CHAPTER 4

DISCUSSION

The National Cholesterol Education Program (NCEP) established the Adult Treatment Panel III (ATP III) for considering the management of high blood cholesterol and the risk of coronary heart disease [64] (Table 16). It should be mentioned that LDL-C is a primary target of lipid lowering therapy [65]. Accumulating evidence suggests that high level of LDL-C is a risk factor for CHD. However, some people with low or normal LDL-C levels still have increased risk of CHD. Therefore, LDL-C levels are not always a foolproof risk marker for CHD. To overcome this problem, a novel indicator has been developed.

Recently, there has been increased interest in sdLDL. Studies have suggested a relationship between the risk of CHD and increased levels of sdLDL in patients independent of variation in plasma lipid concentrations including LDL-C levels [66]. The sdLDL is expected to be a novel marker for CHD according to its high atherogenicity. However, the current methods used for measurement of sdLDL suffer from several limitations, including being technically demanding, time-consuming, high in cost and not feasible in a routine clinical laboratory. In this study, a proteomics analysis was conducted to investigate protein profiles of sdLDL and bdLDL. These findings may be applied to develop novel methods for measurement of sdLDL and may explain why sdLDL is more atherogenic than bdLDL.

Table 16 ATP III guideline for cholesterol management on CHD [64]

LDL cholesterol- Primary target of therapy	
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high
Total cholesterol	
<200	Desirable
200-239	Borderline high
≥240	High
HDL cholesterol	
<40	Low
≥60	High

Table 17 Major risk factors exclude LDL-C for coronary heart disease [64]

Cigarette smoking
Hypertension (BP≥140/90 mmHg or antihypertensive medication)
Low HDL cholesterol (<40 mg/dL)*
Family history of premature CHD (CHD in male first degree relative <55 years; CHD in female first degree relative <65 years)
Age (men≥45 years; woman ≥55 years)
*HDL cholesterol ≥60 mg/dL counts as a negative risk factor; its presence removes one risk factor from the total count.

LDL is a lipoprotein particle with a density of 1.019 to 1.063 g/mL. It can be separated into subfractions by density ultracentrifugation. The largest and most buoyant particle (bdLDL) has a density range of approximately 1.019 to 1.039 g/mL. The remainder particle (sdLDL) is denser with a density 1.040 to 1.063 g/mL. In this study, LDL subfractions were prepared by density gradient ultracentrifugation using a method developed by Guerin *et al*. 1996 [(29)]. Blood samples in EDTA-containing tubes were obtained from healthy volunteers without underlying disease that affected the protein profiles. This technique identifies 5 subclasses of LDL including LDL-I (d = 1.019-1.023 kg/L), LDL-II (d= 1.023-1.029 kg/L), LDL-III (d = 1.029-1.039 kg/L), LDL-IV (d = 1.040-1.050 kg/L), LDL-V (d = 1.050-1.063 kg/L). According to Guerin's method, LDL is classified into sdLDL (LDL-IV, V) and bdLDL (LDL-I, II, III). However, the most important drawback with this technique is that it is a time-consuming procedure. It takes up to 44 hours for the completed isolation of all subfractions. This long period of time may lead to overlap between lipoprotein fractions and the risk of rearrangement of proteins from one to another lipoprotein fraction. A weak binding between lipids and apolipoproteins with noncovalent interactions allows proteins to easily be exchanged between the lipoproteins. However, this circumstance is believed to occur *in vivo*. Moreover, aggregation of proteins might occur in dialysis bag. Protein concentrations in each LDL subfraction were determined in order to simply prove the lipoprotein particle. The sizes of lipoprotein particles are inversely correlated with their protein content. The larger lipoprotein particles contain fewer proteins than the smaller particles. Similar results were observed in this study (Table 10).

This study provided the protein profiles of sdLDL and bdLDL by 1 and 2-DE separation subsequently analyzed with LC-MS/MS. Our findings identified the presence of proteins previously identified in LDL, and newly discovered proteins. For 1-DE, several interesting proteins from sdLDL and bdLDL were identified (Table 12).

