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

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# CHAPTER 3

# CRITERIA FOR GRADING ATHEROSCLEROSIS IN THE APOE DEFICIENT MOUSE MODEL

## 3.1 Introduction

The development of apoE deficient mice by three groups; Piedrahita et al (1992), Plump et al (1992) and van Ree et al (1994) (Daugherty, 2002), has provided a useful model by which mechanisms of atherosclerosis development and its inhibition by therapeutics can be studied. These homozygous apoE deficient ($apoE^{-/-}$) mice, created by gene targeting in embryonic stem (ES) cells, have marked hypercholesterolemia and spontaneously develop lesion patterns characteristic of human atherosclerosis (Plump *et al.*, 1992). Development of atherosclerotic lesions is observed in many regions, including the aortic root, the lesser curvature of the aortic arch, the principal branches of the aorta, and pulmonary and carotid arteries. Signs of early lesion development can be observed in lesion-prone sites such as in the proximal aorta near the level of the aortic valve (Nakashima *et al.*, 1994).

However since its establishment, this model has suffered from a lack of a description of lesion development and criteria for lesion scoring which are required for a range of applications. We have used the Stary classification of atherosclerotic lesions in humans (described in detail in chapter 1), presented by the American Heart Association, as the basis for establishing a grading system in apoE deficient mice.

## 3.2 Characterisation of atherosclerotic lesions

### 3.2.1 Region of the aortic root selected for measurement of plaque area

It has been previously established that the area at the level of the aortic valves produces the most consistent and largest lesions (Plump *et al.*, 1992). To measure the lesion size,

each heart was cut transversely caudal to the atria (Figure 2.1, chapter 2). To measure the plaque area for each mouse 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, chapter 2). This area, which is a lesion prone site, correlated with the area of maximum atheroma in the majority of animals examined. However, for grading the atherosclerotic lesions, all the sections were analysed (Figure 2.1, chapter 2).

### 3.2.2 Histological criteria for grading atherosclerotic lesions in apoE$^{-/-}$ mice

ApoE$^{-/-}$ mice were sacrificed at different ages to enable the generation of tissue sections which would show atherosclerosis development from the early to the late stage. The sections were stained with Haematoxylin and Eosin (H&E). Some spare sections were stained with Elastic Van Gieson and Masson Trichrome and others were used for immunohistochemical staing for muscle actin. The cellular composition and structure of the lesions were examined by light microscopy and confirmed by electron microscopy. The lesions could be classified into the following types (Figure 3.1 and Figure 3.2).

**Type I lesion.** The plaque was comprised of monocytes adherent to the endothelial layer and small isolated groups of macrophages without foamy cytoplasm or a small number of isolated groups of foamy macrophages in the subendothelial region of the intima. The structure of the media remained intact.

**Type II lesion (fatty streak).** As the lesions developed they contained more macrophages, with and without lipid droplets, than seen in type I lesion. The lesions were two or more cells thick and some had a thin cap that was a thick endothelial layer (Figure 3.3). Muscle Actin staining showed that smooth muscle cells are not present in the cap of the lesion at this stage.

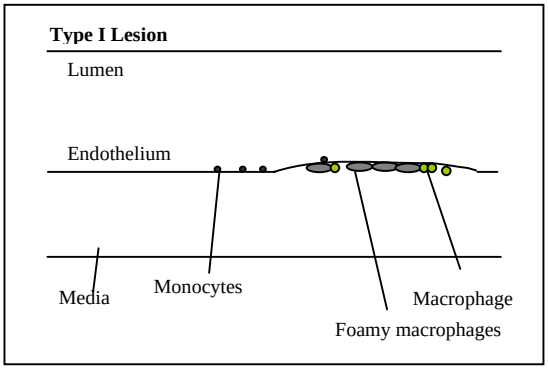
**Type III lesion (preatheroma).** By this stage the lesions contained cholesterol clefts (extracellular lipid pools) surrounded by macrophages, foamy macrophages and a thin cap on top. No smooth muscle cell or elastic fibres could be seen in the cap. Figure 3.4 shows cholesterol clefts in the plaque taken by electron microscopy.

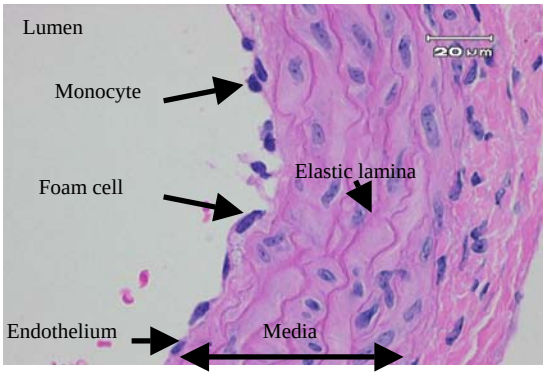
**Type IV lesion (atheroma).** These lesions contained a confluent lipid core surrounded by macrophages, foamy macrophages and/or connective tissue. Fibrous cap formation was first seen at this stage. This thin fibrous cap contained very little collagen, which was confirmed by Elastic Van Gieson staining and Masson Trichrome technique stains, and a few smooth muscle cells, which was confirmed by immunohistochemical staining for muscle specific actin. Penetration of plaque into the media was seen in this lesion and thinning of the media was also evident. Figure 3.3 shows the detail of the confluent lipid core in the plaque by electron microscopy.

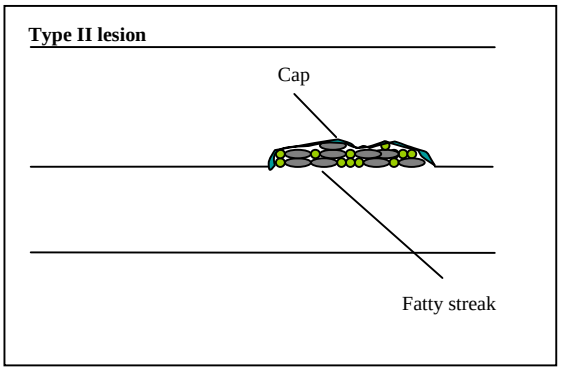
**Type V lesion (fibroatheroma).** The lesions became more advanced and were characterised by a well-developed fibrous cap and/or calcification or ossification. This type could be further subdivided into type Va, type Vb and type Vc. *Type Va lesion* was characterised by a fibrous layer or cap above the lipid core consisting of collagen (Elastic Van Gieson staining and Masson Trichrome technique staining), elastic fibers

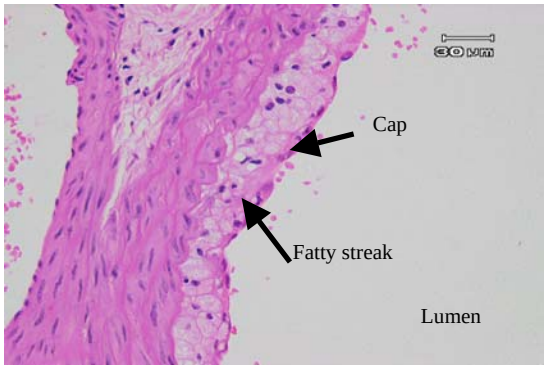
(Elastic Van Gieson staining) and smooth muscle cells (immunohistochemical staining for muscle specific actin) (Figure 3.5). The presence of elastic fibers and smooth muscle cells in the fibrous cap was also confirmed by electron microscopy (Figure 3.6). *Type Vb lesion* was an advanced lesion which often showed fibrosis in addition to calcification and *type Vc lesion* also showed ossification.

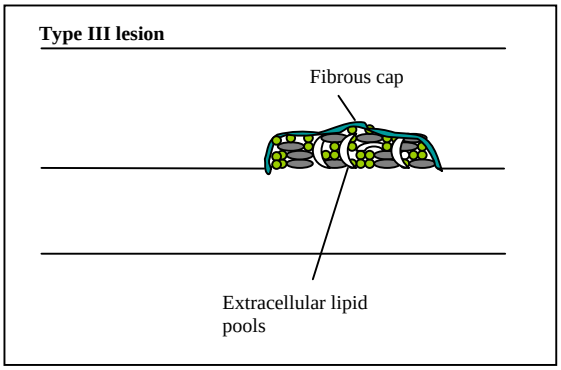
**Type VI lesion (complicated lesion).** This type was initially divided into two subtypes. *Type VIa lesion* with incipient aneurysm formation was characterised by penetration of the vessel wall and associated with severe disruption of the elastic lamina. This lesion was usually associated with inflammation (including neutrophils) both within the plaque and externally on the vessel wall. *Type VIb* was an advanced lesion with actual aneurysm and complete disruption of the elastic lamina. In some cases there was a very severe and advanced plaque in one or both the coronary arteries with partial occlusion. Plaques with haemorrhage and thrombosis previously reported by other research groups (Rosenfeld *et al.*, 2000; Johnson *et al.*, 2005) were not observed by us but can be classified as *type VIc* which was not included in the figure 3.1.

