ROLE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-γ (PPARγ) LIGANDS ON THE FOAM CELL FORMATION DERIVED FROM MNV-1 INFECTED MACROPHAGES

by

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LIST OF SYMBOLS AND ABBREVIATIONS

ABCA1	ATP-Binding Cassette transporter A1
ABCG1	ATP-Binding Cassette transporter A1
AF	Activation Function
apoA	Apolipoprotein A
apoB	Apolipoprotein B
apoC	Apolipoprotein C
apoE	Apolipoprotein E
BAT	Brown Adipose Tissue
CD36	Cluster of differentiation 36
CETP	Cholesteryl Ester Transfer Protein
CM	Chylomicron
CMV	Cytomegalovirus
CVD	Cardiovascular Diseases
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
LDL	Low Density Lipoprotein
EC	Endothelial Cell
FA	Fatty Acid

FC	Free Cholesterol
FFA	Free Fatty Acid
HDL	High Density Lipoprotein
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
ICAM-1	Intracellular Adhesion Molecule
IDL	Intermediate Density Lipoprotein
LBD	Ligand Binding Domain
LCAT	Lechithin-cholesterol Transferase
LDLR	Low Density Lipoprotein (LDL) Receptor
LPL	Lipoprotein Lipase
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MMP	Matrix Metalloproteinase
MNV	Murine Norovirus
OxLDL	Oxidised Low Density Lipoprotein
ORF	Open Reading Frame
PLTP	Phospholipid Transfer Protein
PPAR	Peroxisome Proliferator Activated Receptor

PPRE	Peroxisome Proliferator Response Element
PUFA	Polyunsaturaed Fatty Acid
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
SMC	Smooth Muscle Cell
SR-BI	Scavenger Receptor BI
SRA-1	Scavenger Receptor A-1
TZD	Thiazolidinedione
VCAM-1	Vascular Adhesion Molecule-1
VF1	Virulence Factor 1
VLDL	Very Low Density Lipoprotein
VP1	Viral Protein 1
VP2	Viral protein 2
15d-PGJ2	15 deoxy Δ-prostaglandin J2

PERANAN LIGAND RESEPTOR PEROKSISOM PROLIFERATOR-γ YANG DIAKTIFKAN (PPAR-γ) TERHADAP PEMBENTUKAN SEL BUSA DARIPADA MAKROFAJ YANG DIJANGKITI MNV-1

ABSTRAK

Aterosklerosis adalah penyakit kronik yang berpunca daripada pengumpulan sel busa daripada makrofaj di dalam lapisan intima pada arteri. Kajian yang telah dilakukan menunjukkan MNV-4 dapat mempercepatkan pembentukan aterosklerosis pada tikus. Memandangkan PPARγ kebanyakannya diekspresi di dalam makrofaj, manakala MNV-1 dapat menjangkiti makrofaj, kami membuat hipotesis bahawa PPARγ ligand (ciglitazone and 5d-PGJ2) boleh mengawalselia pembentukan aterosklerosis dengan mengawal pembentukan sel busa daripada makrofaj yang dijangkiti MNV-1. Kajian ini akan mendedahkan pengawalaturan PPARγ ligand pada pembentukan sel busa daripada sel makrofaj tikus (RAW264.7) yang dijangkiti MNV-1. Di dalam kajian ini, MNV-1 rekombinan dihasilkan melalui kaedah sistem genetik songsang dan hasilnya digunakan untuk merawat RAW264.7 bersama oxLDL dan PPARγ ligand. Selepas itu, sel-sel yang dijangkiti disahkan menggunakan tindakbalas rantai polimerase (PCR). Pembentukan sel busa dinilai dengan menggunakan pewarnaan minyak merah yang dianalisis menggunakan visualisasi mikroskopik dan kandungan kolesterol ester dalam RAW264.7 yang dirawat ditentukan dengan menggunakan kit pengukuran kolesterol ester Amplex Red. Aplikasi sistem genetik songsang berasaskan RNA berjaya menghasilkan MNV-1 yang berjangkit dengan hasil sebanyak 4.18 Log10TCID50/ml apabila diukur melalui titrasi menggunakan sel BV2. Keputusan kajian menunjukkan sampel

RAW+oxLDL+MNV+15d-PGJ2 merekodkan titer virus yang tertinggi diikuti dengan RAW+MNV+15d-PGJ2 pada 6, 12 and 24 jam. Manakala titer virus yang terendah direkodkan oleh sampel RAW+MNV+ciglitazone dan RAW+oxLDL+ MNV+ciglitazone. Pemerhatian mikroskopik pewarnaan minyak merah telah menunjukkan bahawa oxLDL boleh merangsang pembentukan sel busa dari masa ke masa dalam sel RAW264.7. Penambahan PPARγ ligand juga meningkatkan pengumpulan titisan lipid. Analisis menggunakan kaedah two-way ANOVA menunjukkan kesemua rawatan, jangka masa dan rawatan berbanding jangka masa mempunyai interaksi yang signifikan untuk semua rawatan. Perbandingan pada 24 jam telah dipilih dan rawatan oxLDL pada sel RAW264.7 didapati meningkatkan kandungan kolesterol ester secara signifikan. Bagaimanapun penglibatan MNV-1 tidak menunjukkan kenaikan kandungan kolesterol ester dalam semua sampel yang dirawat dengan oxLDL. Rawatan ciglitazone menunjukkan pengurangan kandungan kolesterol ester untuk semua rawatan kecuali sampel RAW+oxLDL+ciglitazone manakala 15d-PGJ2 tidak menunjukkan kesan yang ketara dalam pengurangan tahap kolesterol ester dalam semua rawatan. Kesimpulannya, berdasarkan kaedah pemulihan RNA virus yang digunakan dalam kajian ini berjaya menghasilkan MNV-1 yang ditentukan genetiknya dan mampu berjangkit. oxLDL boleh merangsang sel busa dengan mendepositkan titisan lemak di dalam sitosol dan meningkatkan kandungan kolesterol ester. Jangkitan MNV-1 tidak menyebabkan pembentukan sel busa. Selain itu, 15d-PGJ2 dapat menggalakkan pembentukan sel busa. Akhir sekali, MNV-1 dan ciglitazone bekerja secara sinergi untuk merencatkan pembentukan sel busa.