Apolipoprotein B-100 (apo B-100) is expected to be present in LDL. It is a major component of LDL and acts as a ligand for the LDL receptor. It contains an LDL receptor binding domain which allows the specific binding of LDL to cells through the LDL receptor. Apo B-100 is found in LDL, and to a lesser extent in VLDL and IDL [11, 67]. In this study, apo B-100 was found at the equal level in sdLDL and bdLDL from 1-DE separation. Interestingly, in spite of its ligand activity for LDL receptor, apo B-100 is also involved in LDL affinity for arterial proteoglycans (PGs) which are the major component of intima. LDL binds to PGs of the intima by an interaction between the specific positive arginine (R) and lysine (K) segments of apo B-100 and negative sulfate and carboxyl groups of glycosaminoglycans (GAGs) [68]. Previous studies have indicated that subjects with atherogenic lipoprotein phenotype (ALP), which is characterized by high levels of sdLDL, has greater affinity for PGs. This LDL phenotype is associated with the increased risk of CHD due to its prolonged residence time in the arterial wall [69]. Evidence suggests that sdLDL has higher affinity for PGs than bdLDL because it exposes more segments binding for GAGs. This high affinity for arterial PGs could increase its residence time in the intima and provide an opportunity for LDL modification which would subsequently contribute to atherosclerotic formation.

The binding of apo B-100 and GAGs depends on charge-charge interaction with negatively charged GAGs and positively charged apo B-100 on the LDL surface. It should be noted that size of the lipoprotein particles depends on their proportions of lipids and proteins. The larger lipoprotein particles contain more lipids relative to protein. Thus, bdLDL particles contain less area for apo B-100 on the surface, which may result in the hiding of the GAGs segment. For sdLDL particles, the low content of phospholipid and free cholesterol is associated with the increased exposure of apo B segments. The sdLDL contains more area for apo B-100 and leads to the increase in exposed GAGs binding segments. Studies have indicated that segments of 3147-3157 (SVKAQYKKNKHRKH) and 3359-3367 (RLTRKRGLK) act as the binding segments for GAGs [70]. These two segments are separated by 202 amino acids but may be closed to each other as U-turns linked by disulfide bridges between Cys (3167) and Cys (3269). This changing alters the exposure of LDL to GAGs. The smaller particles have greater affinity for PGs due to the merging of two positive segments into larger positive charges, or more exposure of the arginine and lysine side chains [71].

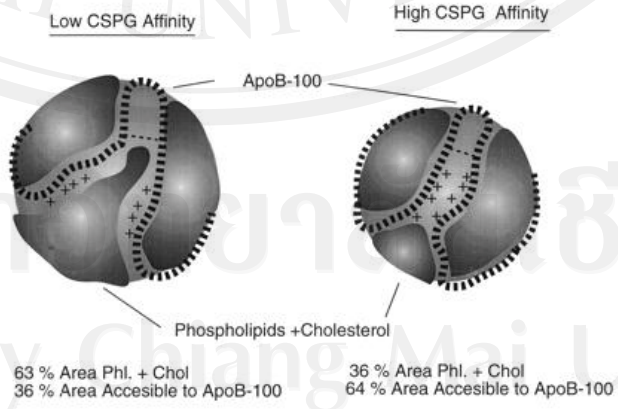

Figure 22 The association between size and content of LDL and the exposure of GAGs binding segments [71]

