CHAPTER TWO

LITERATURE REVIEW

2.1 Human Cholesterol Metabolism

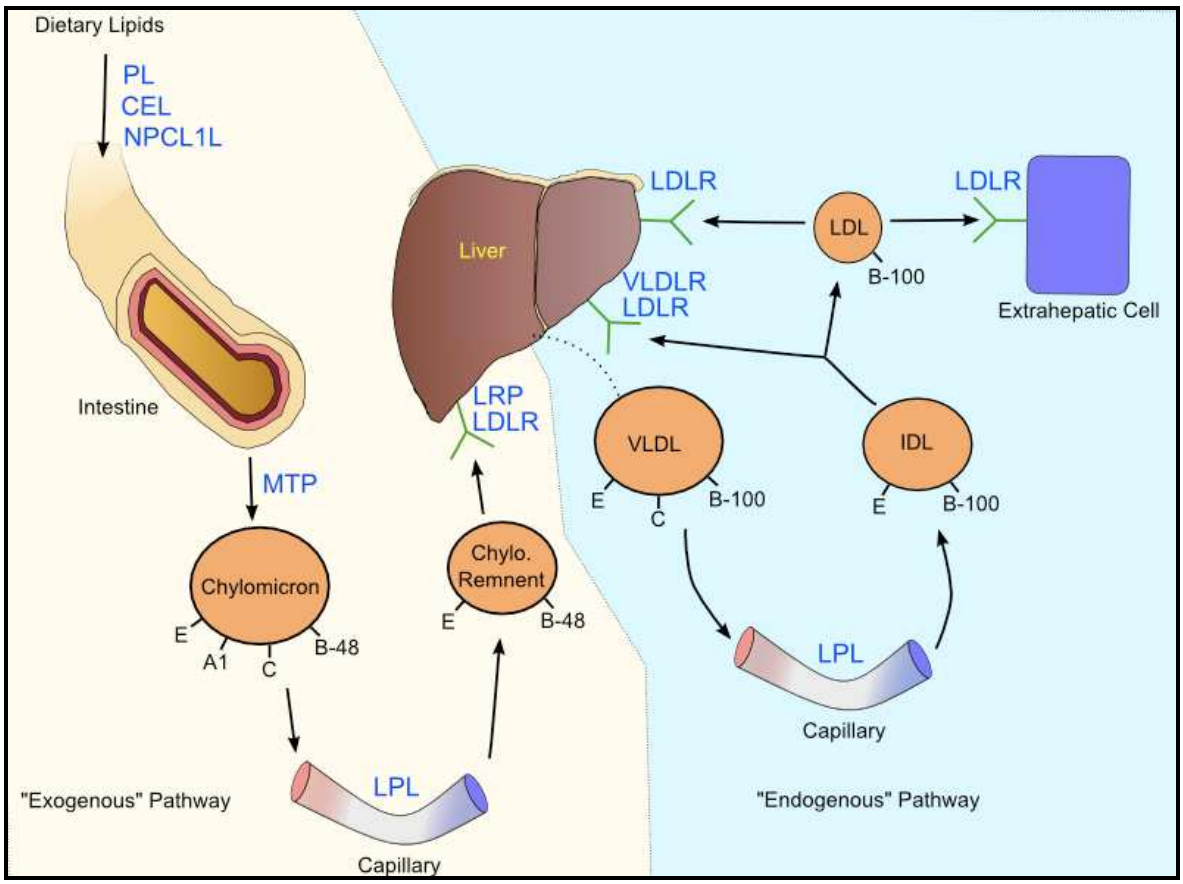
Cholesterol is a rigid, hydrophobic molecule that gives integrity to the structure of plasma membranes of vertebrate cells for the proper establishment of membrane permeability and fluidity (Alberts *et al*., 1994; Rader *et al*., 2003). Despite its popular image as a potent enemy of health and longevity, cholesterol is actually an important substance that performs many vital functions in the body. Cholesterol is required for the synthesis of bile acids, which are essential for the absorption of fats (Steinberg, 2006a). Cholesterol also acts as the precursor for manufacturing steroid hormones such as testosterone, oestrogen, dihydroepiandrosterone, progesterone and cortisol (Hume & Byod, 1978). Combined with sun exposure, cholesterol is required to produce vitamin D (Bouillon *et al*., 1995). Cholesterol plays a crucial role in the formation of the myelin sheath that surrounds the axons for conducting nervous impulse at the synapse level (Goldstein & Brown, 2009; Barres & Smith, 2001) and may even serve as a protective antioxidant (Girao *et al*., 1999).

Cells can obtain cholesterol either from de novo synthesis or from the uptake from circulating lipoproteins (Lagor & Millar, 2010). The typical human diet contains 200-500mg of cholesterol. 800-1200mg of daily cholesterol is derived from bile input and 300mg is from desquamated intestinal epithelial cells. Some 30-60 % of intestinal cholesterol is absorbed while daily faecal loss of cholesterol is 550mg from bile and desquamated cells and 250mg is lost as unabsorbed bile salts (Levy *et al*., 2007). The

cholesterol biosynthesis takes place mainly in the liver and the central nervous system (Charlton-Menys & Durrington, 2008).

In the bloodstream of humans and other vertebrates, cholesterol, due to its hydrophobic property, requires transport vesicle to shield it from the aqueous nature of plasma. These transport particles are known as lipoproteins. The principal plasma lipoproteins are chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Durrington, 2007). The most abundant cholesterol-carrying lipoprotein in human plasma is LDL with approximately 70 % of circulating cholesterol is transported as LDL (Rader *et al*., 2003). Dietary cholesterol has four fates once it reaches the liver: it can be esterified and stored as cholesteryl esters in hepatocytes, packed into VLDL particles and secreted into plasma, secreted directly into bile or converted into bile acids and secreted into bile.

Dietary cholesterol and triglycerides are packed with apolipoproteins B48 (ApoB48) in the enterocytes of small intestines and secreted into the lymphatic system as chylomicrons. Once chylomicrons enter the blood circulation, the core triglycerides are hydrolysed by lipoprotein lipase (LPL) which results in the formation of chylomicron remnants. The cholesterol-rich chylomicron remnants are rapidly removed by the liver by binding to the LDL receptor-like protein (LRP) (Cooper, 1997). The uptake of chylomicron remnants by the LRP completes the transport of intestinal cholesterol to the liver and this pathway is commonly known as the exogenous pathway. In the human body, liver is the most LDLR-abundant organ and account for 70 % of the total LDL clearance in plasma (Spady, 1992; Cooper, 1997). Figure 2.1.1 illustrates the cholesterol metabolism in human (adapted from Daniels *et al*., 2009).