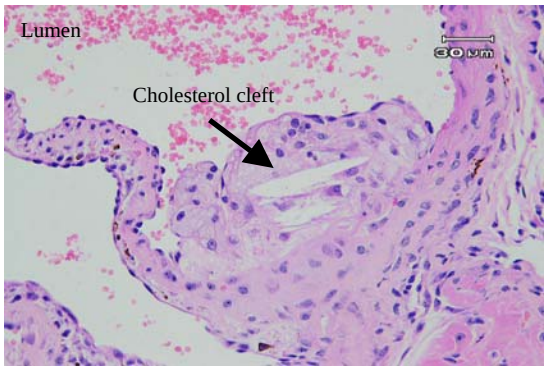

Type I Lesion
Lumen
Endothelium
Media
Monocytes
Macrophage
Foamy macrophages

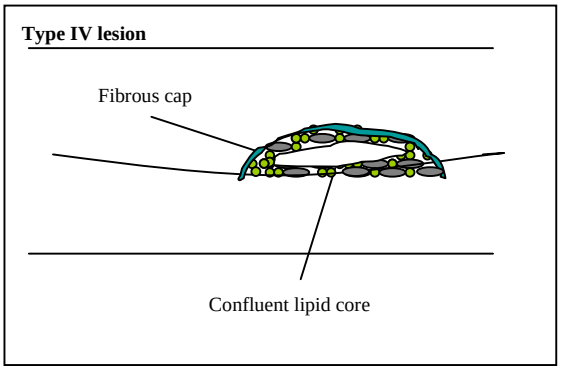

Lumen
Monocyte
Foam cell
Elastic lamina
Endothelium
Media

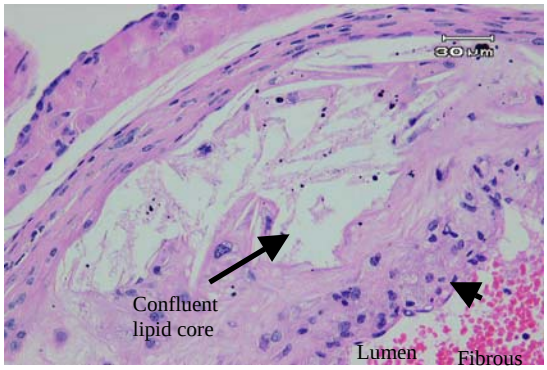

Type II lesion
Cap
Fatty streak


Cap
Fatty streak
Lumen


Type III lesion
Fibrous cap
Extracellular lipid pools


Lumen
Cholesterol cleft


Type IV lesion
Fibrous cap
Confluent lipid core


Confluent lipid core
Lumen
Fibrous cap

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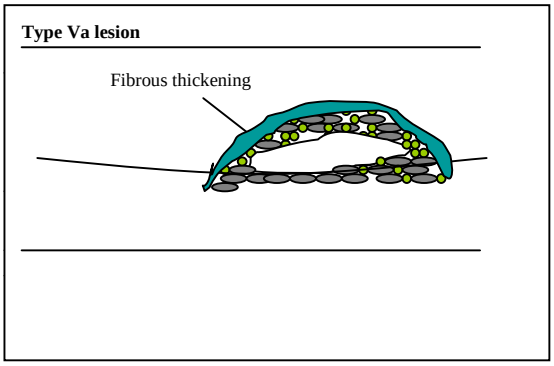
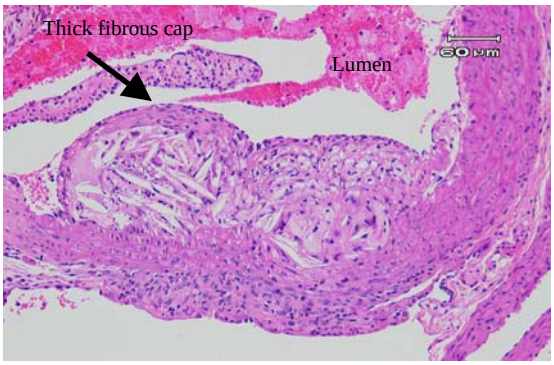
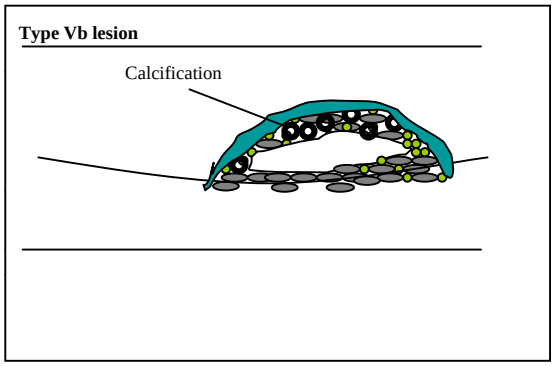
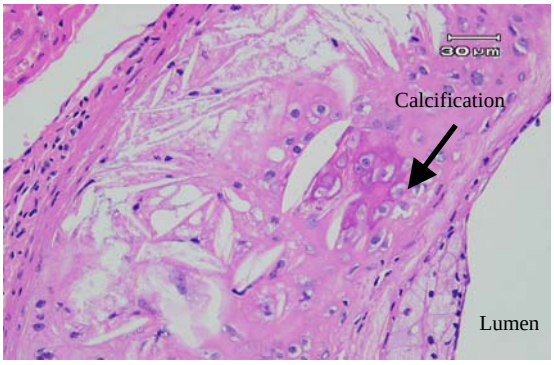
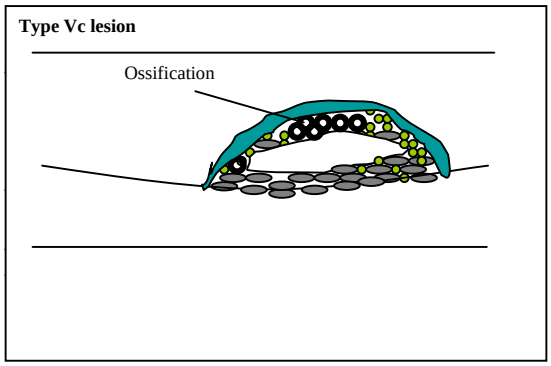
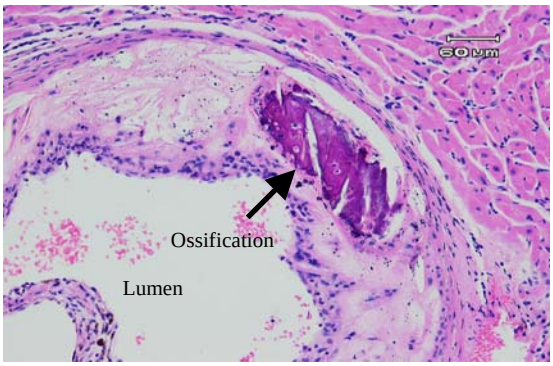

