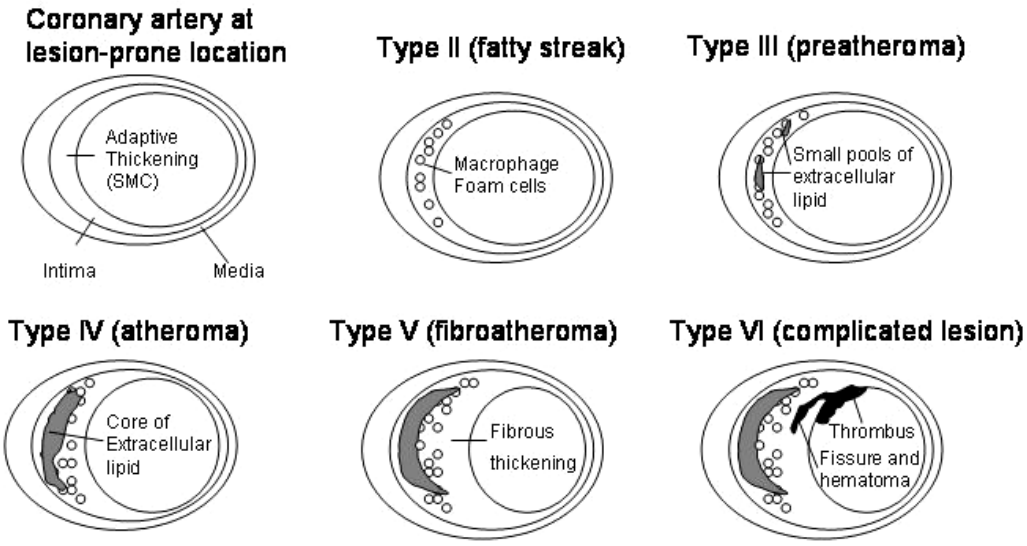


Figure 1.3. Histological classification of atherosclerotic lesions. Drawing of cross sections of a constant arterial location to show the morphology of development of atherosclerosis from adaptive intimal thickening at lesion prone site to advanced lesions (Stary *et al.*, 1995).

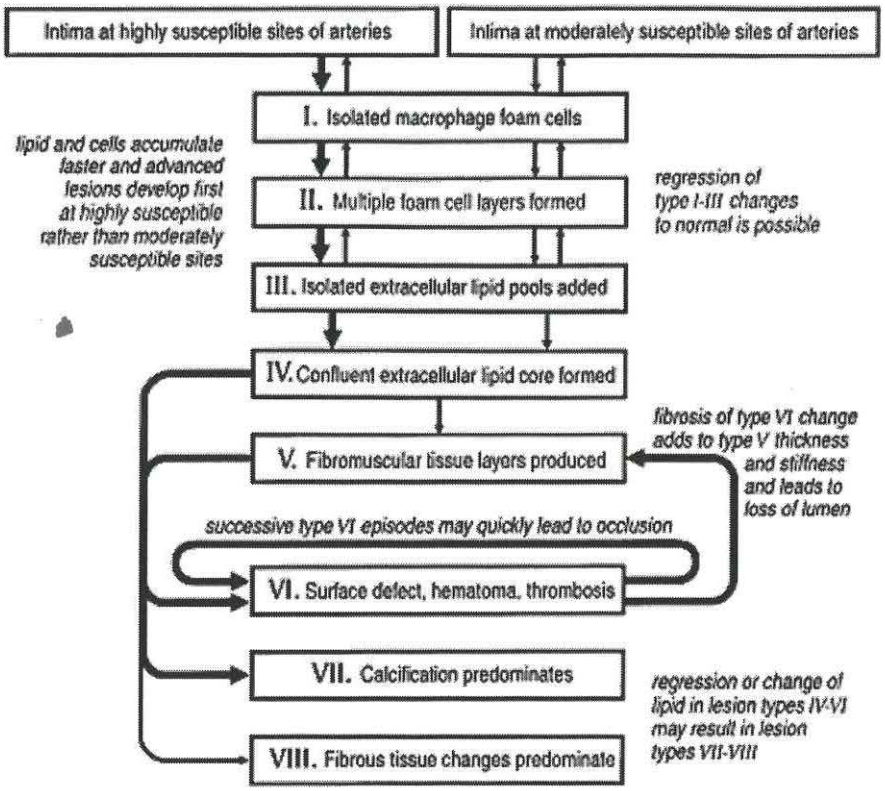


Figure 1.4. Diagram of evolution pathways and histological characteristics of human atherosclerotic lesions. The diagram summarises the sequence in the development of atherosclerotic lesions from type I to type IV and different pathways of progression or regression beyond type IV. The susceptibility of lesion development at specific sites, or frequency and importance of a pathway section is differentiated with either thick or thin arrows. For example, a type IV lesion may develop to type VI without passing through a type V stage (Stary, 2000a).

1.3.4.2 Modifications of AHA classification

Virmani and colleagues (Virmani *et al.*, 2000) reconsidered the Stary classification in 2000. They found that Roman numerals modified by letter codes and the orderly, linear pattern of lesion progression described was difficult to use as it was not clear whether there is a single sequence of events during the progression of all lesions. They proposed some changes to AHA classification which focused primarily on the type IV, V and VI lesions (Table 1.2)(Virmani *et al.*, 2000).

They classified atherosclerotic lesions into seven types based on the accumulation of lipid and its relationship to the formation of the fibrous cap, changes over time in the lipid to form a necrotic core, thickening or thinning of the fibrous cap and thrombosis. These categories include intimal xanthoma, intimal thickening, pathological intimal thickening, fibrous cap atheroma, thin fibrous cap atheroma, calcified nodule and fibrocalcific plaque. Remaining issues such as the culprit lesion associated with thrombus and specific plaque features representing processes critical to changes in the lesion (eg, angiogenesis, intraplaque haemorrhage, inflammation, calcification, cell death and proteolysis) are listed as descriptive terms (Figure 1.5) (Virmani *et al.*, 2000).

Table 1.2. Modified AHA classification based on morphological description. Adapted from Virmani 2000**.**

	Description	Thrombosis
Nonatherosclerotic intimal lesions		
Intimal thickening	The normal accumulation of smooth muscle cells (SMCs) in the intima in the absence of lipid or macrophage foam cells	Absent
Intimal xanthoma, or "fatty streak"	Luminal accumulation of foam cells without a necrotic core or fibrous cap. Based on animal and human data, such lesions usually regress.	Absent
Progressive atherosclerotic lesions		
Pathological intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Absent
Erosion	Luminal thrombosis; plaque same as above	Thrombus mostly mural and infrequently occlusive
Fibrous cap atheroma	Well-formed necrotic core with an overlying fibrous cap	Absent
Erosion	Luminal thrombosis; plaque same as above; no communication of thrombus with necrotic core	Thrombus mostly mural and infrequently occlusive
Thin fibrous cap atheroma	A thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs and an underlying necrotic core	Absent; may contain intraplaque hemorrhage/fibrin
Plaque rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core	Thrombus usually occlusive
Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually nonocclusive
Fibrocalcific plaque	Collagen-rich plaque with significant stenosis usually contains large areas of calcification with few inflammatory cells; a necrotic core may be present.	Absent