Abbreviation:	
PL=Pancreatic lipase,	CEL= Carboxyl ester lipase
NPC1L1= Niemann-Pick C1-Like 1	MTP=Microsomal triglyceride transfer protein
LPL=Lipoprotein lipase	LDLR= Low density lipoprotein receptor
VLDL= Very low density lipoprotein	IDL= Intermediate density lipoprotein
B-48 = ApoB48	E = ApoE
C = ApoC2	LRP = Low density lipoprotein receptor-like protein

Figure 2.1.1: Overview of cholesterol metabolism in humans.

The liver exports cholesterol to the tissues and it is secreted into the plasma as VLDL particles and this pathway is called the endogenous pathway (Kwiterovich, 2000). VLDL secreted into the plasma undergoes similar sequence of events as chylomicron which is the removal of the triglyceride core by LPL to form cholesterol-enriched VLDL remnant particles. Approximately 50 % of the VLDL remnants are cleared from the circulation by LDL receptor-mediated endocytosis in the liver and the remainder mature into LDL. An estimated 70 % of circulating LDL is removed from the circulation after binding to the hepatic LDL receptor (LDLR) via its ligand, apolipoprotein B-100 (ApoB-100) (Rader *et al*., 2003). Upon binding, the LDL-LDLR complex is taken up by cells via clathrin-mediated endocytosis and then delivered into endosomes. In endosomes, the dissociation of LDL-LDLR complex takes place due to low pH environment that trigger the release of the bound lipoprotein particles (Brown *et al*., 1983). The LDLR is subsequently returned back to the cell surface in a process known as receptor recycling (Brown & Goldstein, 1976a; Goldstein *et al*., 1985; Brown & Goldstein, 1986; Soutar, 1996). Figure 2.1.2 illustrates the uptake of LDL particles by the LDLR.

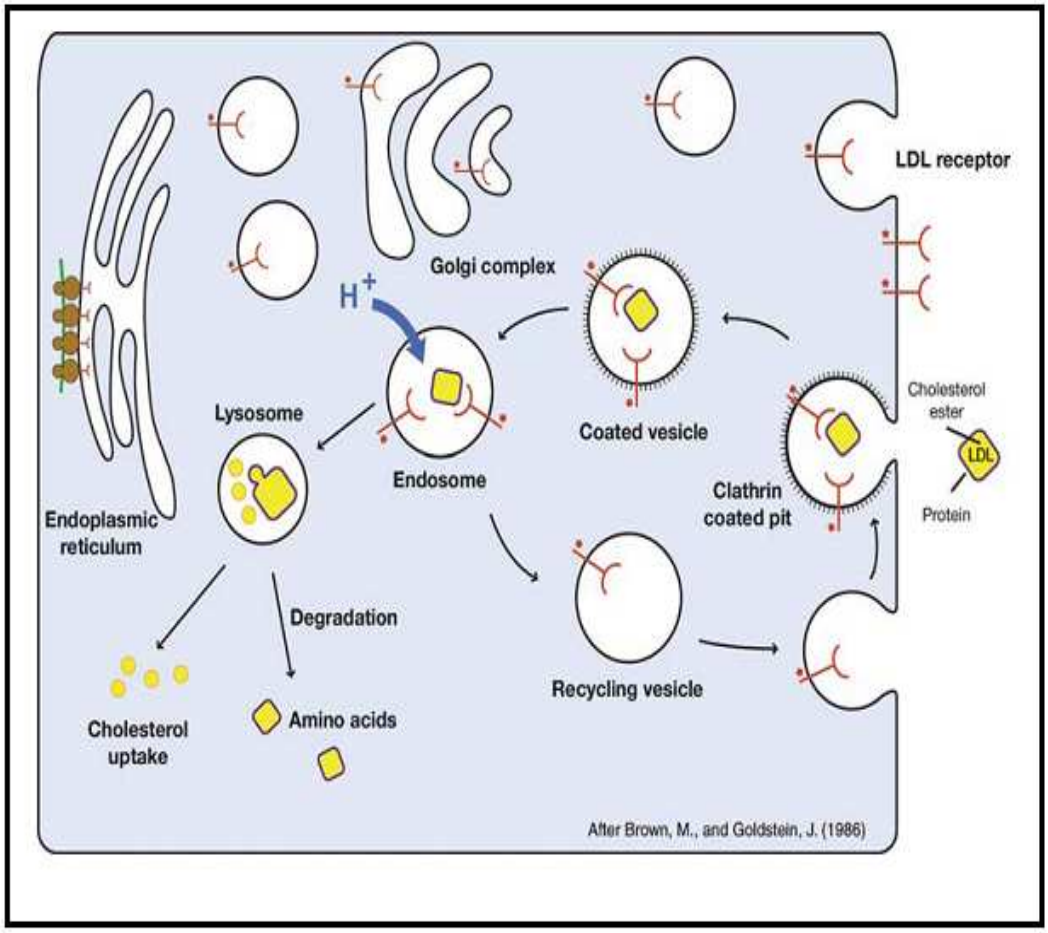


Figure 2.1.2: Schematic of the uptake of LDL particles by the LDLR (adapted from Brown and Goldstein, 1986).

The low density lipoprotein receptor (LDLR) is the primary pathway for removal of cholesterol from the circulation and its activity is governed by intracellular cholesterol levels (Slater *et al*., 1984; Brown & Goldstein, 1986; Goldstein & Brown, 2009). If the LDL is oxidised, it can enter the macrophage through the scavenger receptors, CD36 and SR-A on the surface of macrophage (Kwiterovich, 2000).

2.2 Hypercholesterolaemia

Hypercholesterolemia is a condition characterised by high levels of cholesterol in the blood. The guidelines of the American Heart Association and the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) in 2002, define hypercholesterolemia as a blood cholesterol concentration of greater than or equal to 6.2mmol/L or 240mg/dL with the desirable cholesterol concentrations are less than 5.2mmol/L or 200mg/dL (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, 2002). According to NCEP ATP III guidelines, all adults aged 20 years and above should have a fasting lipid profile determined at least every 5 years to assess the coronary artery disease (CAD) risk.

There are three major mechanisms that may be responsible for the cause of hypercholesterolaemia. The first cause is due to defective clearance of LDL by the LDLR. A reduction in the rate of receptor-mediated clearance of LDL could result from an abnormality in LDLR or defective ligands that bind poorly to the receptors (Brown & Goldstein, 1986). The second cause of elevated LDL is due to overproduction of LDL by the liver (Grundy & Vega, 1990). Increased hepatic secretion of apoB-containing lipoproteins and decreased uptake of VLDL and VLDL remnants can also

contribute to the overproduction of LDL. Finally, low affinity of circulating LDL for receptor may also lead to hypercholesterolemia (Vega & Grundy, 1987).

The primary manifestation of hypercholesterolaemia is increased CAD risk (Lewington *et al*., 2007). Accumulating epidemiological studies have shown a strong relationship between elevated LDL-c concentrations and CAD events and CAD mortality rates and identified LDL-c concentration as a primary target of therapy in patients with hypercholesterolaemia (Anderson *et al*., 1987; Vega *et al*., 1991; Stamler *et al*., 2000; Loria *et al*., 2007).