ROLE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-γ (PPARγ) LIGANDS ON THE FOAM CELL FORMATION DERIVED FROM MNV-1 INFECTED MACROPHAGES

ABSTRACT

Atherosclerosis is a chronic inflammatory disease initiated by the accumulation of macrophage-derived foam cells in the intima layer of artery. Murine norovirus-4 (MNV-4) has been shown to accelerate atherogenesis and increased macrophages content in atherosclerotic lesion in mice. Since PPARγ is predominatly expressed in macrophages whereby MNV-1 has a tropism for macrophages, we hypothesise that PPARγ ligands (ciglitazone and 15d-PGJ2) may regulate atherogenesis by controlling the formation of MNV-1-infected macrophages derived foam cells. In the current study, recombinant MNV-1 was produced using RNA based reverse genetics system and was used to treat the RAW264.7 cells with oxLDL, ciglitazone and 15d-PGJ2. Subsequently, infected cells were assayed for viral infection confirmation using polymerase chain reaction (PCR). Foam cells formation were assessed by microscopic visualisation of lipid droplets using Oil Red O (ORO) staining and measurement of Cholesterol Ester (CE) content in the treated RAW264.7 by using Amplex Red Cholesterol Ester measurement kit. The application of RNA based reverse genetic system had successfully generated infectious MNV-1 at yields of 4.18 Log10TCID50/ml dishes as measured by titration in BV2 cells. The result also showed that RAW+oxLDL+MNV+15d-PGJ2 sample produced the highest viral titres followed by RAW+MNV+15d-PGJ2. Meanwhile, the lowest virus titres recorded by RAW+MNV+ciglitazone and

RAW+oxLDL+MNV+ciglitazone. Microscopic visualisation of lipid droplets showed that oxLDL increased the accumulation of lipid droplets over the time in RAW264.7 cells. Addition of MNV-1, ciglitazone and 15d-PGJ2 in oxLDL-treated RAW264.7 cells also increased deposition of lipid droplets in the cytosol of treated cells. Two-way ANOVA has shown that the test for all treatment, time point and treatment versus time point had significant interaction. At 24 hours, we have found that oxLDL treatment in RAW264.7 cells in the presence of MNV-1, ciglitazone and 15d-PGJ2 significantly increased CE contents. MNV-1 infection did not elicit CE content in oxLDL-treated RAW264.7 cells. In addition, 15d-PGJ2 but not ciglitazone increased CE content in oxLDL-treated RAW264.7 cells. MNV-1 and ciglitazone had synergistic effect in reducing CE content in oxLDL-treated RAW264.7 cells. In conclusion, RNA based virus recovery method used in the current study successfully produced genetically define infectious MNV-1. OxLDL stimulated foam cells formation by depositing lipid droplets in the cytosol areas and increased CE contents. MNV-1 infection may not trigger foam cells formation. On the other hand, 15d-PGJ2 may promote foam cells formation. Finally, MNV-1 and ciglitazone work in synergy in inhibiting foam cells formation.

CHAPTER 1: LITERATURE REVIEW

1.1 Atherosclerosis

Atherosclerosis is defined as hardening and narrowing of the arteries due to accumulation of lipid laden foam cells and it can be considered as a lipid-mediated disease (Collot-Teixeira *et al.*, 2007; Rader and Daugherty, 2008; Samson, Mundkur and Kakkar, 2012; Chung and Vafai, 2013; Annema, Eckardstein and Kovanen, 2015). Aggregation of cholesterol-loaded macrophages in the arterial wall can cause chronic inflammatory disease that derives from an imbalance in lipid metabolism and a maladaptive immune response (Reiss *et al.*, 2004; Moore, Sheedy and Fisher, 2013). Atherosclerotic plaques tend to formed at the branch points of arteries which leads to disturbed or insufficient laminar flow to maintain the normal state of endothelium (Insull, 2009; Moore, Sheedy and Fisher, 2013).

Atherosclerosis can lead to various cardiovascular diseases (CVD) such as myocardial infarction, stroke, coronary revascularization, angina pectoris, ischemic electrocardiographic changes, peripheral artery surgery, intermittent claudication (pain at rest), gangrene and loss of function (Moreno, 2003; Nagy *et al.*, 2012; Frostegård, 2013; Pawlak, Mysliwiec and Pawlak, 2013; de Gaetano *et al.*, 2015). According to World Health Organisation (WHO), CVD is a major cause of global mortality and estimated number of CVD cases will rise to 23.6 million by 2030 (Samson, Mundkur and Kakkar, 2012). Furthermore, CEO of the National Heart Institute (IJN) has reported that there is a 5% increase in the number of cases IJN treats every year, with 10,000 cases of cardiology and hypertension and 4,000 cases of heart surgery. Previous study also has show that coronary artery disease (CAD) is one of the major cause of mortality and morbidity in Malaysia (Seong *et al.*, 2016).

Arterial wall can increasing its external diameter to assist the plaque development without narrowing of the lumen in response to plaque growth thus explaining why atherosclerosis is asymptomatic until the last stage where the artery is totally clot and blocked (thrombosis) (Stocker and Keaney, 2004; Tedgui *et al.*, 2006). Oxidised low density lipoprotein (oxLDL) and recruitment of leukocytes play a pivotal role in initiation of atherosclerosis development (Libby, Ridker and Maseri, 2002; Milioti *et al.*, 2008; Samson, Mundkur and Kakkar, 2012; Pawlak, Mysliwiec and Pawlak, 2013). Modifications of low-density lipoproteins (LDL) *via* oxidation process result in modification of their structure and function that can activate both innate and adaptive immune system (Nagy *et al.*, 1998; Kearney, 2000; Chawla *et al.*, 2001; Stocker and Keaney, 2004; Itabe, 2009). Atherosclerosis also can caused by other risk factors including hypertension, smoking, diabetes mellitus, genetic predisposition, and obesity (Willeit *et al.*, 2000; Hadi, Carr and Al Suwaidi, 2005; Homem de Bittencourt *et al.*, 2007; Insull, 2009; Azhar, 2010). Cigarette smoking and diabetes also can affect vascular biology but unfortunately, the mechanisms is less well understood (Libby, Ridker and Hansson, 2011).

1.2 Lipoprotein

Lipoproteins are particles that compose of hydrophobic core containing phospholipid, antioxidants, cholesterol ester, hydrophilic coat that contains free cholesterol (FC), vitamins, apolipoprotein molecules and phospholipid (Ginsberg, 1998; Hegele, 2009). Insolubility of cholesterol and triglycerol in plasma restrict their movement from the liver to the bloodstream. Therefore, liver will undergo some modification by wrapping the lipid with certain protein namely apolipoprotein to form a new molecule called lipoprotein which act as a carrier protein and it also acts

as a transport vehicles for the lipid in the blood circulation (Ginsberg, 1998; Kumar *et al.*, 2011). Most of the lipoprotein particles have a common structure in which they will form a micellar complexes with a hydrophobic lipid core and an amphipathic surface monolayer (Ginsberg, 1998; Hegele, 2009; Kumar *et al.*, 2011).