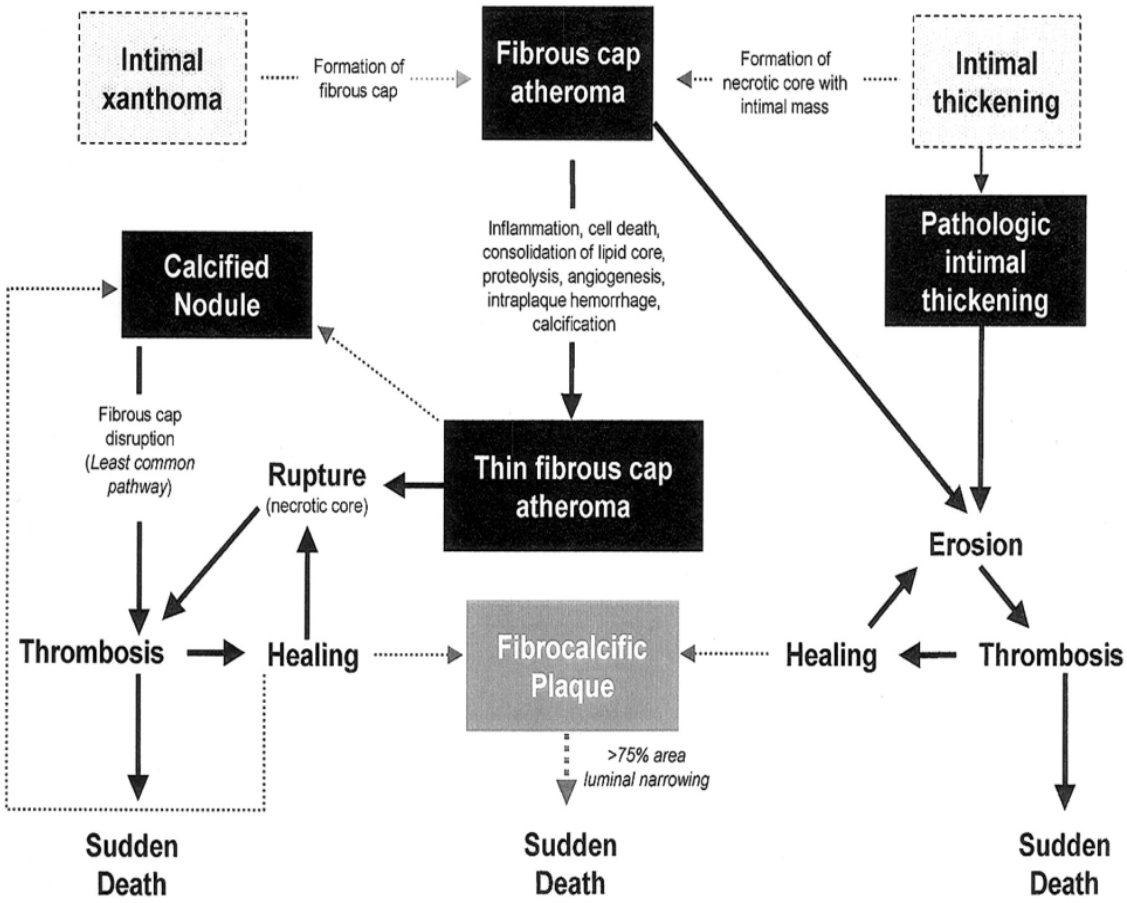


Figure 1.5. Modified classification of AHA recommendation by Virmani. The boxed areas represent the 7 types of lesion. Dashed lines were used for two boxes as there is controversy about their roles in the initial phase of the lesion formation and both lesions can exist without progression to a fibrous cap atheroma. The processes leading to lesion progression are listed between categories. Lines (solid and dotted) represent the progress of one type to another type and the thickness of the line shows the strength of the evidence that these events do happen (Virmani *et al.*, 2000).

1.3.5 The development of calcium deposits in atherosclerotic lesions

Calcium mineral deposits that frequently accompany atherosclerosis act as a substitute indicator for the disease and predict a high risk of myocardial infarction and death. In atherosclerotic lesions, calcification involves the participation of arterial osteoblasts and osteoclasts. Calcification can begin at any stage of development of atherosclerosis and appears to develop more linearly over the time rather that episodically (Doherty *et al.*, 2003).

In humans, microscopic sized calcium granules can be found among the extracellular particles and droplets and in smooth muscle cells isolated among them once a lesion with a lipid core develops. As the lesion progresses to an advanced type, disintegration of arterial structure at the cores leads to tears at the surface, haematoma and thrombosis. As a reparative response, fibromuscular tissue layers are added to a lesion. During this process, accumulation of adjacent extracellular calcium granules leads to formation of calcium lumps and plates (Stary, 2001).

Monckeberg's calcification or medial calcinosis, characterised by ring-like calcification, occurs in medium sized to small muscular and elastic arteries and is most frequently seen in patients with diabetes, renal failure and advanced aging. This calcification is not associated with any inflammatory reaction, does not affect intima and adventitia, and does not narrow the lumen. The vessels most affected are femoral, tibial and ulnar arteries (Moe, 2004; Schoen, 2005).

1.3.6 Role of neutrophils in atherosclerosis

The role of neutrophils in atherogenesis has been neglected probably due to the small number of neutrophils in atherosclerotic lesions.

Neutrophil activation may be one of the inflammatory components of the acute coronary syndrome. Activated neutrophils release a variety of proteolytic enzymes such as neutrophil elastase (NE) that are responsible for tissue destruction and mediate degradation of basement membrane components and endothelial damage. Human NE plays an important role in the digestion of extracellular matrix components such as elastin, fibrillar and non-fibrillar collagens. In addition, NE has the ability to modify lipoproteins including HDL destruction and enhanced LDL uptake and foam cell formation, and modulate metalloproteinase (MMP) and cytokines. NE activates MMP-2, MMP-3 and MMP-9 and inactivates their inhibitor TIMP-1, which leads to matrix breakdown. NE also regulates the activity of cytokines such as IL-β, TNF-α and IL-8. Therefore, neutrophils might play an important role in pathogenesis of plaque destabilisation in human atherosclerotic lesions (Naruko *et al.*, 2002; Dollery *et al.*, 2003).

On the other hand, the presence of NE in macrophages in the atherosclerotic lesion and localised NE in macrophages and endothelial cells in the shoulders of the plaque and the microvessels, respectively was observed by Dollery and colleagues in 2003. Interestingly, NE was reported in vulnerable atherosclerotic plaques, such as plaques with a thin fibrous cap and a macrophage-rich shoulder region, rather than in fibrous plaques or normal arteries. Therefore, the chronic inflammatory nature of atherosclerosis might cause stimulation of other cell types to produce proteases that previously attributed to neutrophils and only considered synthesised in the bone marrow

(Dollery *et al.*, 2003). Alternatively the presence of NE in monocytes/macrophages may have been due to remnant mRNA from apoptotic neutrophils which had been phagocyted by the macrophages.

1.4 Risk factors for atherosclerosis

Both environmental and genetic factors play important roles in the development of atherosclerosis. The major risk factors for atherosclerosis include cigarette smoking, hypertension (BP≥140/90 mmHg), hyperlipidemia, family history of premature coronary heart disease (CHD); CHD in male first-degree relative <55 years and CHD in female first-degree relative <65 years, age (men ≥45 years; women ≥55 years), diabetes mellitus and low high-density lipoprotein (HDL) (<1.0mmol/L (<40mg/dl)). Additional factors include lifestyle risk factors, obesity (>30 percent overweight), physical inactivity, atherogenic diet and stressful psychosocial factors. The non-traditional risk factors or emerging risk factors include lipoprotein (a), homocysteine, prothrombic factors, proinflammatory factors, impaired fasting glucose, subclinical atherogenesis and infectious micro-organisms such as herpes virus or *Chlamydia pneumoniae* (Gotlieb and Havenith, 1991; Altman, 2003; Libby, 2005).

1.5 Treatment and prevention of atherosclerosis

Abnormalities in plasma lipoproteins and lipid metabolism play the most important role in the occurrence of atherosclerotic disease. Therefore preventive aspects of the treatment of lipid disorders is the first aim to prevent atherogenesis (Libby, 2005).

The first step to achieve this goal can be therapeutic lifestyle changes including diet and exercise. The addition of drug therapy to dietary and other non-pharmacologic

measures is the next manoeuvre in patients with established coronary atherosclerosis and in individuals who have not previously suffered CHD events as well (Libby, 2005).

1.5.1 Dietary modification

Dietary modification is essential in treatment of high level of LDL. The ideal diet should cover three purposes. It should allow the patient to reach and maintain ideal body weight, provide a well-balanced diet with fruits, vegetables and grains, and it should be restricted in saturated fats and refined carbohydrates. Dietary management consists of two steps; first limiting saturated fat to 8-10% of total calories (total calories obtained from fat to <30% and intake of cholesterol <300mg per day). If this is unsuccessful, the next step is limiting saturated fat to ≤7% of total calories and cholesterol to <200mg per day (Ridker *et al.*, 2001; Bryant and Knights, 2003).

1.5.2 Treatment guidelines

In the absence of satisfactory effects of exercise, diet and lifestyle modification on reduction of a high level of lipid in plasma, lipid-lowering drugs can be prescribed for the treatment of hyperlipidemia. The main classes of drugs are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), bile-acid-binding resins, fibrates, and additional agents including nicotinic acid, probucol and fish oil. These agents may be used either individually or in combination if not contraindicated. The choice of drug depends on the plasma lipid profile of the patient with consideration of the main aim, reduction of LDL cholesterol and/or triglyceride (Bryant and Knights, 2003).