Figure 2.2.1 illustrates the curvilinear relationship between cholesterol concentrations and relative risk of CAD mortality in three large cohorts of young men from Chicago Heart Association Detection Project in Industry (CHA), Chicago Peoples Gas Company (CP) and Multiple Risk Factor Intervention Trial (MRFIT) (Stamler *et al*., 2000). This implies that most individuals are likely to benefit from LDL-c levels reduction.

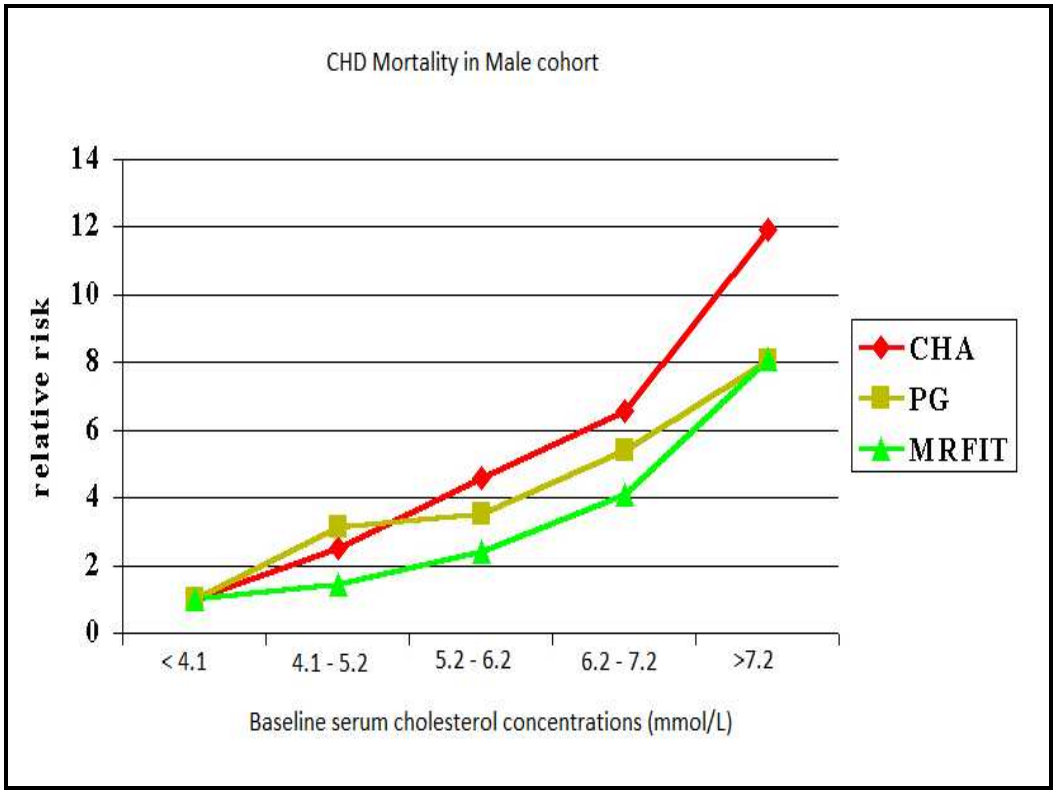


Figure 2.2.1: Relative risk of coronary artery disease mortality versus baseline serum cholesterol concentrations. (adapted from Stamler *et al.*, 2000). CHA – Chicago Heart Association Detection Project in Industry, PG – Chicago People Gas Company, MRFIT – Multiple Risk Factor Intervention Trial.

Hypercholesterolaemias are classified as either primary hypercholesterolaemia, comprising a group of genetically determined disorders or secondary hypercholesterolaemia, in which the abnormalities are the result of an acquired condition (Marshall, 2000). Primary hypercholesterolaemia is attributed by hereditary genetic defects and environmental factors such as high fat diet, obesity, lack of physical activity and inactive life style (Durrington, 2003). Primary hypercholesterolaemia is a relatively common condition that has been associated with the development of atherosclerosis and premature CAD (Ose, 2002). The substantially increased risk of CAD associated with primary hypercholesterolaemia was recorded even before lipid lowering drugs were widely available (Jensen *et al*., 1967; Slack, 1969; Stone *et al*., 1974; Beaumont *et al*., 1976; Heiberg, 1975).

Hypercholesterolaemia does not lead to specific physical symptoms unless it has developed over a long period of time (Durrington, 2003). Some types of hypercholesterolaemia such as Familial Hypercholesterolaemia, lead to specific physical presentations. Among the observed physical presentations are thickening and deposition of cholesterol within tendon (tendon xanthomata), cholesterol deposits around the eyelids (xanthelasma) and cholesterol infiltration around the corneal rim (corneal arcus) (Yuan *et al*., 2006). Longstanding hypercholesterolaemia leads to accelerated atherosclerosis and this can express itself in a number of complications such as angina pectoris, myocardial infarction, stroke and peripheral vascular disease (Grundy *et al*., 1998).

Management of hypercholesterolaemia is targeting to reduce LDL-c concentrations and can be achieved by lifestyle modifications combined with the use of lipid-lowering drugs (Bhatnagar *et al*., 2008). Lifestyle modifications such as smoking cessation,

decreasing alcohol consumption, increasing physical activities, weight loss program and low saturated fat diet regime will all contribute to the lowering of LDL-c concentrations (Vogel, 1998).

According to the NCEP ATP III and American Heart Association guidelines, dietary modifications and other lifestyle changes are recommended as first-line therapy (Lichtenstein *et al*., 2006; Executive Summary of the NCEP ATP III). Since 2001, phytosterol-enriched functional food has recommended as part of an optional dietary prevention of cardiovascular diseases (Executive summary of NCEP ATP III).

Phytosterols are natural constituents of plants that are structurally related to cholesterol (Weingartner *et al*., 2009). This health claim for reducing the risk of CAD of phytosterol food supplements has also been approved by the US Food and Drug Administration in year 2000 (www.fda.gov). Recent meta-analyses of more than 40 clinical studies indicate that dietary supplements containing plant sterol esters can induce the reduction of LDL-c levels up to 10-15% (Hendriks *et al*., 1999; Katan *et al*., 2003).

However, if the treatment goals cannot be achieved through non-pharmacological measures, drug therapy should be initiated (Davidson *et al*., 2002). Among the available lipid-lowering agents, 3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are the commonest choice of lipid-lowering agents prescribed to decrease LDL-cholesterol levels (Davidson *et al*., 2002). HMG-CoA reductase is the rate limiting enzyme that catalyses the conversion of HMG-CoA to mevalonic acid in the cholesterol biosynthesis pathway (Figure 2.2.2).