The cholesterol and triglyceride are most important plasma lipids (Brown *et al.,* 1986; Hegele 2009). Cholesterol has numerous roles such as to trigger the activation of neuron signalling molecules, component of cell membranes, the precursor for steroid hormones, vitamin D, oxysterols and bile acids (Crockett, 1998; Hegele, 2009). On the other hand, triglyceride synthesised in intestinal and liver cells, transported through the plasma and undergoes lipolysis in the endothelial cell surface (Brown *et al.,* 1986; Hegele 2009). Triglycerides are mainly building up by FFSs and linked by ester bond to a glycerol backbone and it is play an important role in production of energy (Ginsberg, 1998; Hegele, 2009).

1.2.1 Lipoprotein physiology

Lipoprotein can be divided into different types such as chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Each lipoprotein types (CM, VLDL, LDL and HDL) are distinguished based on their size, electrophoretic mobility, density, function and composition (Ginsberg, 1998; Hegele, 2009). Affinities of the apolipoproteins in the surface components of the lipoprotein will alter and it often diffuse from one lipoprotein and bind to another during lipoprotein metabolism (Itabe, 2009; Yoshida and Kisugi, 2010). Lipoprotein metabolism as shown in Figure 1.1 is a complex

network of assembly, processing, secretion and catabolism (Rader and Daugherty, 2008; Hegele, 2009).

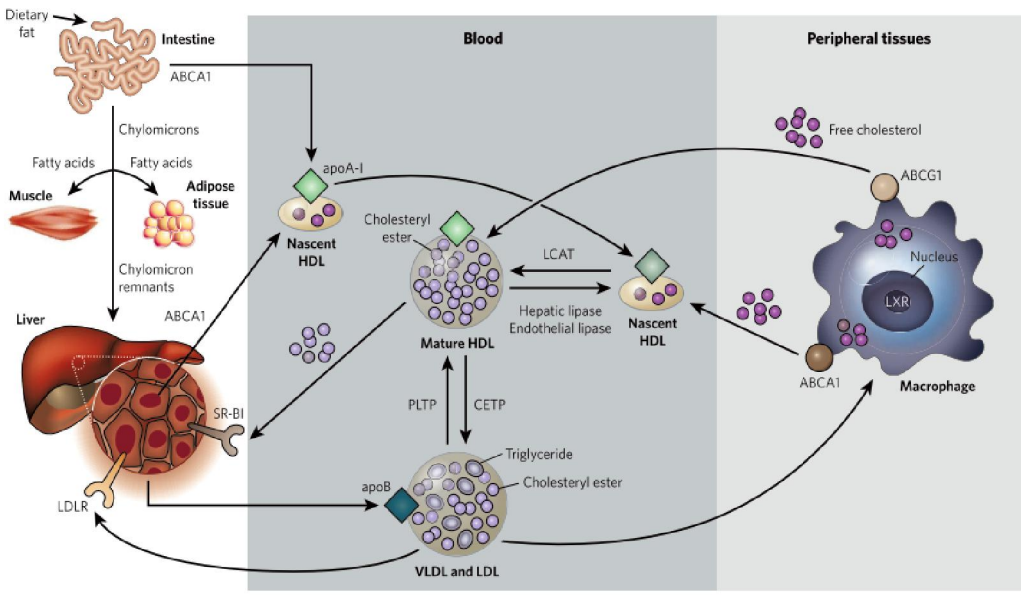

Figure 1.1: Lipoprotein metabolism. Lipoprotein metabolism plays a key role in atherogenesis. It involves the transportation of lipids, particularly cholesterol and triglycerides, in the blood circulation. The intestine absorbs dietary fat and packages it into CM (large triglyceride-rich lipoproteins), which are transported to peripheral tissues through the blood. In muscle and adipose tissues, the enzyme lipoprotein lipase (LPL) breaks down CM, and fatty acids enter these tissues. The CM remnants are subsequently taken up by the liver. The liver loads lipids onto apoB and secretes VLDLs, which undergo lipolysis by LPL to form LDLs. LDLs are then taken up by the liver through binding to the LDL receptor (LDLR), as well as through other pathways. In contrast, HDLs are generated by the intestine and the liver through the secretion of lipid-free apoA-I. ApoA-I then recruits cholesterol from these organs through the actions of the transporter ABCA1, forming nascent HDLs, and this protects apoA-I from being rapidly degraded in the kidneys. In the peripheral tissues, nascent HDLs promote the efflux of cholesterol from tissues, including from macrophages, through the actions of ABCA1. Mature HDLs also promote this efflux by the actions of ABCG1. The free (unesterified) cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme LCAT, creating mature HDLs. The cholesterol in HDLs is returned to the liver both directly, through uptake by the receptor SR- BI, and indirectly, by transfer to LDLs and VLDLs *via* the CETP. The lipid content of HDLs is altered by the enzymes hepatic lipase and endothelial lipase and by the transfer proteins CETP and phospholipid transfer protein (PLTP), affecting HDL catabolism (Rader and Daugherty, 2008). (Adapted from Rader & Daugherty, 2008)

1.2.2 Chylomicrons (CM)

CM is lipoproteins that transports dietary lipids from the intestinal cells to another tissue (Ginsberg, 1998; Desvergne and Wahli, 1999; Zingg, Ricciarelli and Azzi, 2000; Jain *et al.*, 2007). Nascent CM is wrapped with a few types of apolipoprotein such as apoB-48, apoA-I, apoA-II and apoA- IV (Ginsberg, 1998; Hegele, 2009). In the blood circulation, the nascent CM acquires apoE and apoC from plasma HDL in exchange for phospholipids (Ginsberg, 1998; Hegele, 2009). Triglycerols to be hydrolyzed into fatty acids when CM bind to membrane bound lipoprotein lipases (LPL) located in adipose and muscle tissues (Ginsberg, 1998; Lusis, Fogelman and Fonarow, 2004; Jain *et al.*, 2007; Hegele, 2009). Fatty acids are resynthesised once again into triglycerols and stored once it is transported into the adipose cell while in the muscle, fatty acids are oxidised to provide energy (Desvergne and Wahli, 1999; Jain *et al.*, 2007).

1.2.3 Very Low Density Lipoproteins (VLDL)

VLDL is synthesised in the liver and made up of fatty acids and cholesterol (Ginsberg, 1998; Lusis and Pajukanta, 2008). Liver secretes VLDLs *via* exocytosis and primary apolipoprotein of VLDL is apoB-100 (Ory, 2004; Hegele, 2009; Itabe, 2009; Libby, Ridker and Hansson, 2011; Paik *et al.*, 2015). Similar to CM, VLDL undergoes changes in the plasma in which the nascent VLDL acquires apoC and apoE from HDL (Ginsberg, 1998; Ory, 2004; Annema, Eckardstein and Kovanen, 2015). Triglycerols are hydrolyzed into fatty acids when VLDL binds to the LPL in the adipose and muscle tissues (Ory, 2004; Hegele, 2009; Itabe, 2009).