HMG-CoA reductase inhibitors (Statins). These gents include atorvastatin, fluvastatin, pravastatin and simvastatin. They are competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme necessary for cholesterol biosynthesis. These drugs specially decrease total cholesterol by 10-45% and increase HDL by 2-13% (Bryant and Knights, 2003). Both experimental and clinical outcome data also support the hypothesis that statins attenuate plaque inflammation and influence plaque stability. Some can decrease macrophage content within experimental atherosclerotic plagues (pravastatin and cerivastatin), whereas others are able to decrease intimal inflammation and suppress the expression of tissue factor and matrix metalloproteinase both *in vivo* and *in vitro* (simvastatin, fluvastatin and atorvastatin). Statins may also inhibit expression of adhesion molecules critical for monocyte attachment and adhesion to the vascular endothelium (Libby *et al.*, 2002).

Bile-acid-binding resins. This family includes cholestyramine and colestipol, which are non-absorbable anion-exchange resins, also called bile-acid sequestrants and are used mainly as adjunct to statins. They have cholesterol-lowering effects. Cholesterol is the major precursor of bile acids, which are secreted from the gallbladder into the small intestine. Bile acids perform two functions in the small intestine including emulsification of fat from food to facilitate chemical digestion and absorption of lipids (e.g., fat-soluble vitamins, A, D, E and K). Bile acids are returned to the liver via enterohepatic recirculation.

These drugs bind bile acid in the intestine leads to reduce exogenous cholesterol absorption. As a compensatory reaction for the loss of bile acids removed by the resins and excreted in the faeces, the liver increases the rate of endogenous metabolism of

cholesterol into bile acids and increases the expression of hepatic LDL receptors and hence uptake of LDL cholesterol from plasma which causes a reduction of plasma cholesterol concentration. An increase in plasma triglycerides limits the use of these drugs and cannot be used in patients with hypertriglyceridemia (Bryant and Knights, 2003).

Fibrates. The available drug in Australia is gemfibrozil. The mechanism of action is not completely clear but fibrates increase lipoprotein lipase activity (increasing triglyceride hydrolysis), up-regulate LDL cholesterol gene expression and cause different effects on apolipoprotein gene expression (Bryant and Knights, 2003). Fibrates are synthetic ligands for peroxisome proliferator-activated receptor α (PPARα); one of the members of transcription factors family, which mediates the lipid-lowering activity of these drugs. These drugs also inhibit the vascular inflammatory response via PPARα by interfering with NFκB and AP-1 transactivation capacity involving direct protein-protein interaction with p65 and c-Jun (Delerive *et al.*, 1999). This family of drugs is usually used for the treatment of severe hypertriglyceridemia, mixed hyperlipidaemia and as second-line treatment for hypercholesterolemia (Bryant and Knights, 2003).

Nicotinic acid or niacin. This water-soluble vitamin inhibits mobilisation of free fatty acids from peripheral tissue and leads to reduction in the hepatic synthesis of triglycerides and secretion of VLDL. Nicotinic acid also increases plasma HDL concentration by 20-30%. It is used mainly as an adjunct to other therapies like fibrates and bile-acid-binding resins (Bryant and Knights, 2003).

Probucol. This drug is used as a final choice for patients with hypercholesterolaemia who have failed to respond to other treatment. The mechanism of action is not completely clear but might be through its antioxidant properties. It has some disadvantages, including decreasing both LDL-cholesterol and HDL-cholesterol levels, and prolongation of the cardiac action potential and is therefore contraindicated for patients with cardiac abnormalities (Bryant and Knights, 2003).

Nutritional antioxidants. Antioxidants such as vitamin E, vitamin C and selenium can affect the susceptibility of cells (e.g. endothelial cells, macrophages and smooth muscle cells) by preventing oxidative damage and formation of oxidised LDL which has an important role in pathogenesis of atherosclerosis (Wang *et al.*, 2004).

1.6 Apolipoprotein E

ApoE is a member of the apolipoprotein family which consists of apoA-I, apoA-II, apoA-IV, apoB-100, apoB-48, apoC-I, apoC-II, apoC-III, apoD, apoE, apoJ and apo(a) (Ridker *et al.*, 2001), that covers the surface and mediates the metabolism of lipoprotein particles. It is synthesised predominantly in liver, although synthesis has been documented in almost every tissue. Human apoE is produced as a 317 amino acid protein which is cleaved to a 18-amino acid signal peptide remnant yielding the 299-amino acid mature apoE in plasma. The mature human apoE is a 34 kD glycoprotein that is found in plasma at 3-5 mg/dl and resides mainly on very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), high density lipoprotein (HDL), in addition to chylomicrons and their remnants (Plump *et al.*, 1992; Zhang *et al.*, 1992; Plump and Breslow, 1995).

1.6.1 ApoE in lipoprotein metabolism

There are three interrelated pathways in lipoprotein metabolism:

Exogenous or dietary fat transport. This pathway is responsible for the transport of fat from the gastrointestinal tract to the liver. The fat is secreted from intestinal cells on chylomicrons. ApoB-48 has an essential role in this process. Lipoprotein lipase (with apoC-II as a cofactor) hydrolyses the triglyceride in the core of the chylomicrons into free fatty acids and glycerol, which leads to the production of a smaller chylomicron remnant. These remnants are removed mainly by a chylomicron remnant receptor or LDL-like receptor protein in the liver (Kwiterovich, 2000).

Endogenous or hepatic fat transport. Triglyceride rich VLDL is synthesised and secreted by the liver in a process in which apoB-100 plays an important role. Free fatty acids and glycerol are formed as a result of VLDL break down by lipoprotein lipase and its cofactor apoC-2. This leads to formation of IDL that either can be removed by the LDL receptor on the surface of the liver through interaction of apoE, or hydrolysed further by hepatic lipase to form LDL that can be removed via the LDL receptor with the aid of apoB-100. If LDL is oxidised it can enter the macrophage through the scavenger receptors; CD36 and SR-A, on the surface of macrophage (Kwiterovich, 2000).

Reverse cholesterol transport. The nascent HDL particles that mainly contain phospholipids and apoA-1, are made in the liver and intestine. These remove unesterified cholesterol through the ABC1 transportor protein by interacting with peripheral cells such as macrophages. The esterification of the cholesterol in nascent

HDL by lecithin cholesterol acyl transferase and its cofactor, apoA-1, produces HDL particles. This cholesteryl ester in the core of HDL can be returned to the liver by interaction of HDL with SR-B1 receptor or transferred to the apoB-containing lipoproteins by cholesteryl ester transfer protein (Kwiterovich, 2000).

ApoE plays important roles in the lipoprotein metabolic pathways, including both dietary fat transport and endogenous fat transport. ApoE via the LDL receptor and the chylomicron remnant receptor / LDL receptor-related protein (LRP), acts as a ligand for the receptor-mediated removal of chylomicron remnants and intermediate density lipoprotein (IDL) particles from the circulation. ApoE-enriched lipoprotein particles, depending on their size, are cleared by receptors. Larger ones like VLDL and β-VLDL are cleared by the LDL receptor and smaller chylomicron remnants and IDL particles can be cleared either by the LDL receptor or by the LRP. In addition to lipid transport, apoE has been postulated to be involved in reverse cholesterol transport (Plump and Breslow, 1995).

1.6.2 Clinical importance of apoE as a factor for atherosclerosis

The three clinical conditions which provide evidence that apoE is involved in the development of dyslipidemia and atherosclerosis are genetic variation, type III hyperlipoproteinemia and apoE deficiency syndrome (Plump *et al.*, 1992; Plump and Breslow, 1995).

Genetic variation is the most common of these three. There are three major structural isoforms of apoE; E2, E3 and E4. In general, individuals with the apoE4 allele have a total cholesterol level higher than those with the apoE2 allele and coronary artery

disease is significantly more common in individuals with the apoE4 isoform (Plump and Breslow, 1995).

Secondly, an important role of apoE in determining susceptibility to atherosclerosis is seen in type III hyperlipoproteinemia. This disease affects about 1 in 5000 individuals and is characterised by abnormal triglyceride and cholesterol metabolism. In patients with type III hyperlipoproteinemia, fasting plasma cholesterol levels are between 300 and 600 mg/dl and fasting triglyceride levels are often equal to or greater than plasma cholesterol levels by an associated increased in plasma VLDL, which is diagnostic for the disease. Individuals with this disorder have severe premature coronary and peripheral vessel atherosclerotic disease and xanthomas (Plump *et al.*, 1992; Plump and Breslow, 1995).

Thirdly, a role for apoE is seen by the atherosclerosis susceptibility in individuals with apoE deficiency, a rare condition. Individuals with this disorder have lipoprotein pattern like type III hyperlipoproteinemia, and develop atherosclerosis and xanthomas (Plump *et al.*, 1992; Plump and Breslow, 1995).

1.6.3 ApoE knock out mice

ApoE deficient mice were created by gene targeting or homologous recombination in embryonic stem (ES) cells (Plump *et al.*, 1992; Zhang *et al.*, 1992).