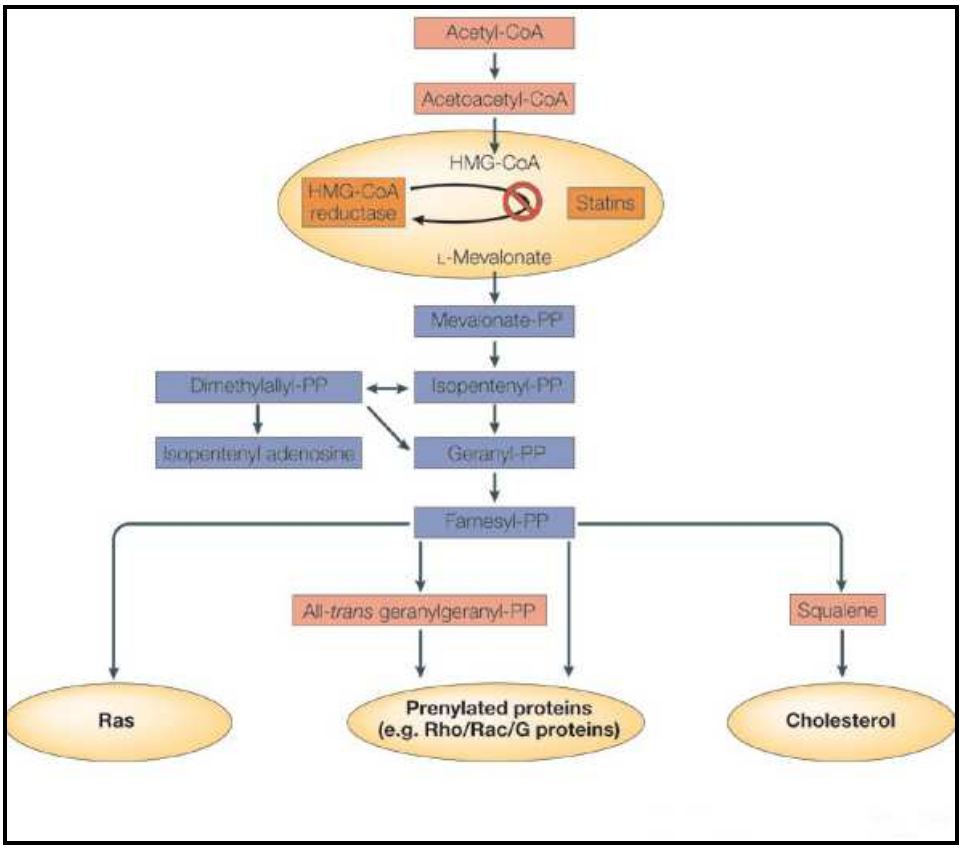


Figure 2.2.2: Simplified schematic diagram of cholesterol biosynthesis pathway.

Other pharmacological agents available for the management of hypercholestrolaemia include bile-acid sequestrants, inhibitor of intestinal cholesterol absorption (ezetimibe), inhibitors of squalene synthase, ApoB mRNA antisense oligonucleotides, microsomal triglyceride transfer protein (MTP) inhibitors and cholesteryl ester transfer protein (CETP) activity inhibitors (Charlton-Menys & Durrington, 2008).

2.3 Familial Hypercholesterolaemia

Familial hypercholesterolemia (FH) is a disorder of cholesterol metabolism with an autosomal dominant mode of inheritance (Goldstein *et al*., 2001). It is characterised by elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) levels. FH is caused primarily by mutations of the low density lipoprotein receptor (*LDLR*) gene (Muller, 1938; Goldstein *et al*., 2001; Austin *et al*., 2004a). Mutations in two other genes that cause the clinical FH phenotype have also been reported but much less frequent (Austin *et al*., 2004b; Soutar & Naoumova, 2007; Minhas *et al*., 2009). One of these is the apolipoprotein B-100 (*APOB*) gene, located on chromosome 2p23-24 that encodes for the protein component of LDL particles (Knott *et al*., 1985; Law *et al*., 1985a; Law *et al*., 1985b; Innerarity *et al*., 1990). However, only a small number of functional mutations have been identified in *APOB* (Austin *et al*., 2004b; Tybjaerg-Hansen and Humpries, 1992).

The third gene, proprotein convertase subtilisin/kexin type 9 (*PCSK9*), has been identified on chromosome 1p32-1p34.1 (Austin *et al*., 2004b; Varret *et al*., 1999; Hunt *et al*., 2000; Abifadel *et al*., 2003; Varret *et al*., 2008). The presence of other candidate genes has been postulated, but these are rare (Soutar and Naoumova, 2007). In 2002, Pullinger *et al*., found a recessive trait in a single family showing recessive mutation in 7alpha-hydroxylase (CYP7A1) gene that encodes the enzyme that catalyses the first

step in the hepatic catabolism of cholesterol. Other groups found variants in the genes for regulatory proteins such as sterol regulatory element binding protein-2 (SREBP-2) and sterol cleavage-activating protein (SCAP) (Muller and Miserez, 2002; Durst *et al*., 2006).

FH occurs clinically in two forms, heterozygous FH and homozygous FH (Goldstein & Brown, 1986). Heterozygous FH patients were found to have one copy of a mutated LDLR gene and the estimated frequency of heterozygous FH in Caucasian population is often reported as 1/500 or 0.2% (Patterson and Slack, 1972; Goldstein *et al*., 1973), making FH among the most common single gene disease. In certain communities, the frequency of FH can be higher due to founder effects (Varret *et al*., 1997; Austin *et al*., 2004a). These communities include French Canadian (Leitersdorf *et al*., 1990; Betard *et al*., 1992), Christian Lebanese (Lehrman *et al*., 1987), Druze (Landsberger *et al*., 1992), Finns (Koivisto *et al*., 1992; Aalto-Setala *et al*., 1992), Afrikaners (Kotze *et al*., 1991) and Ashkenazi Jews of Lithuanian descent (Meiner *et al*., 1991).

In FH homozygous individuals, who inherited two mutant LDLR alleles are rare, with frequency about 1 in a million people (Hobbs *et al*., 1992). Patients with a mutation in both LDLR alleles are more severely affected than patients with a single mutant allele. Heterozygous FH have a two to three fold elevation in plasma LDL and typically begin to develop premature CAD at the age of 30 to 40 years (Goldstein & Brown 1986; Goldstein & Brown, 2009). In contrast, homozygous FH have six to ten times higher than the normal concentration of plasma LDL from birth and often have heart attacks during the first two decades of life or during childhood (Goldstein *et al*., 1985; Hobbs *et al*., 1992).

The FH clinical phenotype is associated with increased risk of CAD and premature death (Austin *et al*., 2004b; Ayyobi *et al*., 2007; Goldstein *et al*., 2001). In the heterozygous FH without effective treatment, the cumulative risk of having coronary event by the age of 60 years is at least 50% in men and about 30% in women (Slack, 1969; Stone *et al*., 1974) with coronary disease occurring earlier in men than women (Gagne *et al.*, 1979). In a cohort of treated heterozygous FH aged 20-39 years, the relative risk of fatal CAD was found to be increased nearly 100-fold (Scientific Steering Committee on behalf of the Simon Broome Register Group, 1991 & 1999).