Type Va lesion
Fibrous thickening


Thick fibrous cap
Lumen
50 µm


Type Vb lesion
Calcification


50 µm
Calcification
Lumen


Type Vc lesion
Ossification


50 µm
Ossification
Lumen

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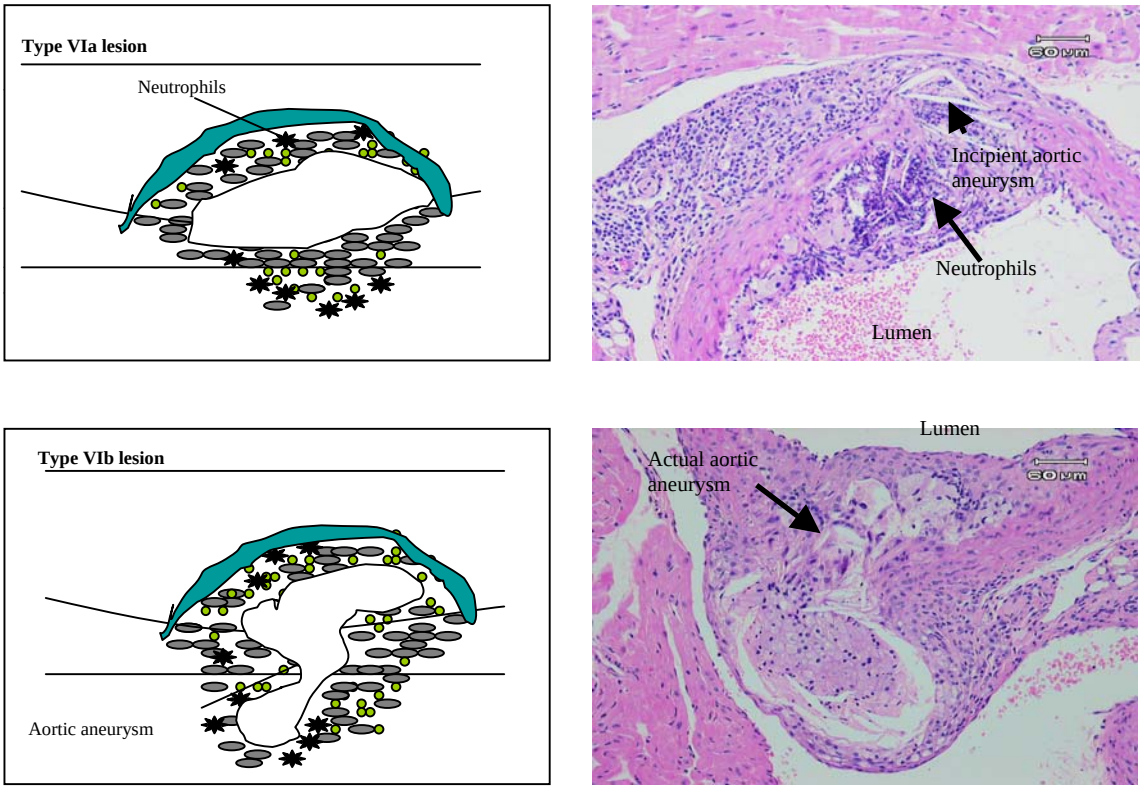
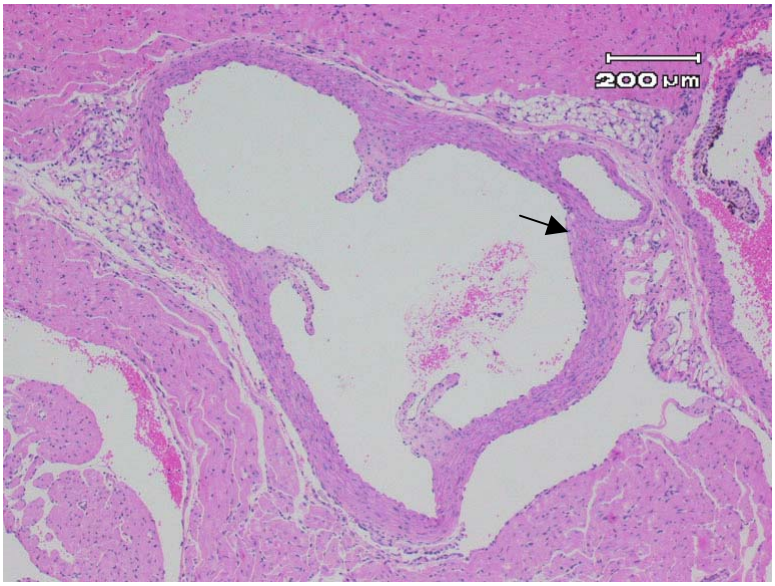
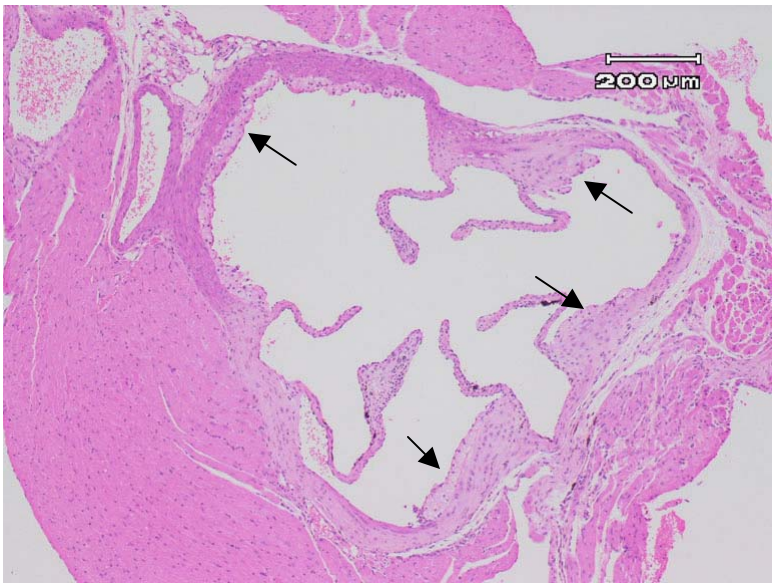



**Figure 3.1. Diagrammatic/photographic representation of grading criteria.** Type I describes lesions containing a sparse macrophage infiltrate and foam cells. Type II lesions contain a greater number of macrophages including foam cells, and are characterised by two or more cell layers, surrounded by a thin cap. Cholesterol clefts surrounded by foam cells and macrophages are a major component of type III lesions. The type IV lesion has a confluent lipid core, a thin fibrous cap and penetration of the plaque into the media. Type V is a more advanced lesion characterised by a well-developed fibrous cap and/or calcification or ossification. The type VI lesion is an advanced lesion with incipient (VIa) or actual (VIb) aneurysm and inflammation including neutrophils.

**Type I Lesion**




**Type II lesion**

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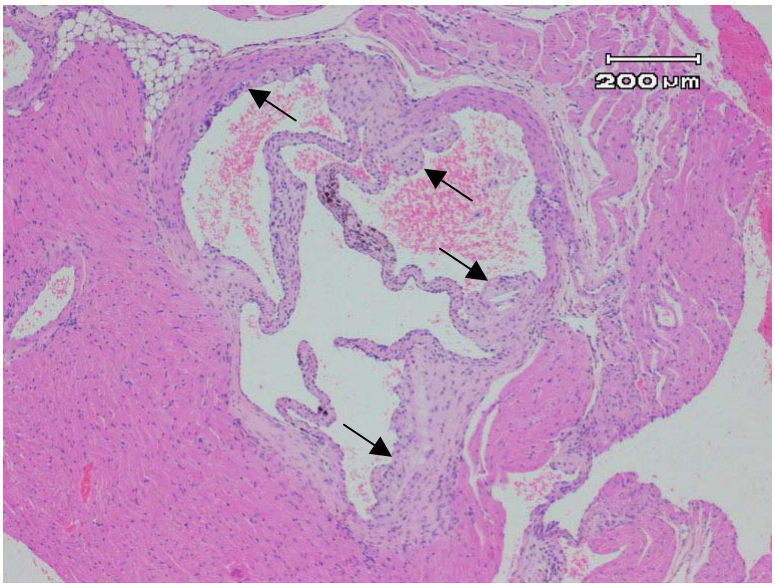
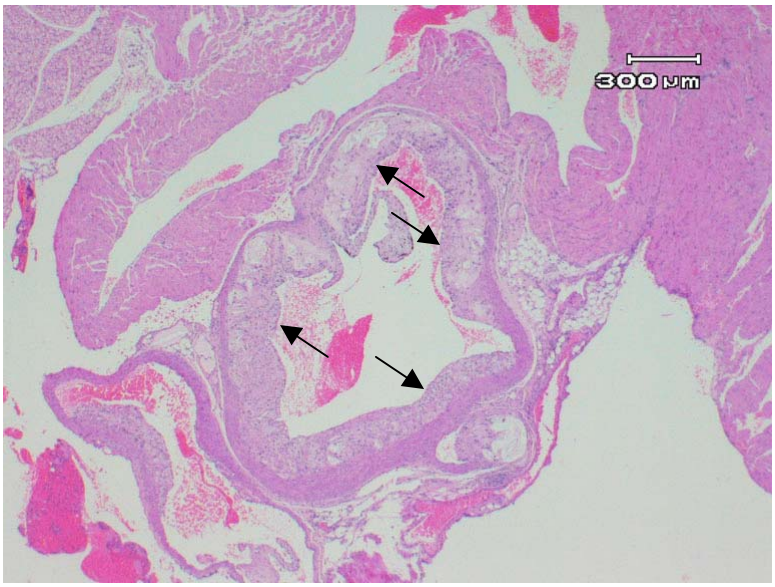
**Type III lesion**