In the muscle, fatty acids oxidation provides energy and transported into the adipose cell, then resynthesised once again into triglycerols and stored (Savage, 2005). Tissues absorb the fatty acids and monoglycerols, causing VLDL to shrink, formed IDLs and transfers its phospholipids and converts apoC to HDL (Ginsberg, 1998; Ory, 2004; Annema, Eckardstein and Kovanen, 2015). IDLs can be further catabolised by LPL in which they will lose apoE to form LDLs when it bind to liver cells receptors (Ginsberg, 1998; Annema, Eckardstein and Kovanen, 2015).

1.2.4 Low Density Lipoproteins (LDL)

LDL is a spherical cholesterol rich lipoprotein which contains mostly apoB-100 and play a critical role as a plasma cholesterol carrier in the blood (Mertens and Holvoet, 2001; Colpo, 2005; Itabe, 2009; Kumar *et al.*, 2011; Pawlak, Mysliwiec and Pawlak, 2013). LDL content consists of cholesteryl esters that form hydrophobic core and triglycerides that are surrounded by phospholipid and cholesterol monolayer with a single molecule of apoB-100 as shown in Figure 1.2 (Mertens and Holvoet, 2001; Milioti *et al.*, 2008; Kumar *et al.*, 2011).

In LDL surface's phospholipids and FC are aligned, while triglycerides and cholesteryl ester are packed in the central core of the LDL particle (Milioti *et al.*, 2008; Itabe, 2009). LDL vary in both diameter (18–25 nm) and density (1.019–1.063 g/ml) (Milioti *et al.*, 2008; Kumar *et al.*, 2011). LDL is known as bad cholesterol and it concentration positively correlates with the coronary heart disease (Ginsberg, 1998; Samson, Mundkur and Kakkar, 2012; Annema, Eckardstein and Kovanen, 2015). Oxidation of LDL is one of the early event in atherosclerosis since oxidized

LDL can contributes to atherogenesis (Mertens and Holvoet, 2001; Itabe, 2009; Yoshida and Kisugi, 2010).

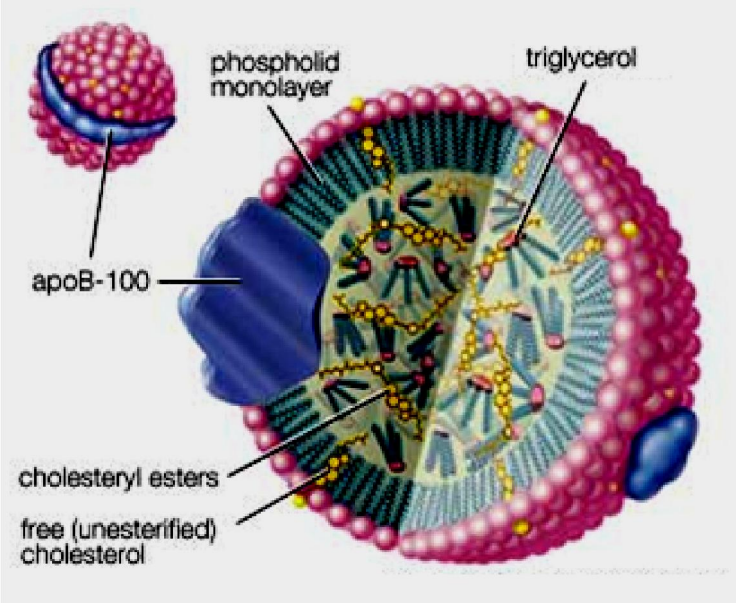

Figure 1.2: Schematic diagram of the low-density-lipoprotein (LDL). LDL acts as transporter of unesterified cholesterol, cholesterol ester, and triglycerides in the blood (Milioti *et al*. 2008). The size of LDL particle is approxiamately 21-24 nm that composed of cholesterol ester and triglycerides. Commonly, the outer layer of LDL contains of phospholipids and unesterified cholesterol in which a single protein called apoB-100 is embedded (Milioti *et al.*, 2008). (Modified from Milioti *et al*. 2008 and https://particulatefouling.wikispaces.com/Low+Density+Lipoprotein).

1.2.5 High Density Lipoproteins (HDL)

HDL is secreted by liver and intestinal cells in disk shaped but they become spherical when acquiring FC from cell membrane and triglycerol from other lipoproteins (Eckardstein, Nofer and Assmann, 2001; Lusis and Pajukanta, 2008; Rader and Daugherty, 2008). It always referred as good cholesterol because of the abilities to remove excess cholesterol (also known as reverse cholesterol transport) and metabolised into bile salts in the liver (Eckardstein, Nofer and Assmann, 2001; Oram, 2003; Rader and Daugherty, 2008; Libby, Ridker and Hansson, 2011; Rosenson *et al.*, 2012). HDL is actually the transporter of plasma cholesterol back to

the liver since it contains Lechithin-cholesterol transferase (LCAT) enzymes that either esterify cholesterol or transfer cholesteryl esters (Eckardstein, Nofer and Assmann, 2001; Rader and Daugherty, 2008; Rosenson *et al.*, 2012). LCAT catalyses the transfer of phospholipid's long chain fatty acids to cholesterol and form cholesteryl ester (Eckardstein, Nofer and Assmann, 2001; Rader and Daugherty, 2008; Rosenson *et al.*, 2012). Exchanged process between cholesteryl esters and lipoproteins is mediated by cholesteryl ester transfer protein (CETP) that promotes transferring process of cholesterol esters from HDL to LDL, IDL and VLDL in exchange triglycerols and this process transforms VLDLs and IDLs into LDLs (Eckardstein, Nofer and Assmann, 2001; Ory, 2004; Jain *et al.*, 2007; Rader and Daugherty, 2008). HDL will acquire apoE which increases their binding affinity towards receptors in the liver and absorbed and catabolised by the liver as it grow in size (Eckardstein, Nofer and Assmann, 2001; Ory, 2004; Jain *et al.*, 2007).

1.2.6 Oxidised Low Density Lipoprotein (oxLDL)

In the subendothelial space, LDL will undergo oxidation process and become the main targets for the engagement of the LDL by scavenger receptor expressed by macrophages (Zingg, Ricciarelli and Azzi, 2000; Milioti *et al.*, 2008; Yoshida and Kisugi, 2010; Ayyappan, Paul and Goo, 2016). OxLDL acts as chemoattractant substances for monocytes and stimulate inflammation in the arterial wall involving all of the atherogenesis phases (Itabe, 2009; Melo *et al.*, 2011; Samson, Mundkur and Kakkar, 2012; Pawlak, Mysliwiec and Pawlak, 2013). OxLDL is composed of heterogeneous modified lipoprotein particles and cause difficulty in analyzing its structure (Itabe, 2009; Pawlak, Mysliwiec and Pawlak, 2013). Furthermore, oxLDL will not be degraded or processed for antigen presentation like other antigens but it

will internalize by the macrophage that will lead to the formation of the foam cell (Hansson, 2001).