2.3.1 Clinical Signs of FH Patients

Increased LDL-c levels often result in cholesterol deposits on the Achilles' tendons and extensor tendons of the hands and feet, and to a lesser extent on the knees and elbows, clinically referred to as tendinous xanthomas (Brown & Goldstein, 1976b). Other obvious clinical signs that develop due to LDL-c elevation include cholesterol deposit on the eyelids (xanthelasma) and white deposit of lipids in the outer rim of the iris (arcus cornealis) (Yuan *et al*., 2006). Xanthomas are composed of monocyte-derived foam cells resulting from intracellular accumulation of lipids and connective tissue (Kruth, 1985). Tendon xanthomas are highly specific for FH in subjects and current recommendations include them as an important clinical diagnostic criterion (Civeira, 2004). It has been reported that 29% of genetically diagnosed FH patients have Achilles tendon xanthomas diagnosed by sonography (Descamps *et al*., 2001; Junyent *et al*., 2005).

2.3.2 Clinical Diagnosis of FH Patients

FH individuals are at higher risk of developing CAD therefore it is important for early diagnosis and management of these patients (Hopkins *et al*., 2001). However, FH has frequently been under-diagnosed and many therefore go untreated (Durrington, 2001). Of the patients identified, there are still many who are not getting appropriate treatment (Umans-Eckenhausen *et al*., 2001). The clinical diagnosis of FH is based on physical signs, laboratory findings of lipid concentrations and the patient's personal and family history (WHO, 1999; Minhas *et al*., 2009). UK Simon Broome Register group (Scientific Steering Committee of the Simon Broome Register Group, 1991), the US MedPed Program (Williams *et al*., 1993) and the Dutch Lipid Clinic Network (Defesche, 2000) have developed clinical diagnostic tools for FH but many clinicians diagnose FH based on the Simon Broome criteria (Live, 1999). The National Institute of Health and Clinical Excellence (NICE) in the United Kingdom has issued a recommendation that the diagnosis of FH should be based on Simon Broome criteria modified for age-adjusted LDL-c levels (www.nice.org.uk; Watts *et al*., 2009; Minhas *et al*., 2009).

The major difference between these criteria is the use of the different cut-offs for premature CAD. The Simon Broome register group recommends the use of cut-off for premature CAD before the age of 60 years for 1st degree relatives and before 50 years in 2nd degree relatives (Scientific Steering Commitee on behalf of the Simon Broome Register Group, 1991). The MedPed criteria recommend a cut-off at age 65 years while Dutch Lipid Clinic Network suggests less than 55 years for men and less than 65 years for women (Austin *et al*., 2004a). The MedPed Criteria use cut-off points for total cholesterol levels specific to an individual's age and family history (Austin *et al*., 2004a). That is, the cut-off points differ for individuals with first-, second-, or third-

degree relatives and for the general population (Table 2.3.1) (Williams *et al*., 1993). In the US MedPed criteria, the total cholesterol cut points were derived from mathematical modelling using published cholesterol levels from FH individuals in the United States and Japan (Kane *et al*., 1990; Yamamoto *et al*., 1989; Williams *et al*., 1986).

Table 2.3.1: The US MedPed Diagnostic Criteria.

Table 2.3.1 US MedPed Program diagnostic criteria for familial hypercholesterolemia*

	Total cholesterol cutpoints (mmol/liter)			
	First-degree relative with FH†	Second-degree relative with FH	Third-degree relative with FH	General population
Age (years)				
<20	5.7	5.9	6.2	7.0
20–29	6.2	6.5	6.7	7.5
30–39	7.0	7.2	7.5	8.8
≥40	7.5	7.8	8.0	9.3
Diagnosis (FH is diagnosed if total cholesterol levels exceed the cutpoint)				

* Williams et al. Diagnosing heterozygous familial hypercholesterolemia using new practical criteria validated by molecular genetics. Am J Cardiol 1993;72:171–6 (8).

† FH, familial hypercholesterolemia.

The criteria used by the Simon Broome Register Group for the diagnosis of FH include total cholesterol levels, LDL-c levels, clinical characteristics, molecular diagnosis and family history (Table 2.3.2) (Austin *et al*., 2004a). Using Simon Broome Register criteria, an individual is diagnosed as either definite FH or probable FH. A 'definite' FH diagnosis is made if an adult patient has elevated LDL-c more than 7.5mmol/L combine with presence of tendinous xanthomas, or if a patient has an identified mutations in the LDLR gene or APOB gene. A 'probable' diagnosis is made if an adult patient has elevated LDL-c more than 7.5mmol/L and a family history of hypercholesterolaemia or premature CAD. The cut-off for LDL-c concentrations for children under the age of 16 years is 6.7mmol/L.

Table 2.3.2: The Simon Broome Register Group Diagnostic Criteria.

Table 2.3.2 Simon Broome Familial Hypercholesterolemia Register diagnostic criteria for familial hypercholesterolemia*

	Description
Criteria	
a	Total cholesterol concentration above 7.5 mmol/liter in adults or a total cholesterol concentration above 6.7 mmol/liter in children aged less than 16 years, or
	Low density lipoprotein cholesterol concentration above 4.9 mmol/liter in adults or above 4.0 mmol/liter in children
b	Tendinous xanthomata in the patient or a first-degree relative
c	DNA-based evidence of mutation in the *LDLR* or *APOB* gene
d	Family history of myocardial infarction before age 50 years in a second-degree relative or before age 60 years in a first-degree relative
e	Family history of raised total cholesterol concentration above 7.5 mmol/liter in a first- or second-degree relative
Diagnosis	
A "definite" FH† diagnosis requires either criteria *a* and *b* or criterion *c*	
A "probable" FH diagnosis requires either criteria *a* and *d* or criteria *a* and *e*	

* Risk of fatal coronary heart disease in familial hypercholesterolemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. BMJ 1991;303:893–6 (13); Mortality in treated heterozygous familial hypercholesterolaemia: implications for clinical management. Scientific Steering Committee on behalf of the Simon Broome Register Group. Atherosclerosis 1999;142:105–12 (14).

† FH, familial hypercholesterolemia.

The Dutch Lipid Clinic Network criteria (Table 2.3.3) are similar to the Simon Broome Register criteria, but uses calculation of numeric scoring (Marks *et al*., 2003). Points are given for family history of hypercholesterolaemia, premature CAD or peripheral vascular diseases, clinical characteristics, elevated LDL-c concentrations and/or an identified mutation in LDLR. A 'definite' FH diagnosis is made if the total points score is greater than eight. Total point score of six to eight is considered as 'probable FH' and score of three to five is 'possible' FH. If the score is below 3 points, a diagnosis is not made. Although the Dutch Lipid Clinic Network criteria are similar with Simon Broome Register criteria, it has one main difference. While Simon Broome Register recognises a molecular diagnosis as an evidence for 'definite' FH, the Dutch Lipid Network requires that at least one other criterion be met in addition to a DNA diagnosis (Minhas *et al*., 2009).