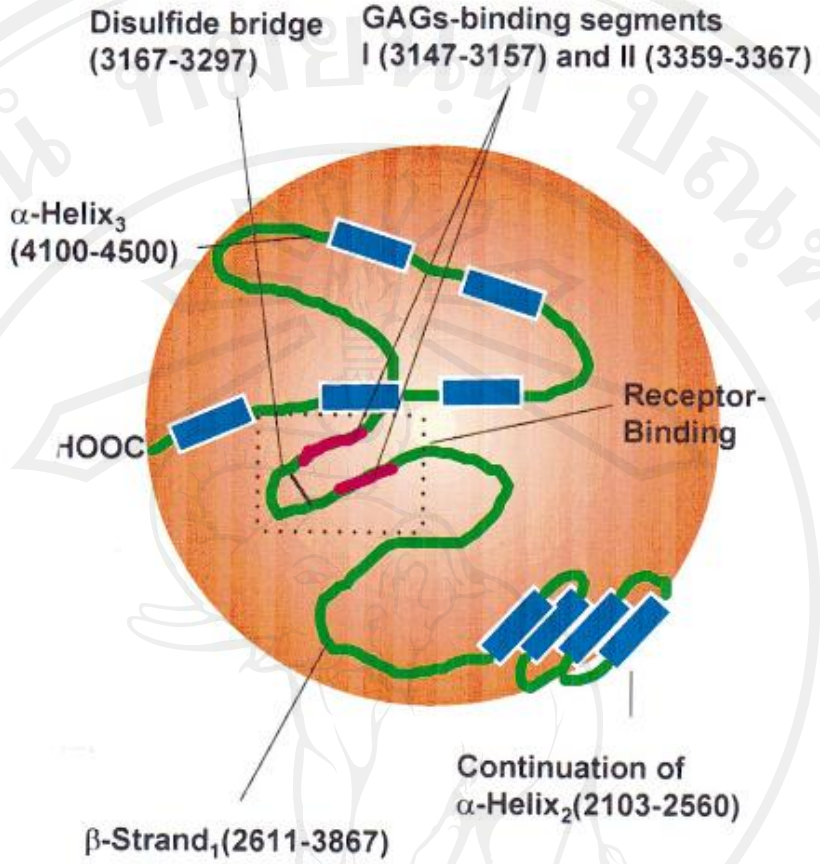

Figure 23 Schematic representation of apo B-100 structure [68]

However, apo B-100 was found only in sdLDL in the 2-DE separation. The absence of apoB-100 in bdLDL might be due to missed spot picking from the gel by visual detection.

In this study, LDL subfractions contained not only apo B-100 but also other lipoproteins such as apo E, apo M, apo L3, apo C-III and LPL. Apo E was another expected apolipoprotein in LDL. Apo E was present in both bdLDL and sdLDL in the 1- and 2-DE separations. It is involved in the clearance of chylomicron remnants from plasma by serving as a ligand for the hepatic (apo E) receptor. It also acts as a

ligand for LDL (apo B/E) receptors. The apo E gene has three alleles, including ε-2, ε-3 and ε-4 which encode three different protein isoforms (apo E-2, apo E-3 and apo E-4). Six genotypes: ε2/2, ε3/3, ε4/4, ε4/2, ε4/3 and ε3/2 have been found. These three protein isoforms have different isoelectric points due to the different amino acid substitution in codons 112 and 158. Arginine and lysine add positive charge to proteins, which provides the different isoelectric points of apo E isoforms. Apo E-4 has two arginine residues in positions 112 and 158. In contrast, apo E-2 has two cysteine residues while apo E-3 has one cysteine and one arginine in positions 112 and 158, respectively [72]. Apo E-3 is the most common isoform and is found in 40-90% of the population. From the 1-DE separation, apo E-3 with Lys146-->Glu mutation and apo E2 with Asp154→Ala mutation were identifed. Moreover, we also identified apo E with pI 5.76 (spot no. 2), 5.98 (spot no. 3), 6.27 (spot no. 4) in sdLDL and apo E with pI 3.12 (spot no. 1), 7.98 (spot no. 2) in bdLDL from the 2-DE separation. Apo E was found in chylomicrons, VLDL, HDL and LDL. Comparative proteomic prolfiling study revealed the equal expression of apo E in VLDL and LDL [67]. In addition, previous proteomics studies have reported similar levels of apo E in sdLDL and bdLDL in healthy controls. However, it has been suggested that sdLDL of patients with metabolic syndrome and type 2 diabetes is decreased in apo E compared with healthy people [9]. The presence of apo ε4 allele results in high LDL-C levels and tends to be a significant risk factor for CHD, whereas apo ε2 appears to be associated with lower LDL-C levels and less atherogenicity. Evidence indicates that apo E influences cholesterol absorption. People with E4 phenotype are associated with an increased ability of cholesterol absorption. In contrast, people with heterozygous or homozygous E2 had lower cholesterol absorption efficiency [73, 74].

Apo Es are recognized by hepatic receptors, except for apo E-2. It has low affinity for hepatic receptors, which results in poor clearance of chylomicron remnants. Apo E 2 is also involved in the reduced changing of VLDL and IDL to LDL and the upregulation of the LDL receptor which subsequently leads to decreased LDL-C levels [75].