Commonly, LDL particle contains a single molecule of apoB as a major apolipoprotein, apoE, paraoxonase, and platelet-activating factor-acetylhydrolase (PAF-AH; also known as Lp-PLA2) (Mertens and Holvoet, 2001; Itabe, 2009; Kumar *et al.*, 2011). Lipid molecules containing polyunsaturated fatty acids (PUFA) in LDL are easily oxidized under oxidative stress conditions and variety of lipid oxidation products is formed and this process subsequently covalently modified apoB protein as well (Itabe, 2009). Meanwhile, copper sulphate ($CuSO_4$) treatment used to convert LDL into oxLDL *in vitro* (Itabe, 2009; Yoshida and Kisugi, 2010).

1.3 Atherosclerosis development (atherogenesis)

1.3.1 Structure of arterial wall

Arterial wall can be divided into three layers and it composed of tunica intimal, tunica media, and adventitia layer (Lusis, 2000; Holzapfel *et al.*, 2005; Shirai *et al.*, 2006; Libby, Ridker and Hansson, 2011; Chung and Vafai, 2013) (Figure 1.4a). Inner layer or tunica intima, is lined by EC monolayer and elastic fibres sheet, extracellular connective tissue matrix (proteoglycans and collagen) the internal elastic lamina, on the peripheral side that has a contact with blood overlying a basement membrane (Lusis, 2000; Libby, Ridker and Hansson, 2011). During atherogenesis, monocytes will proliferate and migrate into the intima where they were further differentiated into macrophages and engulf the lipoproteins, forming foam cells (Smith *et al.*, 1995; Newby and Zaltsman, 1999; Lusis, 2000; Milioti *et al.*, 2008). The middle layer, or tunica media, contains mostly smooth muscle cells

(SMC) embedded in a complex extracellular matrix (Lusis, 2000; Libby, Ridker and Hansson, 2011; Frostegård, 2013). SMCs migrate from the media into the intima during atherogenesis, and proliferate in response to mediators such as platelet-derived growth factor (Libby, Ridker and Hansson, 2011; Nagy *et al.*, 2012). Outer layer of arteries called tunica adventitia, contains mast cells, fibroblasts, dendritic cells, monocytes/macrophages, T-cells, small vessels (also known as vasa vasorum that provide an important source of nutrition to arteries and veins), endothelial progenitor cells nerve endings and microvessels (Libby, Ridker and Hansson, 2011; Frostegård, 2013). Each layer of artery have a specific histological features and mechanical functions as shown in Figure 1.4a (Holzapfel *et al.*, 2005; Libby, Ridker and Hansson, 2011).

1.3.2 Fatty streaks formation

In the normal condition LDL acts as a plasma cholesterol carrier in the blood but after undergo oxidization process, it will initiate the atherogenesis (Stocker and Keaney, 2004; Boullier *et al.*, 2005; Corcoran *et al.*, 2011; Kumar *et al.*, 2011). As shown in Figure 1.4b, 1.4c and 1.4d, atherogenesis is initiated by lipid retention, oxidation, and modification that lead to the chronic inflammation, leading to formation and development of thrombosis or stenosis (Witztum and Steinberg, 1991; Chen *et al.*, 1997; Koopman, Schaart and Hesselink, 2001; Stocker and Keaney, 2004; Milioti *et al.*, 2008; Insull, 2009; Itabe, 2009; Samson, Mundkur and Kakkar, 2012). Normally, endothelium does not support binding of the white blood cells but during early initiation of an atherogenesis, oxLDL stimulates arterial EC to express selective adhesion molecules on their surface such as VCAM-1, ICAM-1 that bind to various receptor expressed on the leukocytes surface as shown in Figure 1.3 and 1.4b

(Lusis, 2000; Libby, Ridker and Maseri, 2002; Rader and Daugherty, 2008; Libby, Ridker and Hansson, 2011; Samson, Mundkur and Kakkar, 2012). In addition, monocyte chemoattractant protein-1 (MCP-1) is the main factor that is responsible to trigger the migration of monocytes into the intima (Van Lenten *et al.*, 1995; Lusis, 2000; Libby, Ridker and Maseri, 2002; Samson, Mundkur and Kakkar, 2012; Ayyappan, Paul and Goo, 2016). Inside the intima, macrophage colony-stimulating factor (M-CSF) is the growth factor that contributes to the differentiation of the blood monocyte into the macrophage as shown in Figure 1.3 and 1.4b (Smith *et al.*, 1995; Qiao *et al.*, 1997; Libby, Ridker and Maseri, 2002; Rader and Daugherty, 2008; Libby, Ridker and Hansson, 2011).

In atherosclerotic arteries, macrophages involved transformed into foam cells through a process that regulated by the balance process between modified LDL uptake and cholesterol efflux (Ley, Miller and Hedrick, 2011; de Gaetano *et al.*, 2015). Formation of foam cells as shown in Figure 1.3 are comprising complex and multistep mechanisms that depend on different signalling pathways (Collot-Teixeira *et al.*, 2007; Rader and Daugherty, 2008; Melo *et al.*, 2011).

Macrophages express scavenger receptors such as SRA-1 and CD36 that can recognise and internalise oxLDL (Mertens and Holvoet, 2001; Akiyama *et al.*, 2002; Collot-Teixeira *et al.*, 2007; Corcoran *et al.*, 2011; Ley, Miller and Hedrick, 2011). The engagement of oxLDL by macrophages also will release various pro inflammatory mediators resulting in a chronic inflammatory reaction as shown in Figure 1.4b (Libby, Ridker and Hansson, 2011). Previous studies have showed various strong evidences that linked atherosclerosis with the immune response alteration and chronic inflammation (Insull, 2009; Kapoor *et al.*, 2012; Samson, Mundkur and Kakkar, 2012; Matsuura *et al.*, 2014). In order to maintain the

homeostasis, macrophages try to clean up cholesterol that has been deposited in the arteries (de Gaetano *et al.*, 2015). However, the accumulation of cholesterol-loaded macrophages namely foam cells will form lipid streaks in the arteries which then trigger various inflammatory responses as shown in Figure 1.4b (Nagy *et al.*, 1998; Ricote *et al.*, 1999; Ley, Miller and Hedrick, 2011; Libby, Ridker and Hansson, 2011; Samson, Mundkur and Kakkar, 2012; de Gaetano *et al.*, 2015). Fatty streak can be considered as a precursor of advanced lesions that can be distinguish by the accumulation of lipid-rich necrotic debris, lymphocytes and SMCs (Lusis, 2000; Tedgui *et al.*, 2006).