**Type IV lesion**

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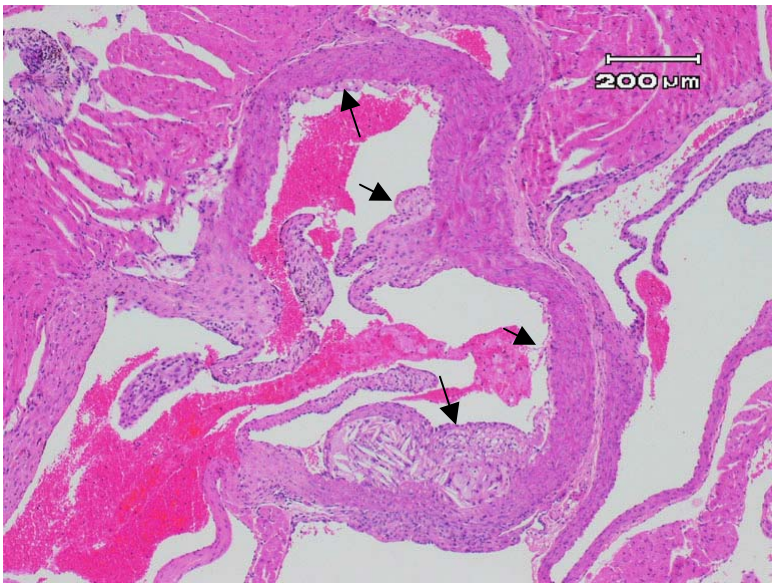
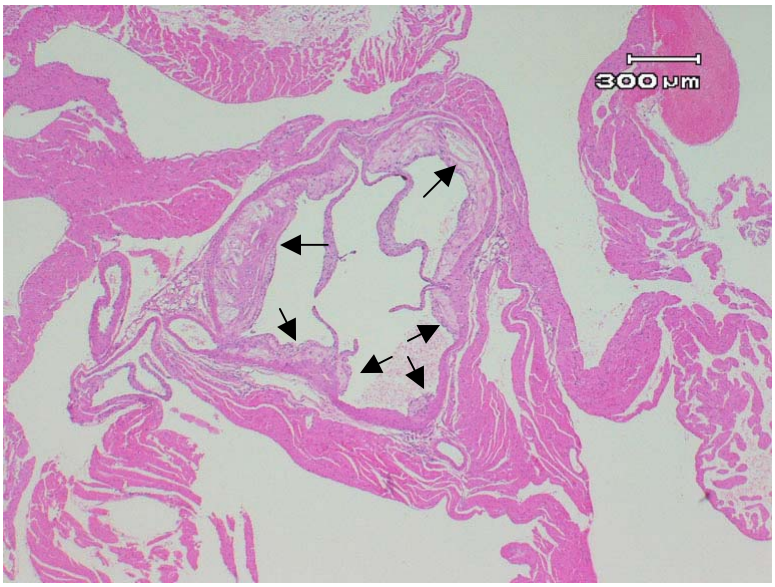
**Type Va lesion**




**Type Vb lesion**

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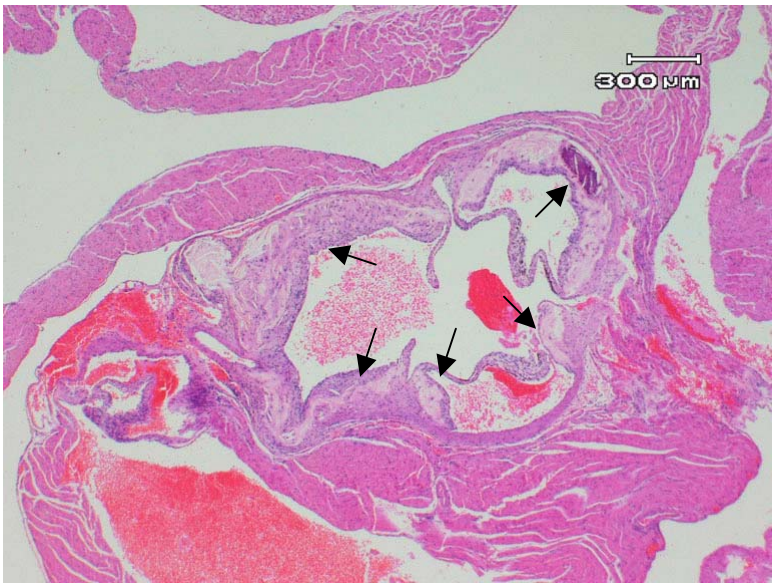
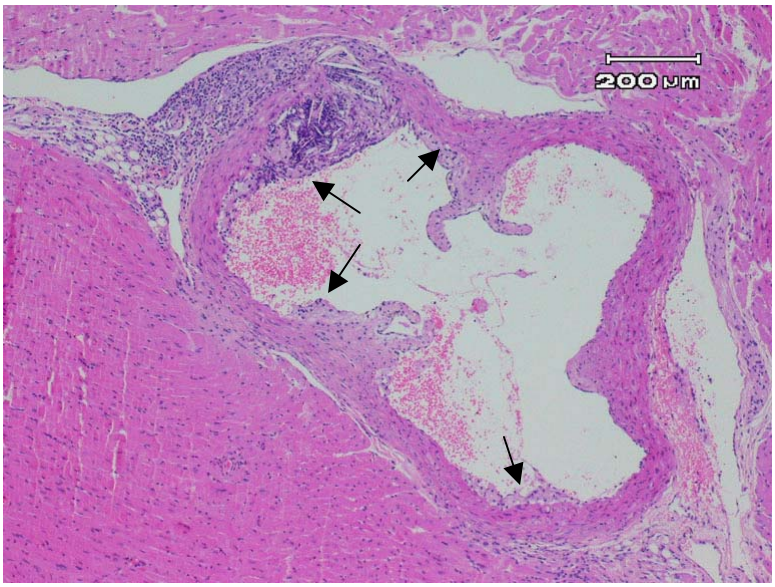
**Type Vc lesion**




**Type VIa**

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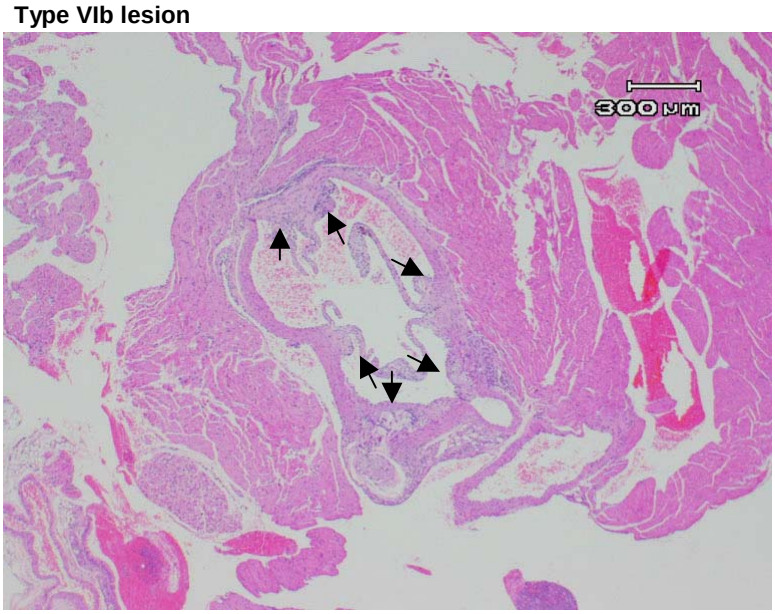


**Figure 3.2. Photographic representation of cross section of aortic root with different types of atherosclerotic lesion.** Lesions are classified into six types. The arrows indicate the plaques. The lesion size usually increases as the plaque progresses to a more advanced type however the components of plaque play more important roles in grading of the atherosclerotic lesion.