$ApoE^{-/-}$ mice display spontaneously increased plasma cholesterol and triglycerides and decreased HDL (Reddick *et al.*, 1994). These 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. The apoE$^{-/-}$ mouse contains the entire spectrum of lesions observed during atherogenesis and is the first mouse model to develop lesions similar to humans (Nakashima *et al.*, 1994). The reductions in the expression of P-selectin, ICAM-1, or E-selectin (by genetic deficiency) in apoE$^{-/-}$ mice provide direct protection from atherosclerotic lesion formation in this model. Therefore, CAMs play essential roles for the emigration of leukocytes during the inflammatory response involved in development of atherosclerosis in this model (Collins *et al.*, 2000). This model develops arterial lesions in a time-dependent manner while being fed regular (low fat) mouse chow (Reddick *et al.*, 1994; Daugherty, 2002). Feeding apoE$^{-/-}$ mice a diet enriched in saturated fat greatly accelerated the progression of atherosclerotic lesions (Plump *et al.*, 1992; Nakashima *et al.*, 1994; Plump and Breslow, 1995; Daugherty, 2002).

Although deficiency in apoE leads to development of hyperlipidemia, the severity of atherosclerotic lesions depends on the strain of the mice as atherosclerotic lesion area in the aortic root of apoE$^{-/-}$ mice in a C57BL/6 strain background is eight times greater than seen in a FVB/NJ strain background (Daugherty, 2002).

1.7 Polyunsaturated fatty acids (PUFA)

Polyunsaturated fatty acids (PUFA) have been identified as having significant biological functions in the pathogenesis and prevention of disease. PUFAs are critical components of cell and organelle membranes and more importantly regulate critical biological activities (Gill and Valivety, 1997).

1.8 Structure of fatty acids

Fatty acids are characterised by an alkyl chain and carboxyl group with the basic formula shown below:

$$CH_3\text{-}(CH_2)_n\text{-}COOH$$

The degree of unsaturation in the molecule is determined by the number of double bonds, which is normally in *cis* configuration and separated by a methylene group (-CH_2-) in the fatty acid backbone. The positions of the double bounds are numbered from the carboxyl group, with the carboxyl carbon atom as carbon 1. The *n-3* polyunsaturated fatty acids have their first double bond between the 3rd and 4th carbon atom counting from the ω or methyl end of the chain, while the *n-6* polyunsaturated fatty acids have their first double bond between the 6th and 7th carbon atom respectively. Short chain fatty acids are classified as those with <6 carbon atoms, medium chain have 6-12 carbon atoms, long chain have 14-22 carbon atoms and very long chain have >22 carbon atoms. One standard nomenclature of fatty acids is the number of carbon atoms followed by the number of double bonds and finally the series, e.g. 20:4*n-6* refers to a 20 carbon fatty acid with 4 double bonds and the first double bond being between the 6th and 7th carbon atoms (Wallis *et al.*, 2002; Ferrante *et al.*, 2005a).

1.9 Sources of fatty acids

Fatty acids in the body are obtained either through diet or *de novo* synthesis in tissues. Dietary fatty acids can be obtained from meat, fish, green vegetables and from oils derived from these. For example, marine oils and fish are rich in *n-3* fatty acids such as eicosapentaenoic acid (EPA; 20:*5n-3*) and docosahexaenoic acid (DHA; 22:*6n-3*), and grain-fed animals are rich in arachidonic acid (AA; 20:*4n-6*) (Figure 1.6) (Ferrante *et al.*, 2005a).

De novo synthesis of fatty acids in the human body from acetyl coenzyme A produces fatty acids up to 16 carbon atoms or palmitate. The carbon chain (16:0) is elongated by two carbon atoms per cycle to form stearate (18:0) which is desaturated to form oleate (18:*1n-9*). Palmitate also can be desaturated to palmitoleate (16:*1n-9*) which can be elongated further to oleate (18:*1n-9*). Mammalian cells are unable to synthesis essential longer chain fatty acids such as linoleic acid (LA; 18:*2n-6*) and α-linoleic acid (ALA; 18:*3n-3*) due to the absence of specific desaturase enzymes necessary for introducing double bonds beyond carbon-9. These essential fatty acids can be obtained through the diet and are required for the synthesis of longer chain fatty acids like eicosapentaenoic acid (EPA; 20:*5n-3*) and docosahexaenoic acid (DHA; 22:*6n-3*). As there is competition among enzymes involved in the elongation and desaturation of LA and ALA, a balanced intake of both *n-6* and *n-3* is necessary (Simopoulos, 1999; Ferrante *et al.*, 2005a).

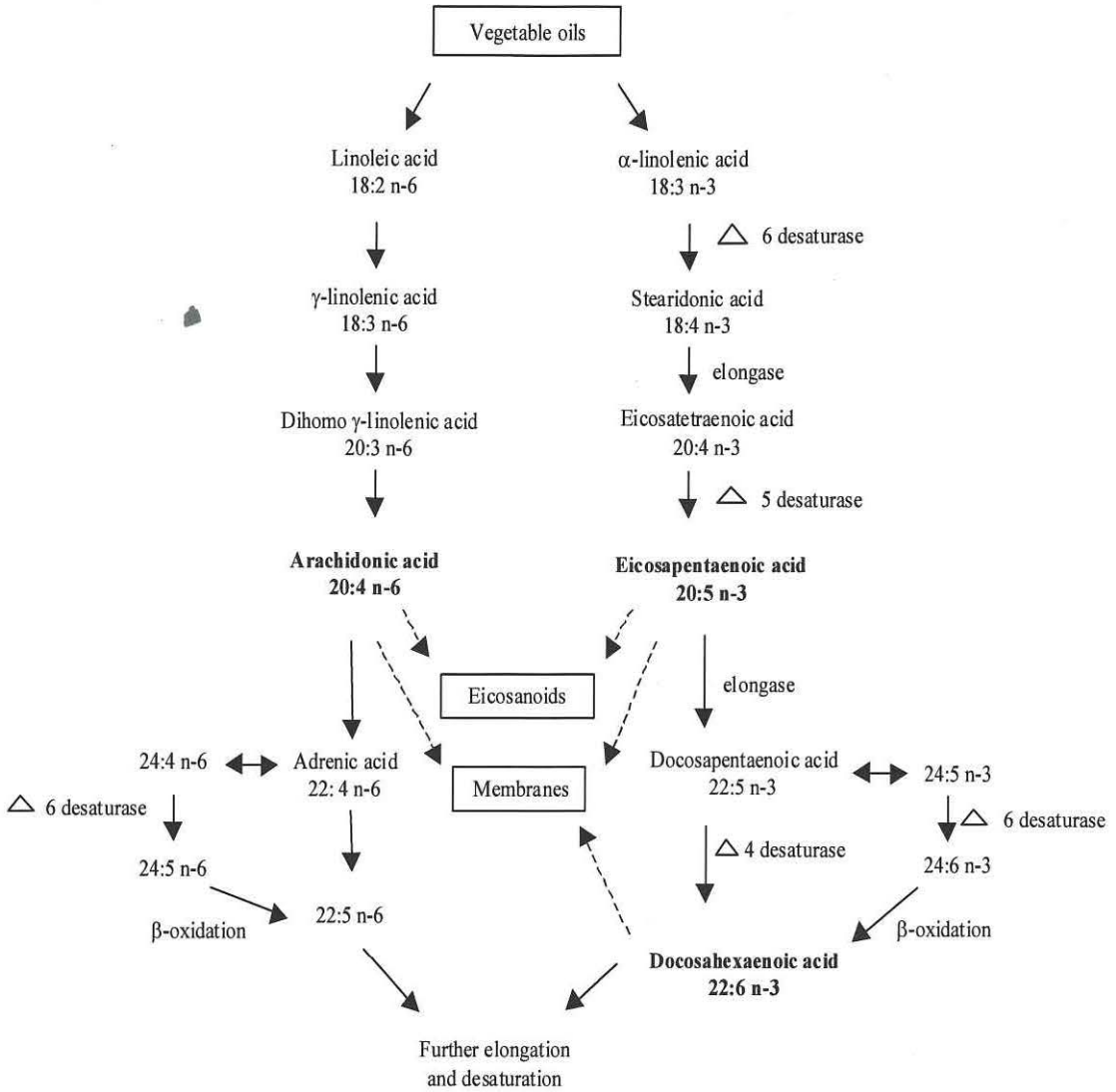


Figure 1.6. Synthesis of essential omega-6 and omega-3 PUFAs. Essential fatty acids such as linoleate and linolenate are obtained from dietary fats and are elongated and desaturated to form longer chain fatty acids. Key products are shown in bold print. The dotted arrows show metabolism to eicosanoids and esterification into membrane phospholipids (Ferrante *et al.*, 2005a).