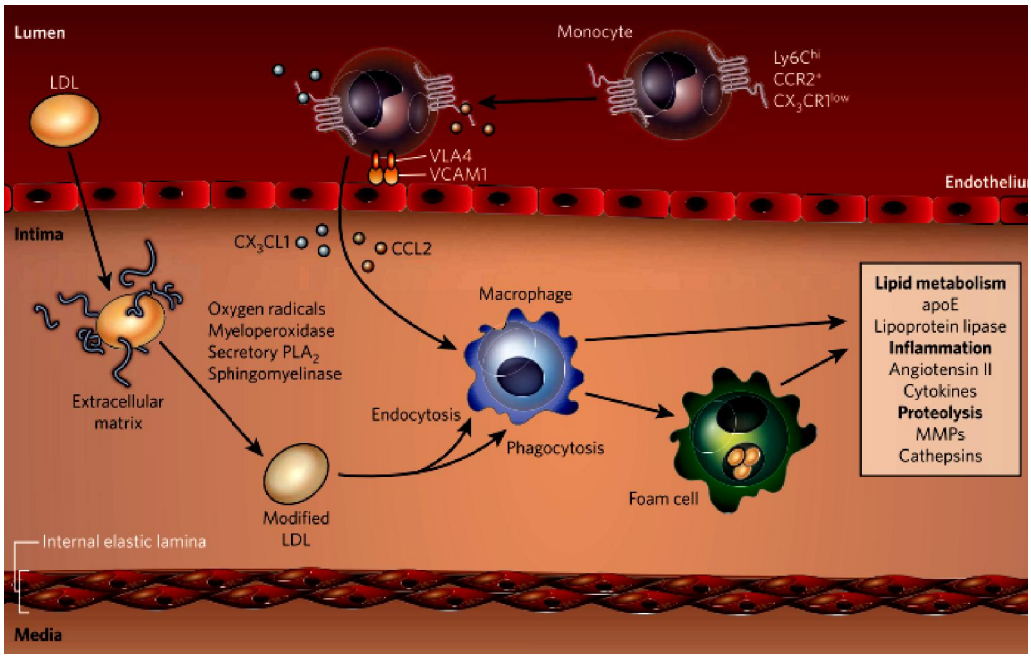

Figure 1.3: Recruitment of monocytes and formation of foam cells. LDL in the blood enters the intima, where they are retained through binding to the extracellular matrix. LDLs are then modified by oxygen radicals, myeloperoxidase, secretory phospholipase A2 and sphingomyelinase. This results in the generation of pro-inflammatory biologically active lipids that initiate and maintain an active inflammatory process in the intima (not shown). This inflammation process will generate chemokines such as CX3CL1 and CCL2, which recruit monocytes to the intima. These monocytes then differentiate into macrophages under influence of MCF, which take up modified LDL through endocytosis or phagocytosis and become foam cells (lipid laden macrophages). Macrophages also secrete various factors involved in propagating the atherosclerotic plaque, including factors involved in lipid metabolism, inflammation and proteolysis (Rader and Daugherty, 2008). (Adapted from Rader & Daugherty. 2008).

1.3.3 Formation of the fibrous cap (early fibro-atheroma or complex lesions)

Fibrous cap formation can either progress to a complex atheroma or regress to a simpler plaque and its formation can remain asymptomatic (Tabas, 1997; Libby, Ridker and Maseri, 2002; Rader and Daugherty, 2008; Ellertsen, Petri and Bäck, 2011; Frostegård, 2013). This stage initiated by the migration and proliferation of SMC into the intima or sub-endothelial space to produce a distinct fibrous cap (Newby and Zaltsman, 1999; Libby, Ridker and Maseri, 2002; Libby, Ridker and Hansson, 2011). Once the lesion reaches this stage, there are two possibilities; either the fibrous cap remains intact which stabilise the plaque, or plaque development continues and becomes more vulnerable and prone to rupture as shown in Figure 1.4c (Libby, Ridker and Maseri, 2002; Insull, 2009; Libby, Ridker and Hansson, 2011).

1.3.4 Thrombosis

Plaque erosions or rupture and thrombosis are depends on the composition and plaque weakness caused by chronic inflammatory condition that can be the most critical clinical aspect of atherosclerosis as shown in Figure 1.4d (Tabas, 1997; Lusis, 2000; Collot-Teixeira *et al.*, 2007; Libby, Ridker and Hansson, 2011). Thrombosis is the process that responsible for clinically observable events affecting peripheral vascular, coronary and cerebrovascular (Tedgui *et al.*, 2006). Susceptibility to atherothrombosis differs not only in individual susceptibility but also in arterial susceptibility (Falk, 2006).

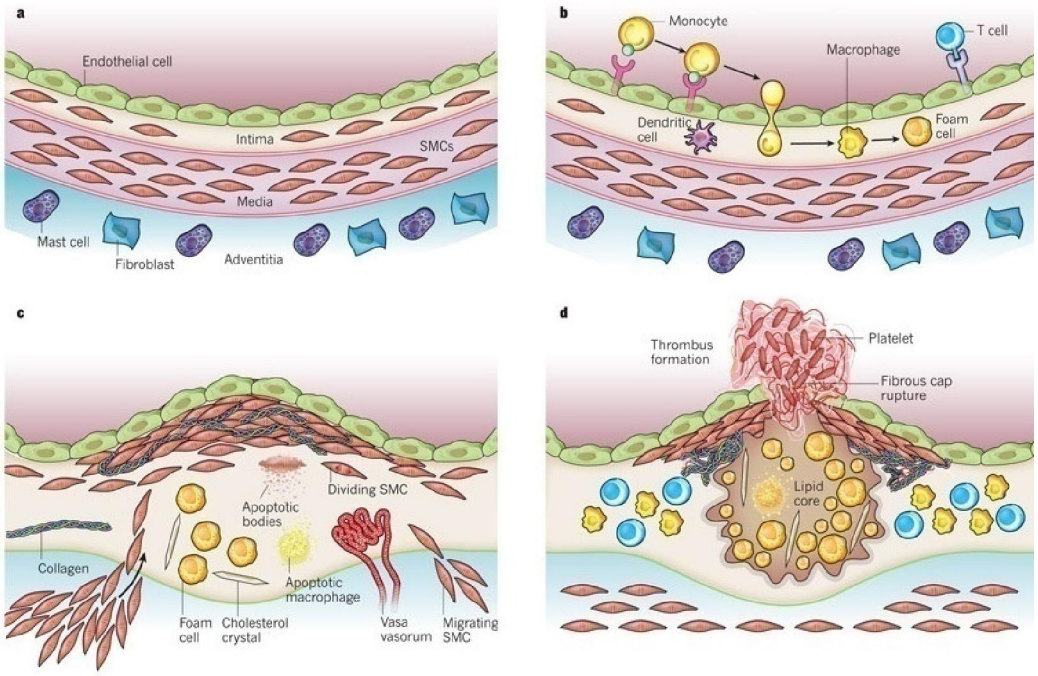

Figure 1.4: Stages in the development of atherosclerotic lesions. a) The normal artery contains three layers namely tunica adventitia, tunica media and tunica intima b) The atherogenesis initiated by the adhesion of blood leukocytes to the activated endothelial monolayer, facilitated the migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid which convert the lipid laden macrophages into foam cells. c) Lesion progression involves the migration of SMC from the media to the intima, the proliferation of SMC and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. d) Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. The burst of the plaque's fibrous cap will cause the blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus to extend into the vessel lumen that can block blood circulation (Libby, Ridker and Hansson, 2011). (Adapted from Libby *et al*. 2011).