Table 2.3.3: The Dutch Lipid Clinic Network Diagnostic Criteria.

Table 2.3.3 Dutch Lipid Clinic Network diagnostic criteria for familial hypercholesterolemia*

	Points
Criteria	
Family history	
First-degree relative with known premature (men: <55 years; women: <60 years) coronary and vascular disease, or	
First-degree relative with known LDLC† above the 95th percentile	1
First-degree relative with tendinous xanthomata and/or arcus cornealis, or	
Children aged less than 18 years with LDLC above the 95th percentile	2
Clinical history	
Patient with premature (men: <55 years; women: <60 years) coronary artery disease	2
Patient with premature (men: <55 years; women: <60 years) cerebral or peripheral vascular disease	1
Physical examination	
Tendinous xanthomata	6
Arcus cornealis prior to age 45 years	4
Cholesterol levels (mmol/liter)	
LDLC, ≥8.5	8
LDLC, 6.5–8.4	5
LDLC, 5.0–6.4	3
LDLC, 4.0–4.9	1
DNA analysis	
Functional mutation in the *LDLR* gene	8
Diagnosis (diagnosis is based on the total number of points obtained)	
A "definite" FH† diagnosis requires more than 8 points	
A "probable" FH diagnosis requires 6–8 points	
A "possible" FH diagnosis requires 3–5 points	

* World Health Organization. Familial hypercholesterolemia—report of a second WHO Consultation. Geneva, Switzerland: World Health Organization, 1999. (WHO publication no. WHO/HGN/FH/CONS/99.2). (15).

† LDLC, low density lipoprotein cholesterol; FH, familial hypercholesterolemia.

2.3.3 Management and Treatment of FH

Once an individual is diagnosed with FH, the management and treatment is usually life-long. Treatment options for FH patients involve the combination of healthy lifestyle and cholesterol lowering medications. In some severe cases such as in homozygous FH, LDL-apheresis, liver transplantation and gene therapy have also been considered (Marks *et al*., 2003).

Among lifestyle modifications that can be employed are heart-healthy diet, regular exercises, ideal weight control, reduction in alcohol intake and cessation of smoking. There are currently plant sterol and stanols products that can be used in the healthy diet to help improve cholesterol lowering. These products work by blocking cholesterol absorption in the gut and can lower LDL cholesterol by up to 15% (Hendriks *et al*., 1999; Katan *et al*., 2003).

Lipid lowering agents are widely prescribed to FH patients and maximum benefits can be obtained if medication is started early (Umans-Eckenhausen *et al*., 2003). The commonly prescribed classes of lipid lowering drugs are statins, fibrates, bile acid sequestrants (resins), intestinal cholesterol absorption inhibitors (Ezetimibe) and nicotinic acids (Charlton-Menys & Durrington, 2008). Since the discovery of compactin (Mevastatin) by Akira Endo in 1976 (Steinberg, 2006a) and the introduction of lovastatin as the first statin in the late of 1987, statins have become the most widely prescribed therapeutic agent for patients with FH (Tobert, 2003). They are by far the most powerful, consistent, best tolerated and complied-with other agents of cholesterol reducers (Stein, 2002). Mevastatin and lovastatin are natural products derived from *Penicillium citrinum* (Endo *et al*., 1976a; Endo *et al*., 1976b) and *Aspergillus terreus* (Alberts *et al*., 1980) respectively. Currently, there are six statins approved for

treatment of hypercholesterolaemia; lovastatin (Havel *et al*., 1987), simvastatin (Pederson *et al*., 1996), pravastatin (Shepherd *et al*., 1996), fluvastatin (Serruys *et al*., 2002), atorvastatin (Sever *et al*., 2003) and rosuvastatin (Rubba *et al*., 2009). Although, lovastation is a natural product, the other statins are either semi-synthetic or synthetic derivatives (Stein, 2002; Tobert, 2003). Simvastatin is a semi-synthetic derivative of lovastatin, while pravastatin is derived from the natural product of compactin (Mevastatin) by biotransformation. The remaining statins are completely synthetic products (Tobert, 2003).

The safety and efficacy of statins have been tested in a large double-blind placebo controlled randomised trials (Sacks *et al*., 1996; Shepherd *et al*., 1995; Downs *et al*., 1998; The Scandinavian Simvastatin Survival Study, 1994). These trials demonstrated the effectiveness of statin therapy in general population with elevated cholesterols levels (Steinberg, 2006b). Simon Broome Steering Committee has reported in 1999 that a decline in the relative risk for coronary mortality was seen in FH patients aged 20 to 59 years when statins use became more widespread. Monotherapy with statins often fail to reduce LDL-c concentrations adequately (Marks *et al.,* 2003). Combination therapy with cholesterol lowering agents of different mechanism of action is often needed to achieve target LDL-c levels. Combination of statin with exetimibe can reduce LDL-c by 18% (Leitersdorf, 2001; Leitersdorf, 2002; Gagne *et al*., 2002).

The use of LDL-apheresis has become increasingly important in the treatment of FH in recent years (Gordon & Saal, 1996; Thompson, 2003). LDL-apheresis is usually reserved for homozygous FH patients not responding to drug therapy (Park *et al*., 1998). LDL-apheresis is an invasive and expensive but safe procedure (Bambauer, 2002; Hudgins *et al.*, 2002). It has been shown that LDL-apheresis can be used safely

in pregnant FH women (Cashin-Hemphill *et al*., 2000). A few studies suggest that aggressive lipid-lowering therapy using a combination of LDL-apheresis and statin drugs can cause regression of coronary plaques in patients with FH (Matsuzaki *et al*., 2002; Barter, 2002).

2.4 Low Density Lipoprotein Receptor (LDLR)

In 1973, Goldstein and Brown have discovered the LDLR on cultured human skin fibroblasts (Goldstein & Brown, 1973). LDLR is a cell-surface glycoprotein that binds the apolipoprotein B on the LDL particle (Defesche, 2004). The clearance of cholesterol and cholesteryl ester-containing LDL particles from blood into cells is mediated by the LDLR (Goldstein & Brown, 1985). The LDLR is produced in the endoplasmic reticulum and is present at the surface of most cell types (Varret *et al*., 2008). The glycosylated mature LDLR reaches the cell's surface and is directed towards clathrin-coated pits where it binds to ApoB-enriched and ApoE-enriched lipoproteins via its extracellular domain (Anderson *et al*., 1977). The complex is endocytosed and migrates to the endosomes. Acidic environment of the endosome triggers the release of the bound LDL particle and later degraded in lysosomes (Davis *et al*., 1987a; Costet *et al*., 2008). The LDLR returns to the membrane and enter a new cycle (Brown *et al*., 1977; Brown *et al*., 1983). The LDLR pathway is shown in Figure 2.4.1.