1.10 Metabolism of fatty acids

n-6 and *n-3* PUFAs are parent fatty acid for the production of eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes. Eicosanoids derived from *n-6* fatty acids have opposing properties to metabolites of *n-3* fatty acids (Simopoulos, 1999).

The *n-6* PUFA, arachidonic acid (AA), is metabolised via the lipoxygenase and cyclooxygenase pathways. The products of AA metabolism via the cyclooxygenase pathway are prostaglandins (PG) and thromboxanes (TX). These eicosanoids play a major role in the inflammatory reaction in rheumatoid arthritis and psoriasis, as well as fever, pain and thrombosis. The metabolism of AA via the lipoxygenase pathway leads to formation of either 5-, 12- or 15-monohydroperoxy-eicosatetraenoic acid (HPETE); which are the precursors of 5-, 12- or 15-hydroxy-eicosatetraenoic acid (HETE), and leukotrienes (LT), some of which are pro-inflammatory and others showing anti-inflammatory activity. For example, LTB_4 and 5-HETE stimulate neutrophil chemotaxis, degranulation, respiratory burst, adherence to endothelial cells and transmigration of neutrophils across vascular barriers while 15-HETE inhibits LTB_4-induced neutrophil activation (Ferrante *et al.*, 2005a).

The *n-3* PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can also be metabolised by the lipoxygenase and cyclooxygenase pathways. They generate eicosanoids with lower inflammatory properties whilst decreasing the production of the pro-inflammatory AA metabolites. The switching of metabolic products from *n-6* fatty acids which are pro-inflammatory, to *n-3* which are of lower or have negligible pro-inflammatory actions, has been proposed to give rise to the anti-inflammatory effect of *n-3* fatty acids (Ferrante *et al.*, 2005a).

1.11 Importance of *n-3* fatty acids in health and disease

Dietary fish oils, rich in *n-3* PUFA, are rapidly incorporated into the membrane phospholipids of cells in the body and they can regulate several aspects of cell functions (Simopoulos, 2002).

n-3 fatty acids enrichment can prevent or ameliorate a wide range of diseases such as coronary heart disease and stroke, essential fatty acid deficiency in infancy (retinal and brain development), autoimmune disorders (like, lupus, nephropathy), Crohn's disease, cancers of the breast, colon and prostate, mild hypertension, rheumatoid arthritis, type 2 diabetes (Simopoulos, 1999; Connor, 2000).

1.11.1 Fish oils and Atherosclerosis

There are controversial reports about the cardioprotective effects of *n-3* PUFA in fish oil. While many epidemiological and experimental studies have shown the beneficial effects of PUFA in fish oil on the development of cardiovascular disease and their effects in reducing risk of CHD incidence and total mortality among diabetic women, some studies have reported no beneficial effects association between fish consumption and cardiovascular disease (Hu *et al.*, 2003; Connor, 2004).

Wide ranges of cardiovascular benefits for long chain PUFAs such as EPA and DHA from fish oil have been reported. These include effects on lipoprotein metabolism, haemostatic function, platelet/vessel wall interaction, anti-arrhythmic actions and inhibition of proliferation of smooth muscle cells and therefore growth of atherosclerotic lesions (Abeywardena and Head, 2001). However, the precise

mechanism of cardioprotective effect of fish oil is not clear. It may help humans with atherosclerosis not through any lipid lowering effect but through its effect on antioxidative defence mechanisms. Oxidised-LDL produced by endothelial cells, macrophages, and smooth muscle cells, has an important role in pathogenesis of atherosclerosis. The antioxidant defence system including SOD (superoxide dismutase), CAT (catalase) and GPx (glutathione peroxidase), which can be influenced by nutritional antioxidants, such as vitamin E, vitamin C and selenium can reduce oxidative damage. The antiatherosclerosis role of fish oil may be through gene regulation by induction of antioxidant enzyme expression through PPAR-mediated gene transcription. Activation of PPAR decreases the incidence of cardiovascular disease not only by correcting metabolic disorders, but also through direct actions at the level of the vascular wall. The presence of a PPAR-response element (PPRE) in the 5'-flanking of Cu/Zn SOD1 which is the key enzyme in metabolism of oxygen free radicals, shows the importance of PPAR activation (Wang *et al.*, 2004). On the other hand, effects on vascular endothelial function have been reported as the cardioprotective effect of *n-3* PUFA. These include inhibition of monocyte adhesion and platelet activation, increased nitric-oxide production and improvement of vasodilation and blockage of lipid oxidation (Brown and Hu, 2001). Simpoulos reported that the beneficial effects of *n-3* fatty acids on coronary heart disease are not due to changes in serum lipid concentration, although EPA and DHA do lower triacylglycerol concentrations, but this occurs by reducing blood clotting in vessel walls and ventricular arrhythmias (Simopoulos, 1999).

Despite the favourable effect of fish intake on coronary atherosclerosis in postmenopausal women who have obvious coronary artery disease, fish intake was not

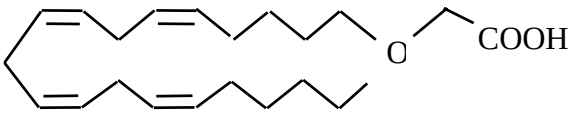
able to prevent atherosclerosis but rather reduced its progression. In other words, atherosclerotic progression occurred more slowly with fish consumption (Connor, 2004). In addition, it is not clear that dietary or supplemental *n-3* PUFA alters total mortality in people with, or at high risk of cardiovascular disease or in a general population. There is no proof that people should be recommended to stop taking rich sources of *n-3* fats however high quality trials are needed to confirm suggestions of a protective effect of this family on cardiovascular health (Hooper *et al.*, 2004).

1.12 Polyunsaturated fatty acids mimetics

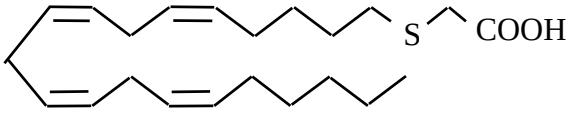
According to the studies undertaken by our group, specific structural elements play an important role in the biological activity of fatty acids. These include the number of carbon atoms, degree of unsaturation, position of double bonds, methylation and esterification of the carboxyl group, and addition of either a hydroxy or hydroperoxy group to the side chain (Ferrante *et al.*, 2005a). This has led to the chemical synthesis of several novel long chain fatty acids with greater selectivity than the natural fats. These contain either an oxygen or a sulphur atom in the β or γ position of the carbon backbone of the PUFA. The β-substituted compounds are not readily β oxidised therefore improving their intracellular stability (Figure 1.7) (Pitt *et al.*, 1997; Pitt *et al.*, 1998).

The engineered PUFA showed a more selective range of actions than the natural PUFA counterpart for biological activity in human umbilical vein endothelial cells (HUVECs) and other cell types (Robinson *et al.*, 1999; Costabile *et al.*, 2001). One of the novel PUFAs is MP3 (β-oxa-23:*4n-6*), which was more effective than fish oil-derived docosahexaenoic acid (22:*6n-3*) at suppressing the upregulation of E-selectin, ICAM-1

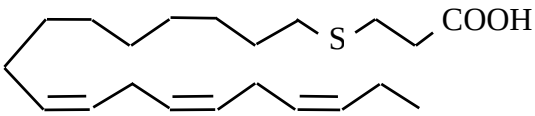
and VCAM-1 expression on HUVEC induced by tumour necrosis factor α (TNF), lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) (Ferrante *et al.*, 2005b). MP3 also suppressed TNF-stimulated adherence of neutrophils to HUVEC (Robinson *et al.*, 1999). Preliminary studies showed that MP3 was effective *in vivo* at suppressing LPS-stimulated upregulattion of E-selectin expression in the aorta of mice and in preventing the infiltration of leukocytes, including monocytes, into the site of inflammation. At the effective doses, MP3 did not cause any observable adverse effects to mice for the duration of the experiment (4 days). These factors indicate that MP3 has the hallmarks of a candidate molecule that may be effective in reducing/preventing the development of atherosclerosis.



β-oxa-23:4n-6 (MP3)



β -thia-23:4n-6 (MP8)



γ-thia-22:3 (n-3)

Figure 1.7. Structure of synthesised novel polyunsaturated fatty acids.