1.4 Role of infectious agents in atherosclerosis

There are several studies showed that the infection of bacteria and viruses are the factor that can contribute to the pathogenesis of atherosclerosis either directly by infection of vascular cells or indirectly by the immune system effects (Cainelli, Concia and Vento, 2000; Willeit *et al.*, 2000; Prediman K. Shah, 2001; Madjid *et al.*, 2003; Reeves *et al.*, 2004; Paik *et al.*, 2011, 2015; Rosenfeld and Campbell, 2011;

Dushkin, 2012; Tall and Yvan-charvet, 2015; Hsu *et al.*, 2016). The findings were supported by *in vivo* studies that revealed an acceleration of the atherogenesis by infection (Reeves *et al.*, 2004; Rosenfeld and Campbell, 2011; Dushkin, 2012; Paik *et al.*, 2015). Infectious agent that has been reported as an accelerator for atherosclerosis are including *C. pneumoniae, Porphyromonasgingi- valis (P. gingivalis), Helicobacter pylori (H. pylori),* influenza A virus, hepatitis C virus (HCV), cytomegalovirus (CMV), murine norovirus (MNV) and human immunodeficiency virus (HIV) (Prediman K. Shah, 2001; Madjid *et al.*, 2003; Reeves *et al.*, 2004; Paik *et al.*, 2011, 2015; Rosenfeld and Campbell, 2011; Dushkin, 2012; Tall and Yvan-charvet, 2015; Hsu *et al.*, 2016). Infectious organisms have shown an ability to involve in the inflammation within the blood vessel either by directly infecting vascular cells or innate immune response activation (Rosenfeld and Campbell, 2011). Currently, there is a concept stated that not only one infectious agent is responsible for the atherogenesis but it caused by the aggregate effects of multiple infectious agents known as the ″infectious burden″ or ″pathogen burden″ (Prediman K. Shah, 2001; Rosenfeld and Campbell, 2011; Frostegård, 2013). Recently, murine norovirus-4 (MNV-4) infection has been shown to accelerate the atherosclerotic plaque development in mouse model of atherosclerosis (Paik *et al.*, 2011, 2015). The subsequent sections described about the MNV and their involvement in atherosclerosis development.

1.5 Norovirus

Norovirus that is earlier known as “Norwalk-like viruses” or “small round structured viruses,” is a non-enveloped viruses that have a positive-sense, linear and single-stranded RNA genome from caliciviridae family (Green *et al*. 2001; Wobus *et al*.

2004; Waugh *et al*. 2014). Human noroviruses are one of the major factor of nonbacterial, epidemic gastroenteritis that typically lasting for 24 to 48 hours all over the world and most of the cases have been reported to occur in schools, nursing homes, hospitals, restaurants, and cruise ships (Hsu *et al.*, 2005; Hwang *et al.*, 2015; Paik *et al.*, 2015). Norovirus infection caused gastroenteritis that develop symptom such as vomiting, diarrhea, low-grade fever, malaise, and abdominal cramping or pain (Wobus, Thackray and Virgin, 2006; Hsu, Riley and Livingston, 2007; Thackray *et al.*, 2007; Kahan *et al.*, 2011; Strong *et al.*, 2012; Waugh *et al.*, 2014; Niendorf *et al.*, 2016). It responsible for more than 90% of all epidemic nonbacterial gastroenteritis outbreaks (Subba-Reddy *et al.*, 2012) and causing about 23 million infections per year in the United States alone (Thackray *et al.*, 2007). Previously, MNV is the only norovirus that efficiently grows in tissue culture (Gonzalez-Hernandez, Bragazzi Cunha and Wobus, 2012; Paik *et al.*, 2015; Hsu *et al.*, 2016). Due to inability of human norovirus to replicate in cell culture or small animal models, it has limited the research in order to have further understanding of the interaction between this virus and their hosts (Hardy, 2005; McFadden *et al.*, 2011; Arias *et al.*, 2012; Thorne, Bailey and Goodfellow, 2012; Waugh *et al.*, 2014; Hwang *et al.*, 2015).

1.5.1 Murine norovirus (MNV)

There are four types of MNV namely MNV-1, MNV-2, MNV-3, and MNV-4 (Wobus, Thackray and Virgin, 2006). MNV-1 caused transient and short duration of infection while MNV-2, MNV-3 and MNV-4 can cause persistent and prolonged fecal shedding in infected immunocompetent mice infection (Wobus, Thackray and Virgin, 2006; Hsu, Riley and Livingston, 2007; Thackray *et al.*, 2007). Genetically,

MNV is the most similar to HuNV and has been widely utilised as a surrogate to study norovirus in the cell culture system. Since first norovirus (MNV-1) was described in 2003, MNV model system has provides the first opportunity to understand the relationship between basic mechanisms and pathogenesis of norovirus in tissue culture and in the natural host (Wobus, Thackray and Virgin, 2006). The identification of the first MNV strain (MNV-1) propagation in the murine macrophage cell line RAW264.7 (Thackray *et al.*, 2007) that originally isolated from the brain of severely immunocompromised mice by serial passage (Wobus, Thackray and Virgin, 2006). Discovery of the MNV-1 enables investigation of the molecular mechanisms that promote norovirus evolution and development of diagnostic assays (including immunohistochemistry) to detect viral antigens, serologic assays for detection of anti-MNV anti- bodies, and molecular assays to detect viral RNA (Paik *et al.*, 2015; Hsu *et al.*, 2016). MNV is one of the virus from the Caliciviridae family that have single-stranded and positive-sense RNA where it infect mice and shown tropism toward antigen-presenting cells such as dendritic cells, macrophages and more recently, has been reported to infect B cells (Paik *et al.*, 2015; Hsu *et al.*, 2016).