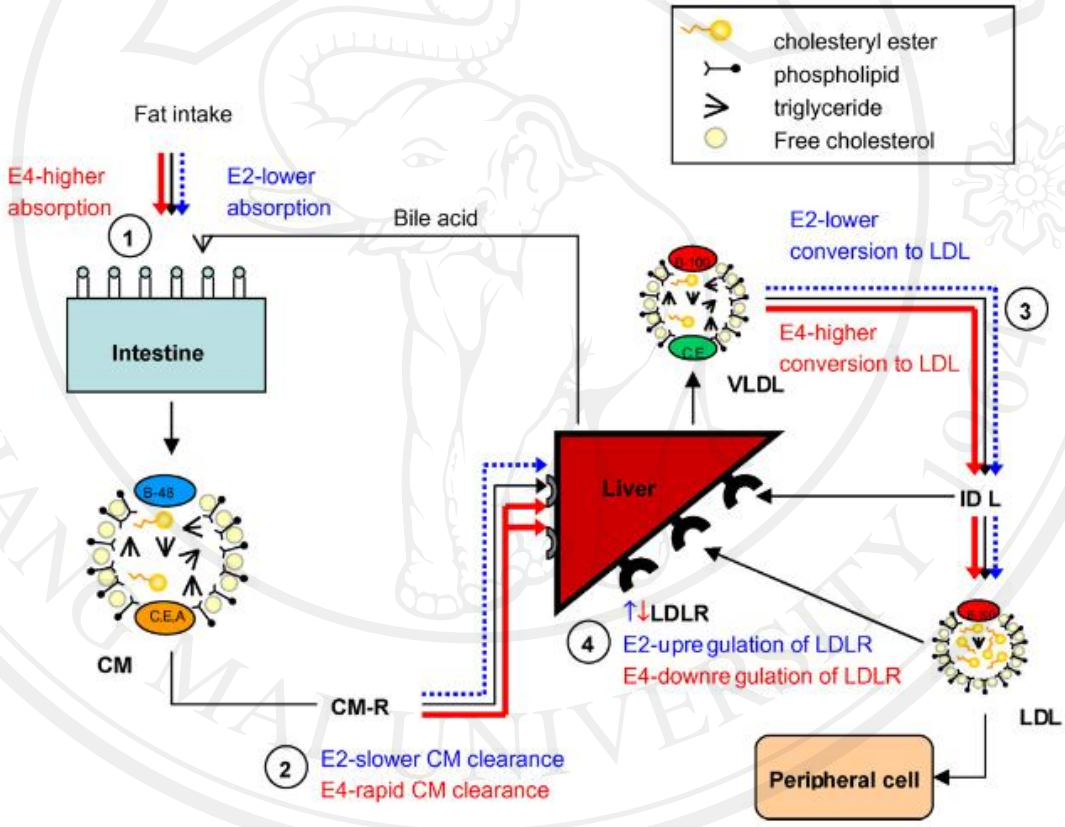

Figure 24 Influence of different isoforms of apo E on plasma cholesterol concentration which affect 1) absorption, 2) CM clearance, 3) formation of LDL and 4) LDLR regulation. CM: chylomicron, CM-R: chylomicron remnant, LDLR: low density lipoprotein receptor [75]

Although apo M is mainly associated with HDL [76], it was also identified in sdLDL and bdLDL in the 1-DE separation in the present study. This result is agreed with the lipoproteomics study described by Karlsson *et al.* 2005 which identified apo M in LDL [77]. Similar results were also observed in a comparative proteomics study of VLDL and LDL, which revealed a higher expression level of apo M in LDL than VLDL [67]. The function of apo M has not been fully elucidated and remains to be investigated. Interestingly, apo M was suggested to have antiatherogenic properties in transgenic mice by increasing the antioxidant effects [78] , enhancing the formation of pre β-HDL and cholesterol efflux from macrophage to HDL [61, 78]. That study reported that apo M-containing HDL was highly resistant to oxidation [78]. In addition, the data suggested that lack of apo M led to impaired formation of pre β-HDL and defective cholesterol efflux. This pre β-HDL is the precursor of mature HDL and is involved in ATP-binding cassette transporter A1 (ABCA1)-dependent efflux of cholesterol [61]. Moreover, overexpression of apo M could prevent atherosclerosis in mice. In spite of anti-atherogenic effect of apo M in mice, recent studies have found similar apo M levels in patients with CHD and healthy controls. There was no association between apo M levels and the risk of CHD. This result implied that apo M might not act as a risk factor for CHD in humans [79]. In addition, we found apo M at equal levels in sdLDL and bdLDL from 1-DE separation in the present study.

Interestingly, apo L3 that had not previously been identified in LDL were found in the present study. We found the presence of apo L3 in sdLDL and bdLDL from 1-DE separation. This new apolipoprotein is mainly associated with HDL [80]. The role of apo L remains unknown. However, it might initiate programmed cell death (PCD). There are six apo L genes in humans, including apo L1, apo L2, apo L3, apo L4, apo L5 and apo L6 [62]. These apo L genes contain a BH3 domain which binds to other Bcl-2 family members that can initiate apoptosis. BH3 proteins play a role as upstream activators of PCD. Moreover, since apo L genes were upregulated by proinflammatory cytokines such as INF-α, IFN-β, IFN-γ and TNF-α, it is believed to be involved in the immune system [63].