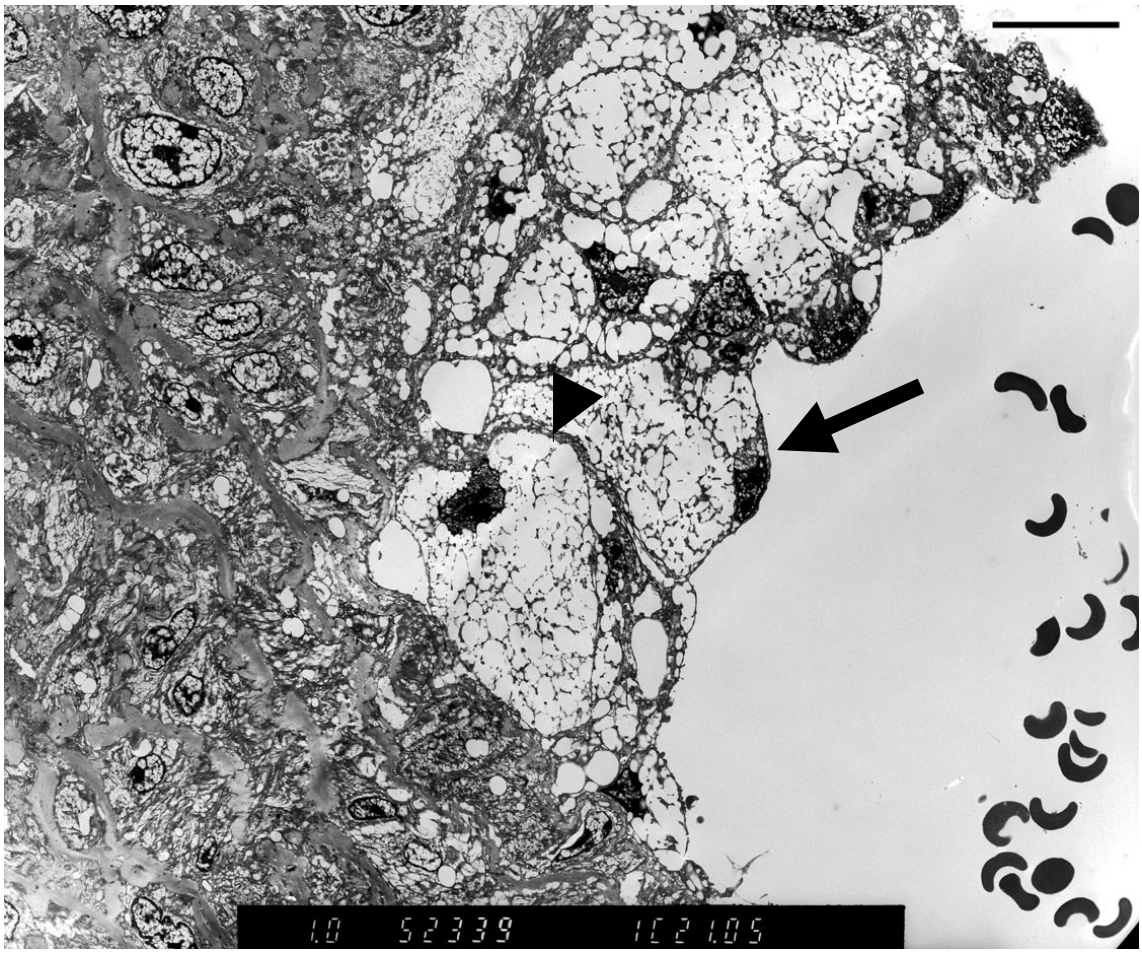


**Figure 3.3. Electron photomicrograph of type II lesion.** The arrow indicates an endothelial cell capping the lesion, the arrowhead indicates a foam cell. The structure of the media including elastic fibers and smooth muscles under the plaque is intact. Scale bar=10μm.

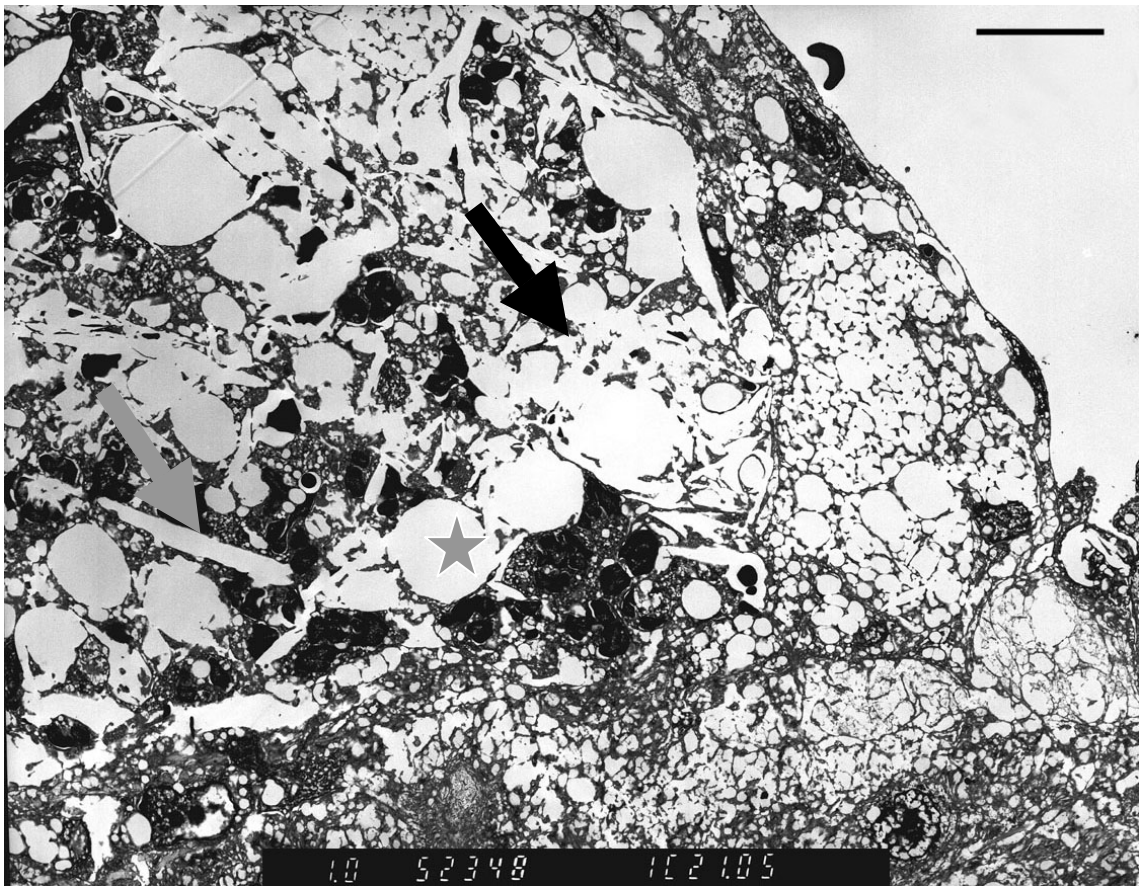

**Figure 3.4. Electron photomicrograph showing cholesterol clefts and confluent lipid in the core of an atherosclerotic lesion.** The grey arrow indicates cholesterol clefts (sharp spindle with pointed ends), the black arrow indicates the confluent lipid cores and the grey star shows a lipid droplet. Scale bar=10µm.