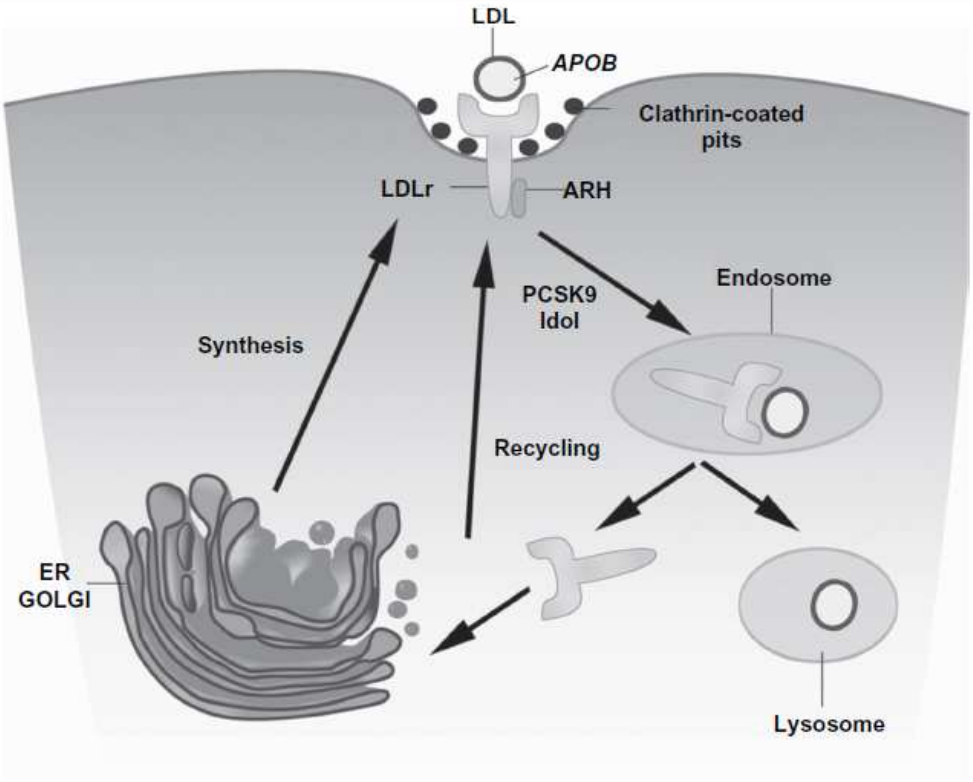


Figure 2.4.1 The LDL receptor pathway.
Notes: The low-density lipoprotein receptor (LDLr) is synthesized as a 120 KDa precursor protein and processed in the golgi apparatus (GOLGI) producing the glycosylated mature receptor that is transported to the cell surface and is directed towards clathrin-coated pits through interactions involving LDL particle, enriched with apolipoprotein B (*APOB*) where it binds to LDL particle. The complex is transported to endosomes where the acidic pH causes a dissociation of the receptor–ligand complex, releasing the LDLr to its recycling so the LDL is degraded in the lysosomal compartment. The proprotein convertase subtilisin/kexin type 9 (PCSK9) and Idol protein participate in a decreasing of receptor recycling and increasing the LDLr degradation.

Figure 2.4.1: Schematic diagram illustrates the LDL receptor pathway.

The mature form of human LDLR protein contains 839 amino acids residues with a molecular mass of 160kDa (Yamamoto *et al*., 1984). It can be broadly divided into five domains. The first domain is N-terminal ligand-binding domain that mediates the binding to LDL, followed with the epidermal growth factor (EGF)-precursor homology domain that is required for the dissociation of lipoprotein from LDLR during receptor recycling. The third domain is the O-linked polysaccharide domain that functions as acceptor sites for O-linked sugar, followed by the hydrophobic trans-membrane domain. Lastly, the C-terminal cytosolic or cytoplasmic tail domain that directs the receptor to clathrin-coated pits (Sudhof *et al*., 1985; Soutar & Knight, 1990).

The domain structure of the human LDL receptor and its relation to the exon organisation of the LDLR gene is shown in Figure 2.4.2.

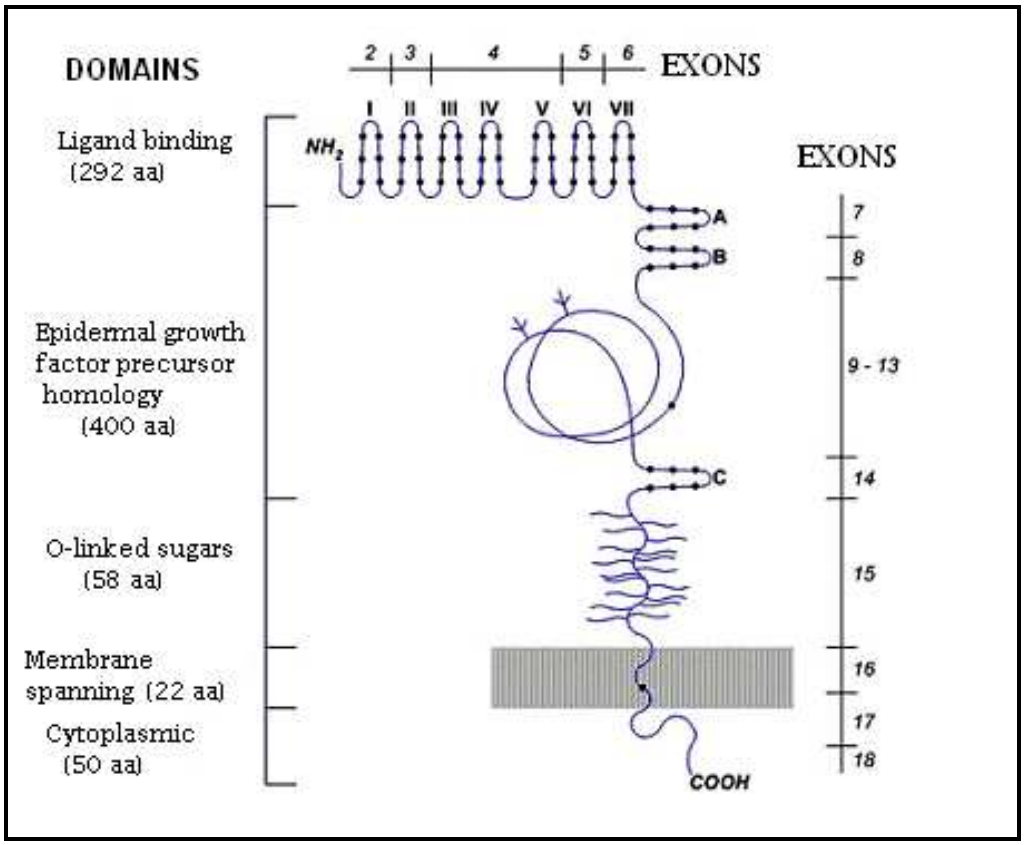


Figure 2.4.2: Schematic diagram represents the domain structure of the human LDL receptor and its relation to the exon organisation of the LDLR gene (adapted from Soutar & Knight, 1990).