In addition, apo C-III was another apolipoprotein identified in the LDL subfractions. It is found mainly in VLDL and HDL, and to a lesser extent in LDL and chylomicrons. Apo C-III inhibits the lipolysis of TG-rich lipoproteins and interferes with their clearance from plasma. It is an inhibitor of lipoprotein lipase which catalyzes triglyceride in TG-rich lipoproteins. Recently, there has been increased interest in high apo C-III contents of LDL due to their higher associated risk of CHD. Studies suggest apo CIII-LDL as a predictor for CHD due to the relationship between LDL containing apo C-III and CHD [81]. Moreover, previous studies have investigated the impact of apo C-III on the binding of LDL to proteoglycans. The results revealed a positive correlation between biglycan binding and apo C-III levels in LDL. The data implied that the apo C-III content of LDL is associated with binding to proteoglycan [82]. Consistent with this finding, previous proteomics studies of patients with the metabolic syndrome and type 2 diabetes, along with subclinical atherosclerosis have suggested that there is an association between high apo C-III in

sdLDLs and their affinity for arterial proteoglycans (PGs). Moreover, the studies showed high levels of apo C-III in sdLDL than in bdLDL in both patients and healthy controls. In addition, sdLDL in patients were rich in apo C-III compared with healthy controls. Thus, these high apo C-III, coupled with the augmented affinity with arterial PGs of sdLDL might contribute to the increased risk of cardiovascular disease [(9)]. However, these protein profiles might be unreliable due to the type of MS used in their study. Proteins were analyzed using surface-enhanced laser adsorption/ionization, time of flight MS (SELDI-TOF MS) which sequence were not presented. However, evidence from our study was contradictory. We identified apo C-III with similar levels of intensity in sdLDL and bdLDL from the 1-DE separation. However, it should be noted that comparative analysis was not performed for the 2-DE separation. Thus, we could not compare protein intensities among two groups of sdLDL and bdLDL. For 2-DE, three spots (spot no. 8, 9, 10) and one spot (spot no. 3) of apo C-III were found in sdLDL and bdLDL, respectively. These different spots might result from modifications of apo C-III such as phosphorylation or glycosylation.

Comparison of protein profiles from sdLDL and bdLDL from the 1-DE separation is shown in Table 13. Of 11 proteins, 3 were predominantly found in sdLDL. An interesting finding was the presence of phospholipase A1. Phospholipase A1 is an enzyme that hydrolyzes phospholipids and triacylglycerol. The members of the family that are involved in lipoprotein metabolism include lipoprotein lipase (LPL) and hepatic lipase (HL). Interestingly, the LPL gene has been reported to be associated with small/medium LDL but not with large LDL [(83)]. Ichikawa *et al*. 2004 reported that overexpression of LPL in transgenic rabbits

developed an aggressive atherosclerosis [84]. LPL is produced by adipose, muscle and heart tissue. It catalyzes the hydrolysis of TG-rich lipoproteins such as chylomicron and VLDL to generate triglyceride-poor lipoproteins, including chylomicron remnants and IDL, respectively. Another family member is HL, which plays an important role in the remodeling of LDL and HDL. It catalyzes the hydrolysis of TG-rich HDL to form TG-poor HDL. HL activity is inversely related to buoyancy and size of LDL and HDL. HL has been linked to atherosclerosis in many studies. It hydrolyzes the TG in the TG-rich LDL leading to smaller, denser LDL, which is pro-atherogenic. It has previously been suggested that the increase in LDL size and buoyancy mediated by a decrease in HL activity was associated with CAD improvement [85]. However, the association between this lipase family and inflammatory cytokines is receiving much attention. It is well known that inflammation plays an important role in atherogenesis. Recent studies have revealed the relationship between LPL and cytokines. Interestingly, the suppression of LPL decreased proinflammatory cytokine expression, including those of interleukin-1β (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 and tumor necrosis-α (TNF-α) [86]. Apart from the previous data, this PLA1 might explain why sdLDL is more atherogenic than bdLDL.