1.13 Concluding remarks

In view of the limitations which our community has experienced in the use of *n-3* fatty acids for cardiovascular disease, an opportunity has arisen to improve the approach by developing novel polyunsaturated fatty acids which have greater selectivity in inhibiting biological systems. One of these, β-oxa 23:4*n-6*, targets pathways that prevent atherosclerosis. In order to examine this possibility we used a mouse model of atherosclerosis, apoE$^{-/-}$ mice fed a high saturated fat/cholesterol diet. As this model has not been appropriately standardised, it was also the objective of this thesis to characterise the atherosclerotic lesions and establish criteria for scoring the severity of those lesions.

1.14 Aim

1. Attempt to develop criteria for grading atherosclerotic lesions in apoE deficient mice.
2. Assess the kinetics of development of these lesions.
3. Examine whether MP3 protects against atherosclerosis in this experimental model.

1.15 Hypothesis

The novel PUFA; MP3, through its ability to inhibit up-regulation of CAM protects against the development of atherosclerosis in the apoE$^{-/-}$ mouse model..

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Materials used for polyunsaturated fatty acid preparation/analysis

Arachidonyl alcohol was purchased from Nu-Chek Prep Inc., Elysian, Minnesota, USA and rodium acetate from Sigma Aldrich Pty. Ltd, Castle Hill, NSW, Australia. Methanol, acetone, chloroform, ethanol, diethyl ether were purchased from Merk Pty. Ltd, Kilsyth, Victoria, Australia. Hexane and acetic acid were from AJAX chemical, Auburn, NSW, Australia.

Thin Layer Chromatography (TLC) aluminium sheets silica gel was from Merk, Darmstadt, Germany. Disposable culture tubes were from Kimble Glass, Vineland, NJ, USA. Parafilm was purchased from Menasha, WI, USA. Hanks balanced salt solution (HBSS) was from JRH biosciences, Lenexa, KS, USA.

2.1.2 Materials used for animal and histopathological studies

Formalin was from Fronine Pty. Ltd, Riverstone, NSW, Australia. Haematoxylin was purchased from Surgipath Medical Industries, Inc., Manitoba, Canada. Lithium carbonate, celestine blue, eosin, phosphotungstic acid, hydrochloric acid, EDTA, disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, xylidine ponceau, acetic acid, saturated aqu. picric acid, hydrogen chloride, aluminium ammonium sulphate, sodium iodate, citric acid, chloral hydrate and tris ([hydroxymethyl] aminomethane) were purchased from Crown scientific Pty. Ltd,

Sydney, NSW, Australia. Paraffin (wax) was from Lomb Scientific, Sydney, NSW, Australia.

Anti human muscle actin, Clone HHF35 (1ml) was purchased from DakoCytomation, Inc., Carpinteria, CA, USA. Immunoperoxidase staining was performed using a Chemicon Detection Kit from Temecula, CA, USA.

Polypropylene cap was purchased from Sarstedt, Nürbrecht, Germany. Spurr's epoxy resin and BEEM capsule were from TAAB, Berkshire, England.

Needles (size 25G5/8 0.5×16mm) and 1ml Tuberculin syringe, were from Becton Dickinson Medical Pty. Ltd, Singapore. Sterile containers (5 and 60ml capacity), 10ml sterile tubes and microfuge tubes (1.5ml), were from Sarstedt, Nürbrecht, Germany. Nine millilitre capacity Lithium-heparin tubes; Vacuette®, were from Greiner bio-one GmbH, Kremsmünster, Austria. Slides were from Menzel-Glaser, Braunschweig, Germany.

2.2 Methods

2.2.1 Polyunsaturated fatty acid

2.2.1.1 Synthesis of engineered polyunsaturated fatty acid

The polyunsaturated fatty acid (MP3: β-oxa 23:4*n-6*) used in this study was synthesised by our lipid biochemist; Dr Neil Trout. A brief outline of the methodology involved in the synthesis of MP3 is provided below.

For synthesis of MP3 (β-oxa 23:*4n-6*), *tert*-butyl (*all*-Z)-(eicosa-5,8,11,14-tetraenyloxy) acetate, a solution of arachidonyl alcohol (510mg, 1.76mmol) and rhodium (II) acetate dimer (9mg, 0.5%mol equiv) were stirred in CH_2Cl_2 (15ml) at room temperature under dry nitrogen. A solution of *tert*-butyl diazoacetate (1.60g, 11.2mmol) in CH_2Cl_2 (15ml) was added dropwise and stirring continued at room temperature for 2h. The mixture was concentrated under a stream of dry nitrogen and the residue was purified by flash column chromatography, eluted with hexane/Et_2O (9:1), to afford *tert*-butyl (*all*-Z)-(eicosa-5,8,11,14-tetraenyloxy) acetate. Trifluoroacetic acid (4ml) was added to afford *tert*-butyl (*all*-Z)-(eicosa-5,8,11,14-tetraenyloxy) acetate (288mg, 712µmol) in CH_2Cl_2 (10ml) under N_2. The solution was stirred at room temperature for 2h. Flash column chromatography on silica was used to purify the crude mixture, eluted with hexane/Et_2O/acetic aid (40:60:2, v/v), affording (*all*-Z)-(eicosa-5,8,11,14-tetraenyloxy) acetic acid as a colourless oil (Pitt *et al.*, 1997).

The MP3 was identified using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (Pitt *et al.*, 1997). The purity of MP3 was assessed by using thin layer chromatography (TLC). 50µl of a 20mM stock solution of MP3 was spotted onto a silica coated aluminium plate and allowed to dry. The plate was developed in a tank containing diethyl ether:hexane:acetic acid:H_2O (65:35:0.5:0.5, v/v). After the solvent front had

travelled 16cm, the plate was air dried and exposed to iodine vapour until the lipid could be visualised. There was no evidence of decomposition or degradation of the MP3.

2.2.1.2 Preparation of fatty acid

Stock of MP3 (purity≥99%) was prepared in chloroform and kept in -70 degrees freezer. Based on the MP3 dose, weight of mice and concentration of MP3 stock, the correct amount of MP3 for each mouse was calculated. On the day of use and immediately before injection to animals, the calculated amount of MP3 was transferred to glass tubes, chloroform was completely evaporated under a constant stream of high purity nitrogen and MP3 was sonicated in calculated amount of Hanks Balanced Salt Solution (HBSS) without phenol red, based on the animal weight, for 10 times 10 seconds bursts on ice (Branson Sonic Power Company, model B-30, Danbury, CONN, USA) (continuous, % Deputy cycle=50, Output control=1.5) giving rise to a clear preparation. The sonicated MP3 was kept on ice until required for injection.

MP3 was prepared at concentration of 4mg/ml in HBSS and animals received 10ml/kg body weight to give 40mg/kg body weight of MP3. For 70mg/kg dose of MP3, the concentration of 3.5mg/ml of fatty acid in HBSS was prepared (as it was not easy to get a homogenous emulsion at 7mg/ml) and animals received 20ml/kg body weight to gain 70mg/kg of MP3.

Control animals received an appropriate amount of vehicle (HBSS) based on their weight and by consideration of amount of vehicle in the test group. For example, animals in control groups of MP3 treatment dose of 40mg/kg received 10ml/kg body weight of vehicle and control animals of test with 70mg/kg received 20ml/kg body weight of HBSS, respectively.

Animals were weighed once a week to calculate the correct amount of MP3 and HBSS that they received.

2.2.2 ApoE deficient mice

Male apoE deficient mice were purchased from the Animal Resources Centre in Perth, WA, Australia. They were created by homologous recombination; gene targeting in embryonic stem (ES) cells on a C57BL/6J background. Animals were received at different weeks of age (see chapter 5).

2.2.2.1 Feeding with high fat diet and normal diet

To accelerate the development of spontaneous atherosclerotic lesions (Plump *et al.*, 1992; Nakashima *et al.*, 1994; Plump and Breslow, 1995), apoE$^{-/-}$ mice were fed a high fat high cholesterol diet containing 21% fat and 0.15% cholesterol manufactured by Specialty Feeds, Glen Forrest, WA, Australia. This will be referred to as a high fat diet in the thesis. In a separate protocol, apoE$^{-/-}$ mice received a normal diet manufactured by Ridley AgriProducts, Wasleys, SA, Australia. Animals were given water ad libitum.

The timing for sacrificing animals in both high fat and normal diet group will be describe in details in chapter 4.

2.2.2.2 Collection of organs and blood samples

$ApoE^{-/-}$ mice were sacrificed by carbon dioxide (CO_2) asphyxiation. After dissection, HBSS was flushed through the left ventricle using a 25G5/8 needle to wash out blood cells from the heart and aorta. The heart and the aorta down to the bifurcation at the common iliac arteries of each mouse were isolated intact. The heart and an approximately 5mm length of ascending aorta were removed from the remainder of the aorta and fixed in 10% buffered formalin [disodium hydrogen orthophosphate 203.7g, sodium dihydrogen orthophosphate 88.5g, formaldehyde 2.5L and distilled water 22.5L] at room temperature (Figure 2.1).