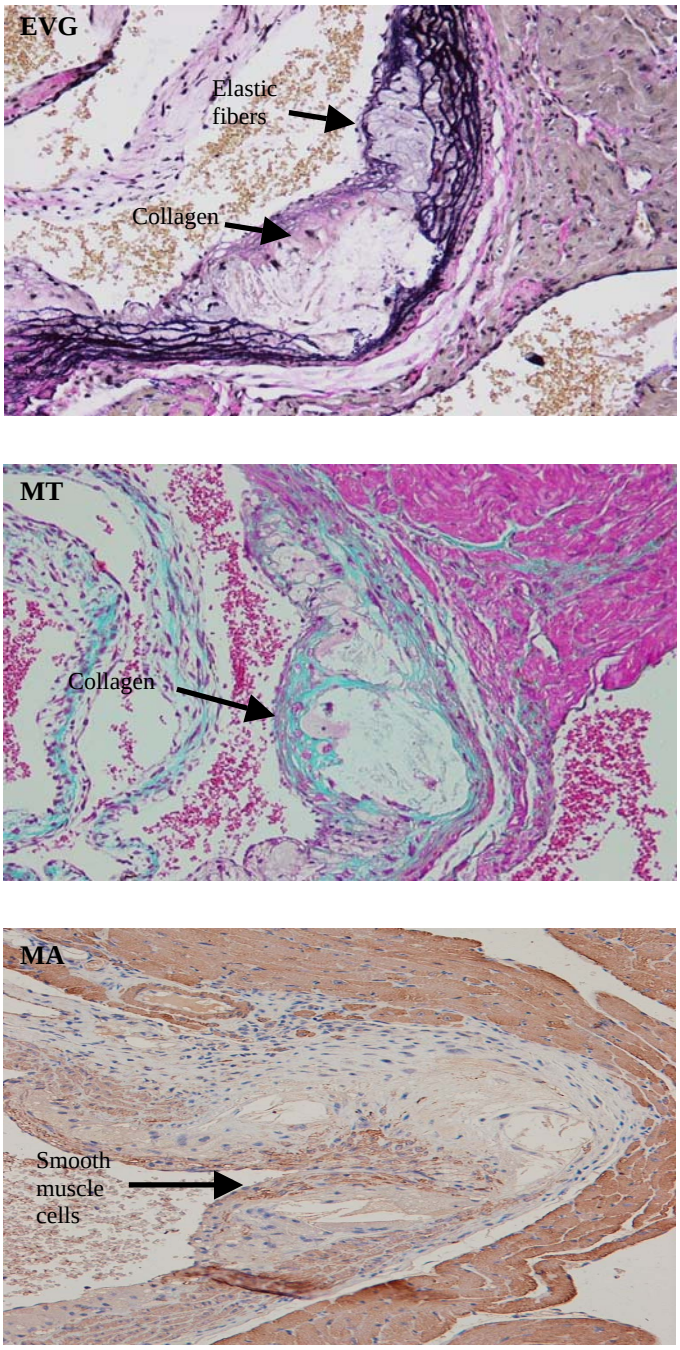


**Figure 3.5. Special staining for lesion components of type Va.** Elastic fibres stained dark violet and collagen stained pink after staining with Elastic Van Gieson (EVG). Masson Trichrome technique (MT) staining confirmed the presence of collagen in the cap and plaque, staining collagen green. The presence of smooth muscle cells as a component of the lesion and fibrous cap was confirmed by Muscle Actin (MA) staining which stained muscle fibers brown.

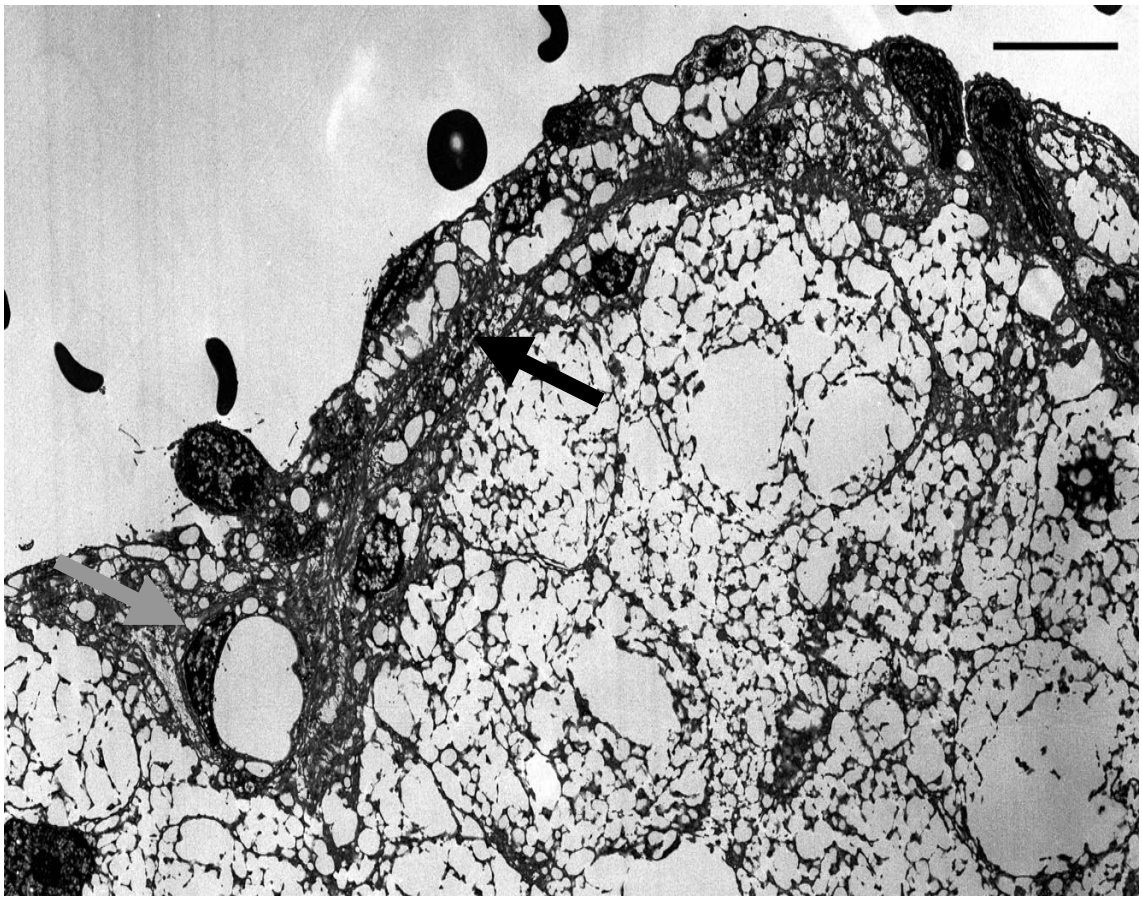

**Figure 3.6. Electron photomicrograph showing elastic fibers and smooth muscle cells in the fibrous cap.** The black arrow indicates elastic fibers and the grey arrow shows a smooth muscle cells with accumulated fat. Scale bar=5µm.

### 3.2.3 Presence of calcification and ossification in atherosclerotic lesion of apoE$^{-/-}$ mice

We observed calcification in the media at the level of aortic valve (Figure 3.7). This is probably the result of deposition of calcium granules in cytoplasm of smooth muscle cells. Intracellular calcium granules become extracellular after disintegration of dead muscle cells and often form aggregates (Stary, 2001) or ossify. However, in apoE$^{-/-}$ mice with large atherosclerotic plaques we also saw calcification and ossification within atherosclerotic plaques in the proximal aorta (Figure 3.8). Apparently, all or most lipidic cell remnants can calcify including the remnants of both macrophage foam cells and smooth muscle cells however elastic fibres were not a nidus for calcium deposition (Stary, 2001). On the other hand, Qiao in 1995 reported that in mice models, including apoE$^{-/-}$ mice some vessel wall cell populations can differentiate to chondrocytes, form cartilage and contribute to calcification. Therefore cartilage metaplasia may be a potential pathway of artery wall calcification in atherosclerotic plaque (Qiao *et al.*, 1995).