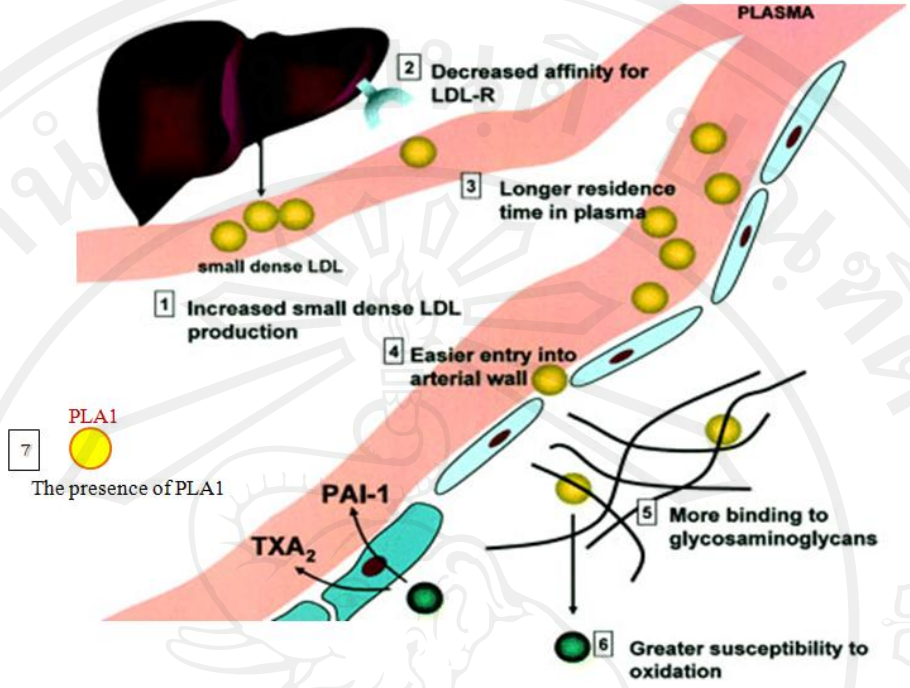

Figure 25 Atherogenicity of small, dense LDL (modified from http://content.onlinejacc.org/content/vol50/issue18/images/medium/coverfig.gif **)**

Our study identified 2 spots of apo A-I (spot no. 1, 6) in sdLDL from 2-DE separation. It should be mentioned that apo A-I was not observed in bdLDL in the present study. On the other hand, previous proteomics studies have found the presence of apo A-I with similar levels of intensity in both sdLDL and bdLDL. Moreover, levels of apo A-I of sdLDL in patients with the metabolic syndrome and type 2 diabetes, both with subclinical atherosclerosis were decreased compared with healthy controls [(9)]. Apo A-I is the major apolipoprotein associated with HDL. It functions as a ligand for ABCA1 and activates LCAT for cholesterol transportation from tissues to HDL. Thus, apo A-I is expected to inhibit atherosclerotic development due to its roles in increased cholesterol efflux and LCAT activation in

the reverse cholesterol transport pathway. Furthermore, apo A-I also has antioxidant properties that can protect LDL from modification [8].

In the present study, we also identified protein phosphatase 2A (PP2A) which had not previously been identified in LDL. The presence of PP2A was observed in sdLDL from 2-DE separation. It is a major serine/threonine phosphatase involved in cellular biology, including signal transduction pathways, cell growth and the cell cycle. Other studies have suggested a relationship between anti-inflammatory effects of apo E and PP2A ; apo E could reduce inflammatory response by binding the SET proteins and subsequently activating PP2A-mediated phosphatase activity. Apo E has been shown to have anti-inflammatory activities by suppressing TNF-α, IL-6, IL-2 production, macrophage activation and T lymphocyte proliferation. Christensen *et al*. 2011 investigated the mechanism by which apo E was able to suppress inflammation. They found that apo E bound to SET protein which act as an inhibitor of PP2A. The binding of apo E and SET protein led to activation of PP2A and subsequently dephosphorylated PP2A substrates including p38 MAPK, ERK, JNK Akt and IKK. These PP2A targets are involved in the NF-κB pathway that regulates NO production. There were decreased levels of phosphorylated p38 MAPK, Akt and reduced production of NO synthase and its products. This provided clues to the mechanisms of anti-inflammatory apo E by binding of SET protein followed by increasing PP2A activities and decreasing levels of phosphorylated kinase, which subsequently reduces NF-κB activation [98]. However, the roles of PP2A in sdLDL remain unknown.

We identified 8 proteins that were predominantly found in bdLDL from 1-DE separation as shown in table 13. An interesting finding was the presence of lysozyme.