Blood was collected by cardiac puncture using a 25G5/8 needle and placed in heparinised tubes. Red blood cells and plasma were separated by centrifugation at 1800 rpm for 10 min, kept in micro tubes and stored at -20ºC. The organs of mice (brain, lung and liver) were collected in 5ml containers, frozen in ice-cold methanol and dry ice and stored at -70 ºC for MP3 extraction at later stage if required. Some organs were fixed in 10% buffered formalin for histology.

The aorta of each mouse was collected, trimmed of extra fat under the dissecting microscope, (Zeiss, West Germany), snap-frozen in liquid nitrogen and fixed in acetone

at -20ºC for 2 min, then air dried and kept at -70 ºC for the measurement of VCAM-1 expression at a later date (Figure 2.1).

2.2.3 Light microscopy

All the sections were examined by light microscopy (Olympus Optical Co. Ltd., model BX51, Tokyo, Japan) and photos were taken by a DP12 camera (Olympus Optical Co. Ltd, Tokyo, Japan) attached to the microscope.

2.2.3.1 Tissue sectioning and staining

Animals' hearts were kept in 10% buffered formalin for at least for 24h to allow adequate fixation. The fixed hearts were cut transversely caudal to the atria (Figure 2.1) and embedded in paraffin overnight. The tissue then underwent sectioning, serially, at 5μm thickness beginning from the transverse cut. The sections were stained with Haematoxylin and Eosin. Where appropriate, sections were also stained with elastic Van Gieson and Masson trichrome technique to detect elastic fibers and collagen. Some sections were also immunostained to detect smooth muscle cells using the anti-human muscle actin antibody, Monoclonal Mouse, HHF35. Where it was necessary sections were decalcified.

Haematoxylin and Eosin staining. Sections were dewaxed and hydrated through graded alcohols to water, stained in haematoxylin for approximately 2min, washed in water, in Lithium Carbonate (Li_2CO_3) until turn to blue, washed in water, stained in Eosin Y

working solution; 50:50 dilution of stock Eosin Y [Eosin Y CI 45380 (10g), potassium dichromate (5g), saturated aqueous picric acid (100ml), absolute alcohol (100ml) and deionised water (800ml)] and deionised water, for about 2 min, rinsed with water and finally dehydrated through alcohol, cleared in xylol and mounted in DPX [Lustrex (80mg), xylol (280ml) and dibutyl phosphate (40ml)].

Van Gieson staining. Sections were dewaxed and brought to water, stained nuclei with Celestine Blue for 3min and Haematoxylin for 3min, washed in water, placed in Li_2CO_3 until the section turn to blue, stained in Van Gieson solution [1% aqueous acid fushion (20ml), saturated aqu. picric acid-sat (180ml) and HCL conc (0.5ml)] for 1min and dehydrated, cleared in xylol and mounted in DPX.

Masson Trichrome staining. Sections were dewaxed and brought to water, mordanted in Bouin's fixative for 30min at 60°C, washed in water, mordanted in Celestine Blue for 3min, rinsed in water, stained in Mayer's Haematoxylin [haematoxylin (4g), distilled water (2000ml), aluminium ammonium sulphate (100g), sodium iodate (0.4g), citric acid (2g) and chloral hydrate (100g)] for 3 min, washed in water and blued up, stained in Fuchsin Ponceau [xylidine ponceau (2g), acid fuchsin (1g), 1% acetic acid (300ml)] for 5min, rinsed in distilled water, treated with phosphotungstic acid for 10min, drained and stained in light green for 3min, dipped quickly in 1% acetic acid, dipped quickly in distilled water and dehydrated, cleared and mounted in DPX.

Decalcification. Decalcification is the process of softening the tissue by removing not only calcium but other minerals. It was done as occasional surface decalcification when it was required during the process of sectioning by using a decalcifying agent; a mixture of a strong acid, hydrochloric, and a chelating agent; EDTA [7% HCL in 2% EDTA: EDTA (20g), distilled water (930ml) and HCL concentrate (70ml)]. The blocks were washed after decalcification and underwent the process of cutting and staining.

Immunohistochemical staining. Some sections were stained for muscle specific actin (β and γ msa) using monoclonal mouse anti-human muscle actin, clone HHF35. Immunoperoxidase staining was performed using a Chemicon Detection Kit. Paraffin fixed sections were deparaffinised and then endogenous peroxidase quenched with 3% hydrogen peroxide for 5min. After rinsing with distilled water, the tissues were placed in Tris buffered saline (TBS) for 5min, the sections were incubated for 20min with normal goat serum (Chemicon 20773, Temecula, CA, USA) for blocking non-specific sites. After removal of the serum, the sections were incubated with monoclonal mouse anti-human muscle actin antibody (primary antibody) (1:600 in TBS) overnight at 4°C. After rinsing with TBS, the sections were incubated for 20-30min with biotinylated goat anti mouse IgG antibody (secondary antibody) (Chemicon 20775, Temecula, CA, USA), rinsed with TBS followed by incubation for 20-30min with Streptavidin HRP (Chemicon 20774, Tmecula, CA, USA). The tissues were rinsed again in TBS, incubated for 5min with 3,3' diaminobenzidine (DAB substrate) solution, rinsed in TBS, counterstained in Mayer's haematoxylin and finally mounted in PIX.

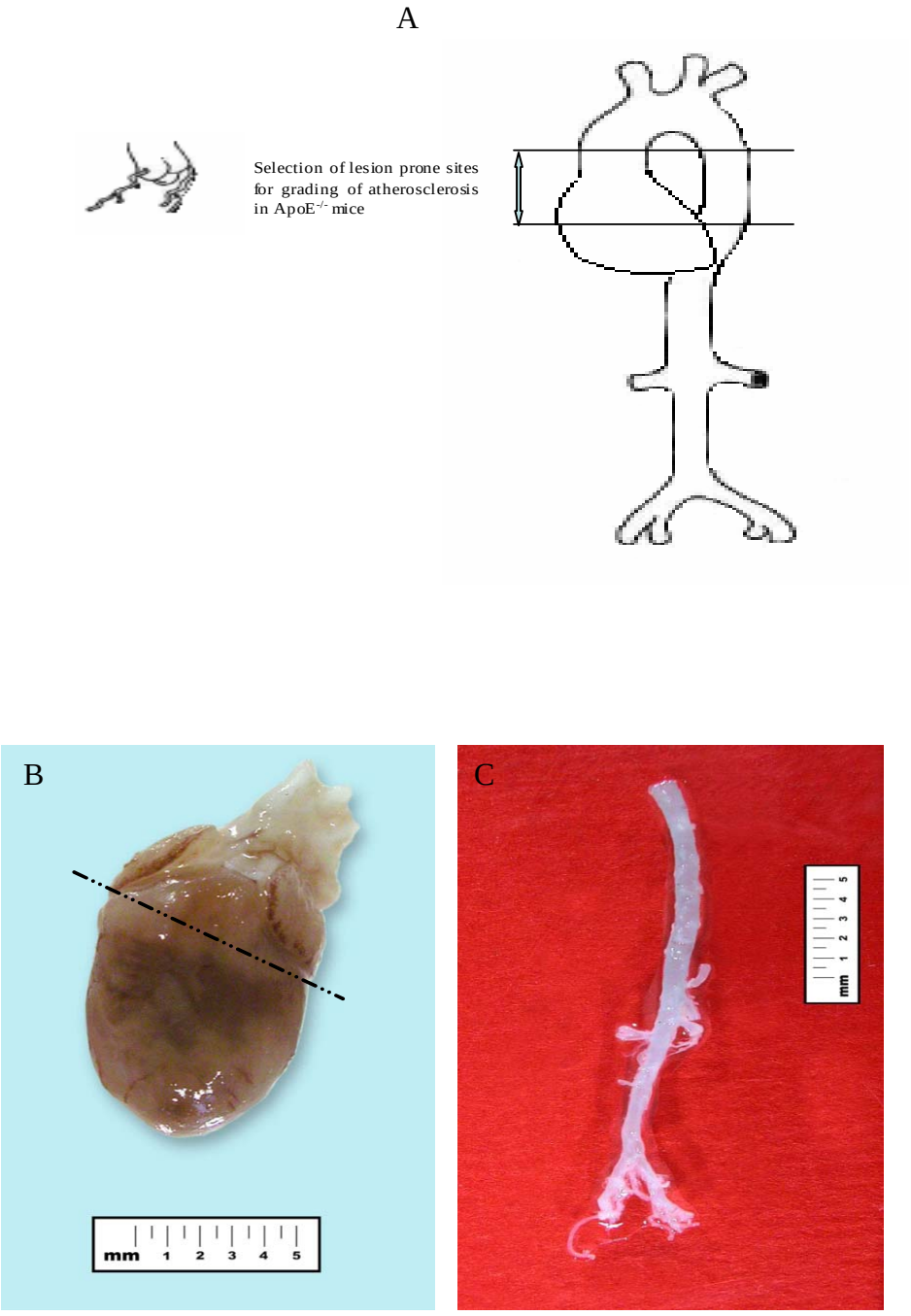


Figure 2.1. Diagrammatic/photographic representation of the heart and aorta of an apoE$^{-/-}$ mouse. Figure A shows the area for grading of atherosclerotic lesions in diagrammatic form. Figure B shows the isolated heart with the area selected for examination located above the dotted line. Figure C shows the dissected aorta from an apoE$^{-/-}$ mouse.