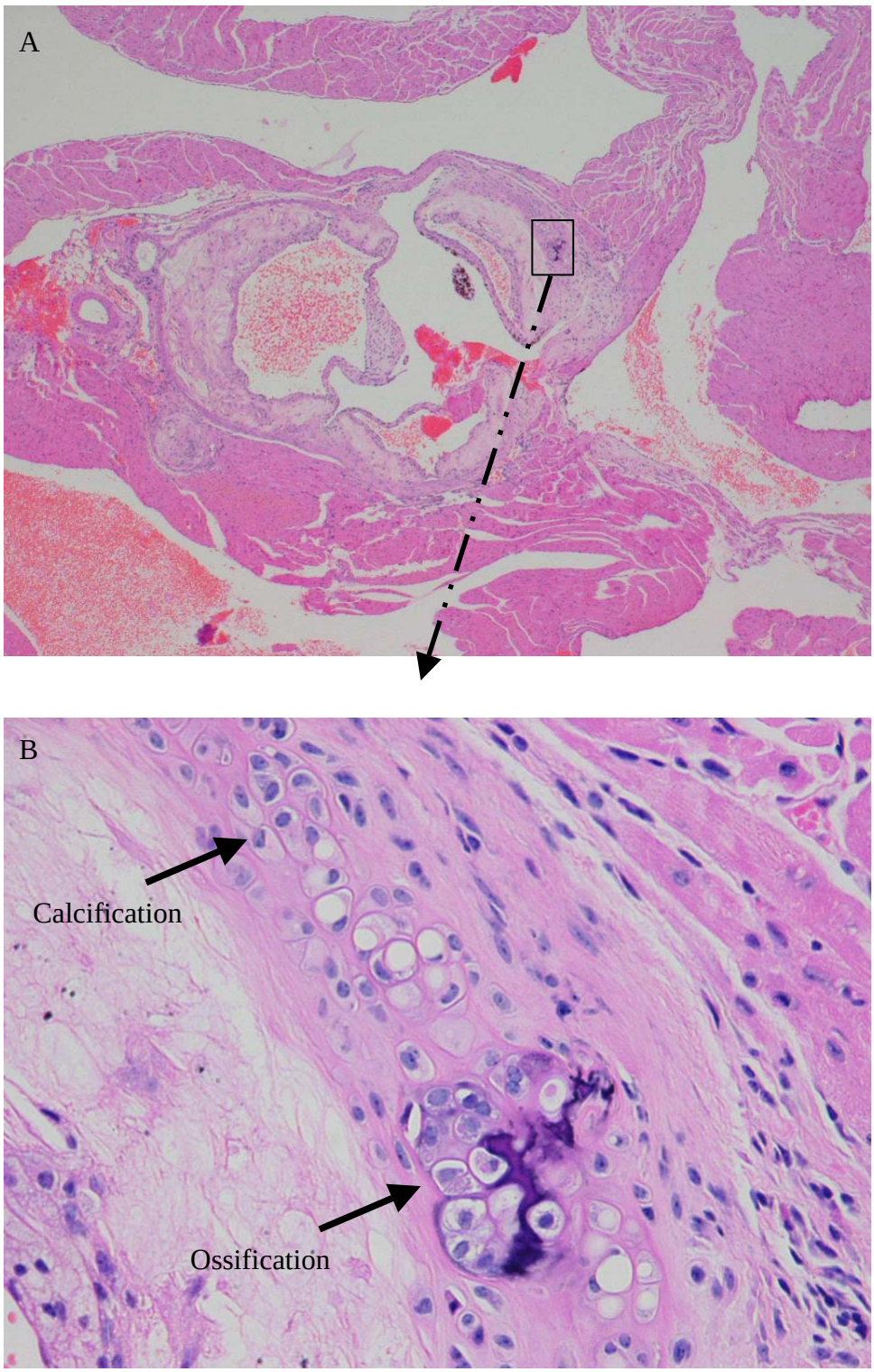


**Figure 3.7. Photographic representation of calcification in atherosclerotic lesions in apoE$^{-/-}$ mice.** (A) Proximal aorta at level of aortic valve and (B) chondrification observed in the media at level of aortic valves.

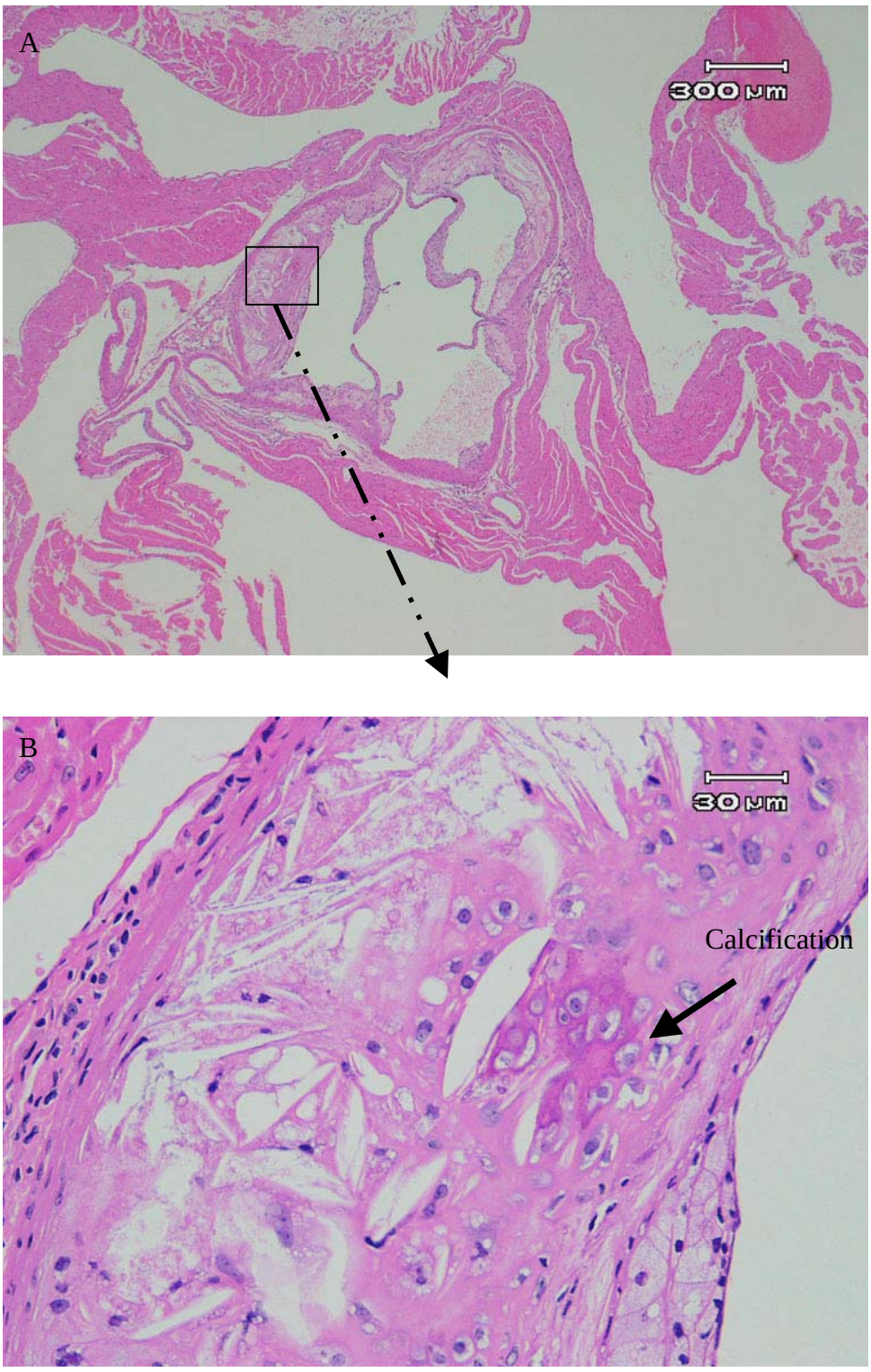


**Figure 3.8. Photographic representation of calcification in atherosclerotic lesions in apoE$^{-/-}$ mice with advanced lesion.** (A) Aortic cross section and (B) calcification and chondrification observed within an atherosclerotic plaque.

### 3.2.4 The presence of neutrophils in atherosclerotic lesions of apoE$^{-/-}$ mice

We observed neutrophils in some very advanced plaques. Figure 3.9 shows the presence of neutrophils in a section of atherosclerotic lesion which was stained by Haematoxylin and Eosin and figure 3.10 confirms the presence of neutrophils by electron microscopy. Activated neutrophils release neutrophil elastase that is responsible for degradation of basement membrane constituents, endothelial damage which leads to plaque rupture, haemorrhage and aneurysm formation (Naruko *et al.*, 2002).