It plays an important role in the prevention of bacterial infection by catalyzing the hydrolysis of β(1-4) glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, which is the major component of the cell wall of bacteria, resulting in the lysis of bacterial cells. Recent study has shown the presence of lysozyme in LDL [77]. Although the role of lysozyme in LDL remains to be elucidated, an increased incidence of CHD in patients with chronic infections such as *Chlamydia pneumonia* has been reported [87]. It has been demonstrated that host response to infection induces LDL oxidation, which may result in atherosclerosis [88, 89]. Therefore, the role of lysozyme in atherogenesis may be act as an antibacterial agent. Moreover, previous studies have suggested an association between lysozyme and advanced glycation end product (AGE). The data indicated an increased level of lysozyme in patients with type 2 diabetes [90]. It is well known that atherosclerosis often occurs in patients with type 2 diabetes. The advanced glycation end product (AGE) present in diabetes has been shown to react with lipoproteins and prevent LDL receptor recognition [91]. Lysozyme has previously been shown to bind AGE in many proteins. Interestingly, studies have revealed greater binding of lysozyme to AGE-LDL than to unmodified LDL [90]. In this study, we found the presence of this protein at high levels in bdLDL compared to sdLDL.

The presence of apo D was also observed in bdLDL from 2-DE separation in our study (spot no. 5). Although apo D has previously been identified in LDL [67], it had not been found in LDL subfractions. Thus, our finding identified the newly discovered apo D in bdLDL for the first time. However, there was a point that deserves attention. It should be mentioned that Mascot's score was used to judge whether a result was significant or not. For example, scores with greater than 43 are

designated to be significant ($p<0.05$). In our study, the score of apo D was not significant due to its lower levels (score = 24). However, insignificant scores might be due to insufficient or too small mass values for a statistically significant match. Further western blot analysis is required to confirm the presence of apo D. Apo D is structurally similar to proteins of the lipocalin family and is present mainly in HDL and to a lesser extent in LDL and VLDL. Apo D transports several hydrophobic ligands including cholesterol, progesterone, bilirubin and arachidonic acid. It is believed that apoD is associated with apo A-II. The fifth cysteine residue of human apo D is responsible for forming the disulfide-link with Cys-6 of apo A-II [(92)]. Genetic mutations in the apoD gene are associated with abnormal lipid metabolism and increased risk for metabolic syndrome. A role of apoD is to enhance the VLDL-TG hydrolysis and clearance. Elevated apoD production resulted in reduction of triglyceride levels. This effect can be described by enhancing the LPL activity and improving catabolism of TG-rich VLDL particles [(93)]. Moreover, recent studies have found elevated apoD in stroke mice during the recovery period. This result is believed to be involved in the transporting function of a remodeling process of brain injury which requires cholesterol and phospholipid [(94)]. In addition, high apoD levels were observed in human failing hearts compared with non-failing hearts [(95)] and atherosclerotic lesions of apoE knockout mice [(96)]. The evidence indicated that apoD also plays an important role in anti-oxidative stress. Overexpression of apoD in the mouse brain prevented the lipid peroxidation [(97)].

The identified proteins were subjected to protein-protein interaction network using the STRING database as shown in figure 27. The interactions include direct physical binding and indirect (functional) interaction such as contributing to the same

metabolic pathway. These associations are derived from genomic context, high throughput experimental data and the literature. There were 3 interesting protein identified in sdLDL including PLA1, PP2A and apo A-I. On the other hand, we found the presence of apo D and lysozyme in bdLDL (Figure 24).

Two-dimensional gel electrophoresis is currently used to separate proteins. However, it is widely recognized that 2-DE also suffers from various limitations. Some problems associated with this technique are that it is a time- consuming, labor-intensive process, low reproducibility, gel to gel variability, poor resolution and limited detection of proteins with extreme pI (below pH 3 and above pH 10) and low abundance proteins due to insufficient quantity for analysis [(99)]. In spite of increasing the protein loading capacity, it is still insufficient to visualize low abundance proteins because high abundance proteins will dominate and hide low abundance proteins. Moreover, overloading causes horizontal streaks. High ridges at the isoelectric point can build up. Proteins diffuse over the surface due to a high quantity of proteins. Furthermore, high molecular weight proteins with sizes above 150 kDa are lost during the isoelectric focusing process [(100)].