2.2.3.2 Slides and tissue quality control

To ensure that each section belonged to the right heart, tissue in each block was checked with the last section of the last slide. This assessment was done by two observers for 70 cases. This checking was done for the rest of the cases by one observer according to normal histological practice.

2.2.3.3 Quantification of atherosclerotic plaque in apoE$^{-/-}$ mice

To measure the plaque area for each heart at a consistent site, a section of proximal aorta caudal to the ostium of the most inferior of the coronary arteries, at a level at which the attachments of the aortic valve leaflets were visible was selected (Figure 2.2). The sections were photographed with a 2× objective using an Olympus BX51 microscope (Olympus Optical Co. Ltd, Tokyo, Japan) and a DP12 camera (Olympus Optical Co. Ltd, Tokyo, Japan). Aortic area (expressed as aortic cross sectional area) and plaque area were measured digitally using Measure Master (Leading Edge, Adelaide, SA, Australia) (Figure 2.2). The percentage of lumen occupied by plaque was calculated by dividing plaque area by aortic area. All the measurements were done in a blinded manner with all the cases having been coded by an independent person and only decoded at the completion of the assessment.

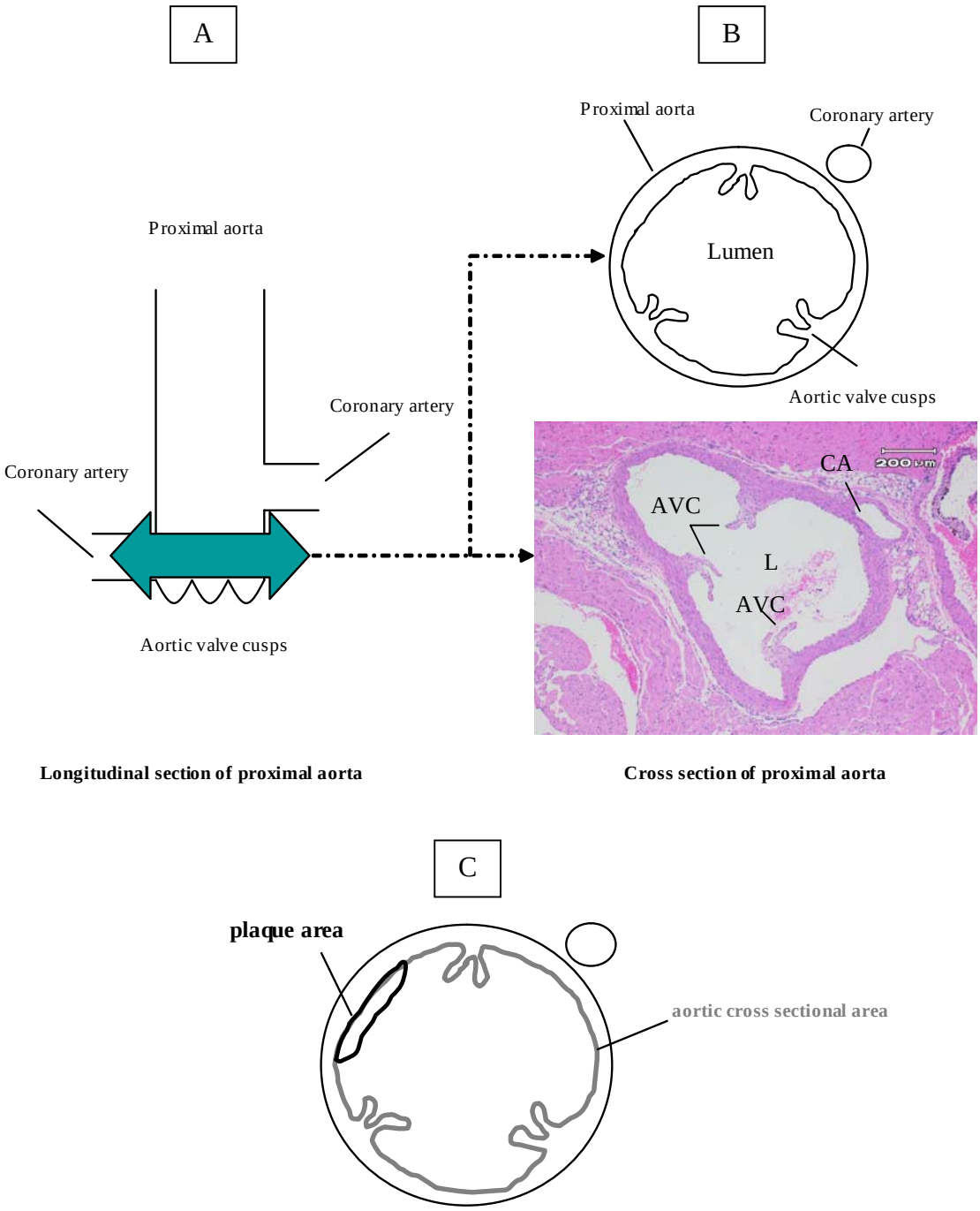


Figure 2.2. Diagrammatic/photographic representation of the proximal aorta of an apoE$^{-/-}$ mouse. The section of proximal aorta at the level close to the aortic valve cusps and coronary arteries was selected for examination and measurement. (A) Longitudinal section of proximal aorta, (B) cross section of proximal aorta and (C) measurement of plaque area and aorta area. L, AVC and CA indicate lumen, aortic valve cusps and coronary artery respectively.

2.2.3.4 Consistency in measurement

To determine intra-observer and inter-observer variability 6 cases were selected randomly for analysis. In this analysis, each section was measured three times by two observers (Table 4.1, chapter 4). This consistency in measurement was determined by using an analysis of variance test and is described in detail in chapter 4.

2.2.4 Electron microscopy

One of the achievements during this study was the development of a novel electron microscopy method. This technique was conducted on a spare unstained formalin fixed paraffin section. Using this method therefore the appropriate area for ultrastructural examination was found by comparing the unstained slide to a serial H&E section facilitating confirmation of plaque component. Although all sections were examined with light microscopy, electron microscopy was used in only a few cases to confirm the cellular composition.

2.2.4.1 Tissue processing

A 5µm unstained paraffin section was compared with a H&E section to find the appropriate area for sampling. The area of interest was scored with a diamond pencil and broken off with pliers. The tissue and surrounding wax were placed into a polypropylene Sartedt cap for processing. The 5µm paraffin section was taken to water then was postfixed in osmium tetroxide, dehydrated and infiltrated with Spurr's low viscosity epoxy resin. Embedding was performed by cutting the end of a BEEM

capsule, inverting the capsule over the area of interest with a resin depth of 5mm, polymerising the resin at 70°C in a vacuum oven overnight and filling the BEEM capsule with resin and polymerised. Material was plunged into liquid nitrogen and the BEEM capsule with the section on the surface separated from the glass slide.

2.2.4.2 Preparation and examination of sections

Thin 90nm sections were cut on Reichert Ultracut S ultramicrotome (Vienna, Austria) and mounted on a 150mesh hexagonal copper grid (Gilder Grid, Grantham, England). Sections were stained with 2% uranyl acetate followed by 1% lead citrate. Specimens were examined with a Hitachi H7000 transmission electron microscope (Hitachi, Katsuta, Japan) operated at an accelerating voltage of 75KV.

2.3 Statistics and data analysis

Data has been expressed in this thesis are either raw data or mean±sem. Statistics were calculated using Graph Pad InStat V2.02 (Graph Pad Software). For multiple comparisons, analysis of variance was used to assess any difference. To calculate mathematical equations data were fitted in SPSS version 12.0.1. Results were considered statistically significant when $p<0.05$.