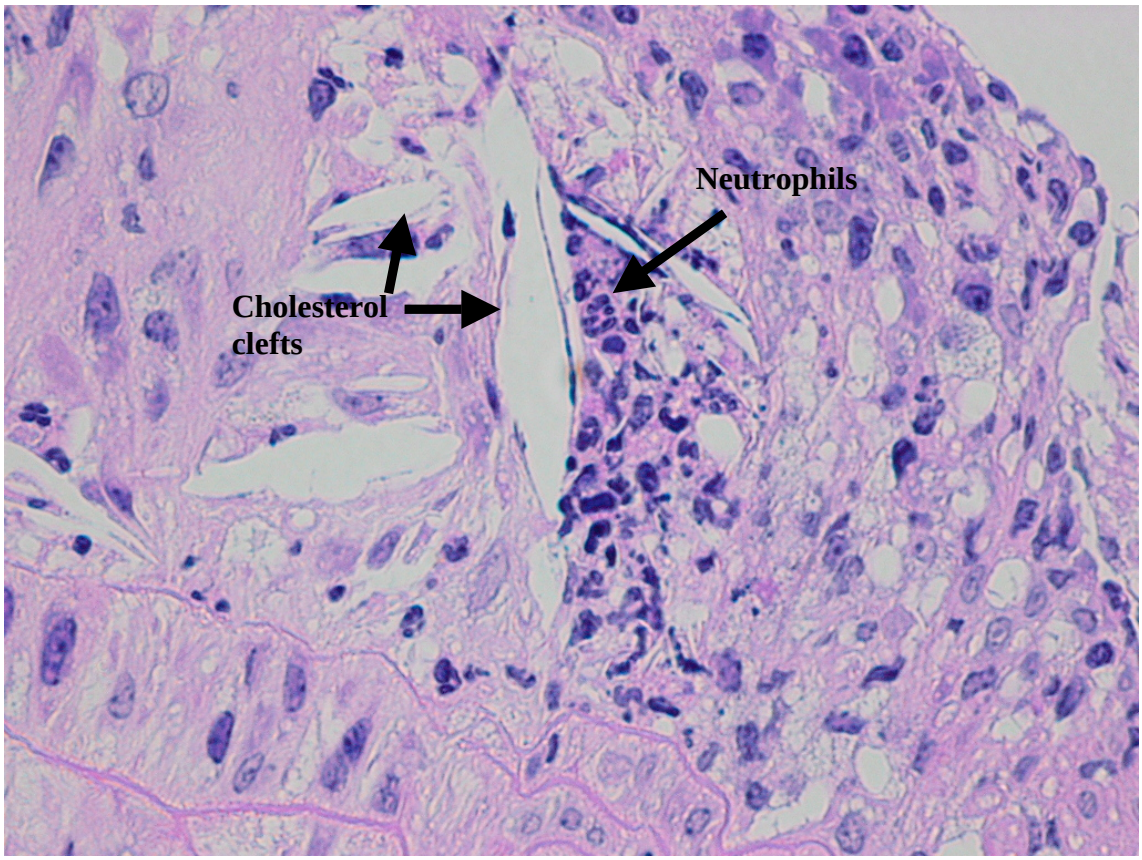


**Figure 3.9. Photograph showing the presence of neutrophils in atherosclerotic lesions in apoE$^{-/-}$ mice.**

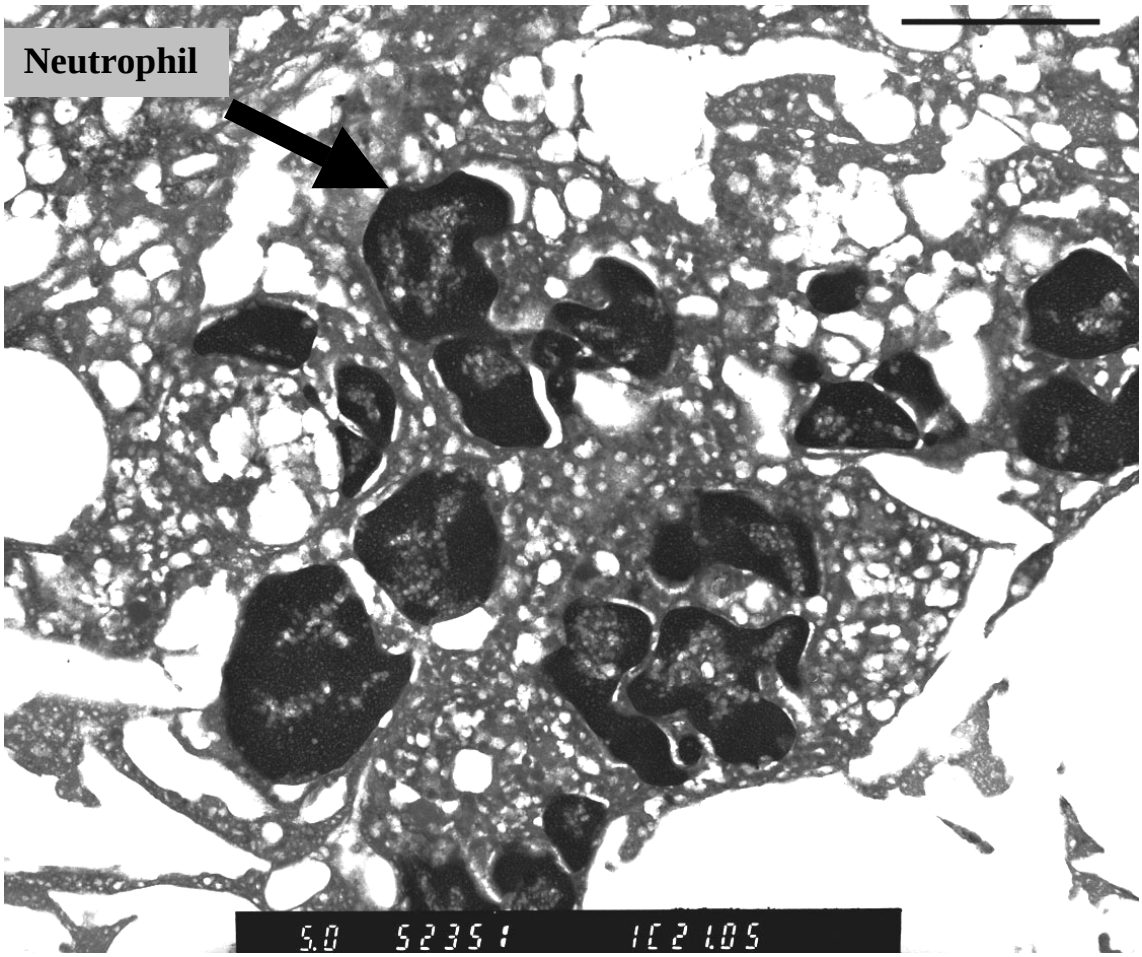


**Figure 3.10. Electron photomicrograph of the atherosclerotic plaque with neutrophils.** Scale bar=3µm.

## 3.3 Summary

ApoE$^{-/-}$ mice are a useful model to study the development of atherosclerosis at different stages. Although there are many similarities between atherosclerotic lesion components in apoE$^{-/-}$ mice and humans, histological classification of atherosclerotic lesions in humans is not completely applicable in the apoE$^{-/-}$ model.

We classified atherosclerotic lesions in apoE$^{-/-}$ mice model into six categories based on the morphologic characteristics. Lesions containing monocytes adherent to the endothelial layer, a sparse macrophage infiltrate and foam cells in the subendothelial layer are classified as type I lesions. Type II lesions have a greater number of macrophages including foam cells, which can consist of two or more cell layers, and are surrounded by a thin cap. Cholesterol clefts surrounded by foam cells and macrophages are major components of type III lesions. The type IV lesions contain confluent lipid cores, a thin fibrous cap and penetration of plaque into media. Type V is a more advanced lesion including a well-developed fibrous cap and/or calcification or ossification. The type VI lesion is an advanced lesion with incipient (VIa) or actual (VIb) aneurysm formation and inflammation. Neutrophils are frequently seen. Plaque with haemorrhage or thrombosis are classified as type VIc.

Calcification in the apoE$^{-/-}$ mice model in the media at the level of aortic valve ring may happen in even very early stages of development of atherosclerosis. In addition, neutrophils can be observed in a few cases and they are usually coincident with severe inflammation within and outside the aorta, especially around the coronary arteries.