MNV genome consists of four open reading frames (ORFs) which are known as ORF1 that encodes a large polyprotein which is cleaved to produce the viral non-structural proteins required for viral genome replication, (NS1-7); ORF2 that encodes the major capsid protein VP1; ORF3 that encodes a minor structural protein VP2 whereas ORF4 encodes for virulence factor 1 (VF1) protein as shown in Figure 1.5 (McFadden *et al.*, 2011; Arias *et al.*, 2012).

Figure 1.5: Illustration of MNV genome organisation. Each protein coding region is illustrated as a single white box. ORF1 is translated into 7 different non-structural proteins (NS1/2 to NS7) that are released from precursor polyprotein after self-proteolytic processing. ORF 2 encodes the major capsid protein VP1, ORF 3 encodes the minor capsid protein VP2, and ORF4 overlapping with ORF2 coding region encodes virulence factor VF1. Genomic and subgenomic RNAs contain a polyA tail at their 3' ends of variable length (Arias *et al.*, 2012). (Modified from Arias *et al*. 2012).

1.5.2 The association of MNV infection with atherogenesis

In the previous study, MNV-4 infection has been shown to carry a potential in suppressing the ABCA1 transporter and increasing of CD36 expression (Paik *et al.*, 2015; Hsu *et al.*, 2016). Possibility of infections in contributing to the development of atherosclerosis was suggested as early as 1800s and 1900s (Prediman K. Shah, 2001). From the study that has been done by Paik *et al*, they have suggest that MNV4 infection may influences cellular cholesterol levels in macrophages by regulating uptake of oxLDL *via* CD36 and cholesterol efflux (ABCA1) pathways in bone marrow derived macrophages. Study that has been conducted also show that MNV infection did not influence glucose metabolism and weight gain, but it increased the size and macrophage content of aortic sinus lesions in Ldlr−/−mice fed an atherogenic diet. On the other hand, they also have suggest that MNV-4 infection have an effect to increases the oxLDL uptake capacity of macrophages since there is an increasing for CD36 protein expression (Paik *et al.*, 2015). While, study that has been conducted by Hsu *et al*. has reported that there are variable influence of MNV infection on the development of atherosclerosis (Hsu *et al.*, 2016).

1.6 Peroxisome Proliferator Activated Receptor (PPAR)

Peroxisome proliferator activated receptors (PPAR) are transcription factors that is ligand-activated from a subfamily of the nuclear receptor gene family (Issemann and Green, 1990; Nagy *et al.*, 1998; Azhar, 2010; Libby, Ridker and Hansson, 2011). PPARs exist in three sub-types namely PPARα, PPARδ/β, and PPARγ that is result from differential splicing (Brun *et al*. 1996; Ray *et al*. 2006; Tyagi *et al*. 2011; Norazmi *et al*. 2012). PPARα (NR1C1) is expressed in tissues with high fatty acid (FA) oxidation, such as heart, kidney, brown adipose tissue (BAT), and liver, where it regulates FA transport, esterification and oxidation *via* transcriptional activation of genes encoding enzymes involved in each step of FA breakdown. PPARγ (NR1C3) is predominantly express in adipose tissue and plays ban essential role in adipocyte differentiation and survival (Desvergne and Wahli, 1999; Evans *et al.*, 2004; Metzger *et al.*, 2005). While PPARβ/δ (NR1C2) is expressed ubiquitously but abundantly detected in skeletal muscle (Escher *et al.*, 2001).

1.6.1 Peroxisome Proliferator Activated Receptor (PPAR) structure

All of the three PPAR isoforms (PPARα, PPARβ/δ, and PPARγ) have five or six structural regions within four functional domains that are A/B, C, D and E/F as shown in Figure 1.6 (Nosjean and Boutin, 2002; Blanquart *et al.*, 2003; Azhar, 2010; Wang, 2010; Grygiel-Górniak, 2014). N-terminal, A/B domain or ligand-independent transactivation domain contains activation function (AF)-1 which is a target of phosphorylation process by kinases C domain of PPARs have a 70 amino acid-long PPAR DNA-binding domain consists of two highly conserved zinc finger motifs (Desvergne and Wahli, 1999; Guan and Breyer, 2001; Azhar, 2010). They stimulate the binding of receptor to a DNA sequence in the promoter region of target

genes known as the peroxisome proliferator response element (PPRE) (Zhang and Young, 2002; Argmann *et al.*, 2003; Khoo *et al.*, 2003; Grygiel-Górniak, 2014). The D domain or hinge region of PPAR acts as a base for cofactors binding (Auwerx, 1999; Debril *et al.*, 2001; Azhar, 2010). While, C-terminal, E/F domain or ligand-binding domain (LBD) playing a main role for ligand specificity and activation of PPAR that lead to PPRE binding, thus increasing target genes expression (Desvergne and Wahli, 1999; Cunard *et al.*, 2004; Azhar, 2010; Lefebvre, Benomar and Staels, 2010). Ligand-dependent transactivation function 2 (AF-2) is responsible for recruitment of cofactors in order to assist the transactivation process (Desvergne and Wahli, 1999; Cunard *et al.*, 2004; Azhar, 2010; Lefebvre, Benomar and Staels, 2010).

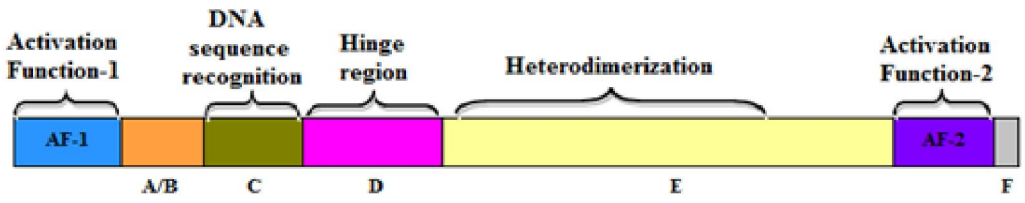

Figure 1.6: Schematic representation of the PPAR structure. PPAR proteins are organized in distinct domains which display specific function. The domain A/B contains the activating function 1 which is independent of the presence of ligand, the domain C is implicated in the DNA binding, the domain D is a hinge region and the domain E is implicated in the ligand recognition, contains an activating function 2 which is dependent of the presence of ligand and is necessary for the heterodimerization with RXR. Concerning the domain F, no function has been identified to date (Blanquart *et al.*, 2003). (Modified from Blanquart *et al.* 2003).

1.6.2 Peroxisome Proliferator Activated Receptor-γ (PPARγ)

PPARγ is a transcription factor that plays a pivotal role in adipogenesis (Kubota *et al.*, 1998; Auwerx, 1999; Debril *et al.*, 2001; Guan and Breyer, 2001; Savage, 2005; Ray, Akbiyik and Phipps, 2006; Wang, 2010; Costa *et al.*, 2010; Nagy *et al.*, 2012; Takenaka *et al.*, 2013; Grygiel-Górniak, 2014; Raman and Koenig, 2014; Annema,