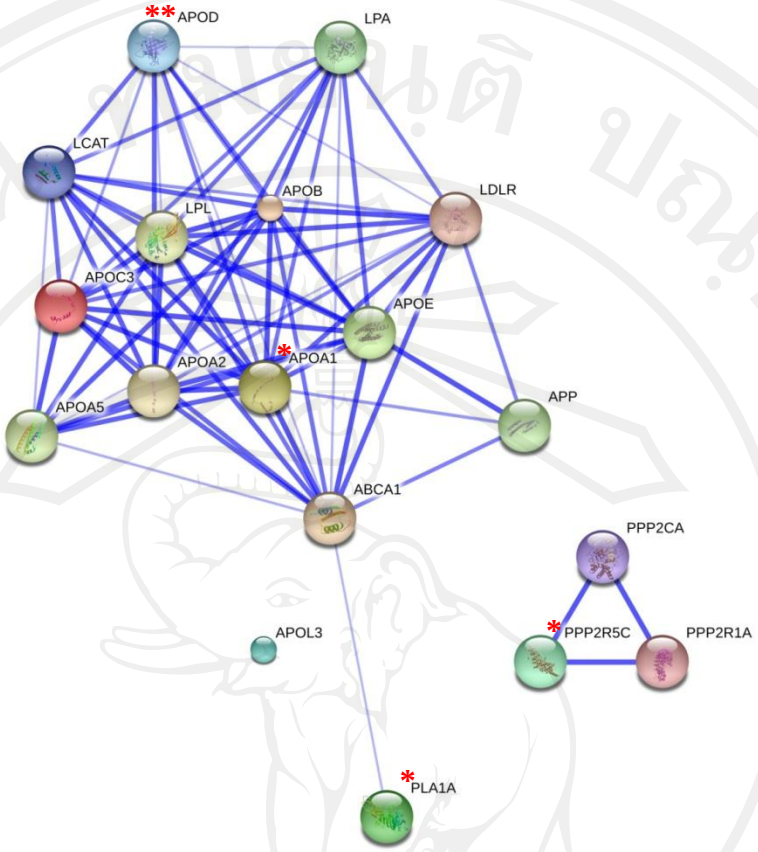

Figure 26 Protein-protein interaction network described by STRING. The high color saturation of the edges represents a strong association. Our identified proteins include APOC3; apolipoprotein C-III, APOB; apolipoprotein B, APOA1; apolipoprotein A-I, APOE; apolipoprotein E, PLA1; phospholipase A1, PPP2R5C; protein phosphatase 2 regulatory subunit B gamma isoform, APOL3; apolipoprotein L3, APOD; apolipoprotein D. The predicted functional partners are LCAT; lecithin-cholesterol acyltransferase, PPP2CA; protein phosphatase 2 catalytic subunit alpha isoform, PPP2R1A; protein phosphatase 2 regulatory subunit A alpha isoform, LDLR; low density lipoprotein receptor, ABCA1; ATP-binding cassette sub-family A member 1, APOA2;apolipoprotein A-II, LPL; lipoprotein lipase, APOA5; apolipoprotein A-V, APP; amyloid beta precursor protein, LPA; lipoprotein Lp(a). (* and ** represent proteins found in sdLDL and bdLDL, respectively.)

This limitation implies the missing of numerous proteins from the analysis. Hydrophobic and membrane proteins are problematic for 2-DE. They have low solubility in isoelectric focusing buffer, leading to minimal extraction [101]. Besides, some visualization methods are not compatible for MS-based protein identification techniques.

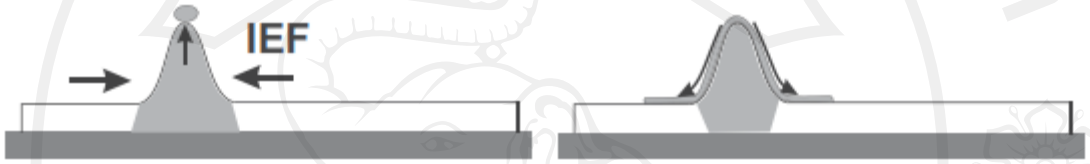

Figure 27 Overloading effect of proteins [44]

Moreover, in spite of silver stain's high sensitivity with 1 ng detection of proteins per spot, it is unfortunately unsuitable for protein quantification. Silver staining has a narrow dynamic range, thus it is difficult for quantification of high and low abundance proteins in complex mixtures. For low abundance protein quantification, the staining process has to be continued until the optimal time for color development, whereas the high abundance proteins have reached saturation. This limitation causes underestimation of high abundance proteins [102]. Moreover, according to the fact that the staining process has to be stopped at the optimal time by visual decision, it is difficult to have exact reproducibility. In practice, spots may show no signal in MS because of too long a development time (too much contact of protein with glutaraldehyde) or too low quantity of proteins [44].