The human LDLR is encoded by LDLR gene located in the short arm of chromosome 19 at 19p13.1-p13.3 (Lindgren *et al*., 1985; Francke *et al*., 1984). The location of the LDLR gene on chromosome 19 is illustrated in Figure 2.4.3.

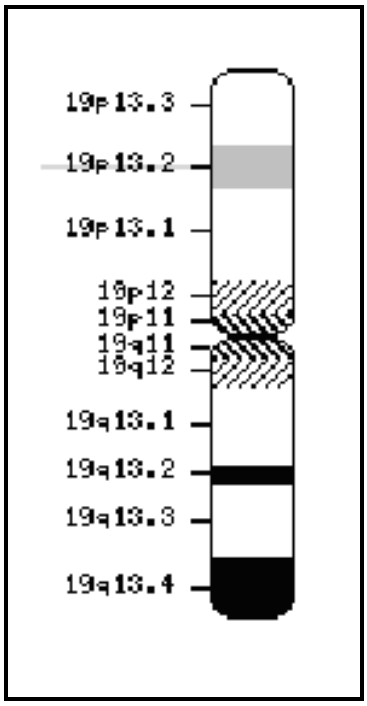


Figure 2.4.3 Ideogram showing the location of LDLR gene on chromosome 19 at 19p13.1-p13.3.

LDLR gene spans approximately 45kb and consists of 18 exons and 17 introns (Lindgren *et al*., 1985; Sudhof *et al*., 1985; Hobbs *et al*., 1990). The promoter region spans 177bp and is located on the 5'-flanking region, within which the majority of the *cis*-acting DNA elements are found between base pairs (bp) -58 and -234, with A of the initiator methionine codon as +1 (Kong *et al*., 2006).

Twenty-one amino acids of the signal sequence which is cleaved from the protein during translocation into endoplasmic reticulum are encoded by Exon 1 of LDLR (Russell *et al*., 1986; Varret *et al*., 1997). The ligand-binding domain of LDLR is encoded by exons 2-6, which is made up of seven repeats of 40 amino acids each

(Sudhof *et al*., 1985; Varret *et al*., 1997). Exons 7-14 encode a 400 amino acids sequence that is 35% identical to a portion of the human EGF precursor gene (Russell *et al*., 1984; Davis *et al*., 1987a; Varret *et al*., 1997).

Fifty-eight serine and threonine residues-enrich amino acids which serve as attachment sites for O-linked sugar chain are encoded by Exon 15 (Davis *et al*., 1986; Varret *et al*., 1997). The 3' end of exon 16 and the 5' end of exon 17 encode the 22 hydrophobic amino acids of the trans-membrane domain that serves to anchor the LDLR within the plasma membrane (Brown *et al*., 1997; Varret *et al*., 1997). A mutant that is truncated in this region has a reduced capacity to remain attached to the plasma membrane and is also found free in the plasma (Lehrman *et al*., 1985).

The remaining of exon 17 and the 5' end of exon 18 encode the 50 amino acids that make up the cytoplasmic domain that serve as the localization of the LDLR in coated pits on the cells surface (Davis *et al*., 1987b; Chen *et al*., 1990). The remaining of exon 18 specifies the 2.6kb of 3' untranslated region of mRNA (Varret *et al*., 1997).

Over 1200 mutations in the LDLR gene have been identified and reported world-wide (Leigh *et al*., 2008, Kolansky *et al*., 2008; Stenson *et al*., 2009a; Stenson *et al*., 2009b, Stenson *et al*., 2008; Stenson *et al*., 2003; Heath *et al*., 2000; Villeger *et al*., 2002; Hobbs *et al*., 1992). The identified mutations to date comprise small deletions, insertions, duplications, missense mutations, major rearrangement, premature stop codons, single amino acid substitutions, mutation of promoter region well as splicing defects (Soutar & Naoumova, 2007; Gent & Braakman, 2004; Villeger *et al*., 2002). The mutations of LDLR gene have been classified into five functional classifications depending on the nature of the receptor defect (Goldstein *et al*., 2001).

Class 1 mutations are known as ‘null-alleles’, which disrupt the receptor’s synthesis in the endoplasmic reticulum. These mutations are characterised by the absence of messenger RNA (mRNA) and can be due to LDLR promoter deletion, by frameshift, nonsense, spilicing mutations or rearrangements (De Castro-Oros *et al*., 2010).

Class 2 mutations are transport-defective alleles which affect the transport of the LDLR from endoplasmic reticulum to Golgi apparatus (Austin *et al*., 2004a). Class 2 mutations can be grouped into class 2A and 2B, indicating either a complete block or a reduced rate of transport respectively (Defesche, 2004). This class of defects are normally caused by missense mutations or small deletions located within the exons that encode ligand-binding and EGF-precursor homology domains (Yamamoto *et al*., 1984).

Mutations that interfere with cell surface binding of the LDL particles are classified as Class 3 mutations. These defects produce LDLR that are transported to the cell surface but fail to bind LDL normally (Varret *et al*., 1997). These defects are due to rearrangements in repeat cysteine residues in ligand-binding and EGF-precursor homology domains (Lehrman *et al*., 1985).

Mutations that prevent the internalization of the ligand-receptor complex belong to class 4. These mutations produce proteins that are not able to group into clathrin-coated pits and internalization of bound LDL particles cannot take place. If the mutations affect cytoplasmic domain alone, they are group into class 4A, however if trans-membrane domain also affected, they are group into class 4B (Hobbs *et al*., 1992).

Mutations that block the acid-dependent dissociation of receptor and ligand in the endosome making the receptor fails to be recycled to the cell surface are categorised as Class 5 mutations (Davis *et al*., 1987b; Hobbs *et al*., 1992). This class of mutations

cluster in the 5' end of the EGF-precursor homology domains (van der Westhuyzen *et al*., 1991).

2.5 Mutation Detection Techniques

Genetic diagnosis of FH depends on the identification of a mutation in the LDLR gene. DNA-based mutation screening methods play an important role in the definitive diagnosis of FH (Marks *et al*., 2000; van Aalst-Cohen *et al*., 2006; Humpries *et al*., 1997a). The diagnosis of FH in approximately 15-20% of known FH family members is made based on a DNA test where measurement of cholesterol levels alone would not have established the diagnosis (Koivisto *et al*., 1992; Koivisto *et al*., 1993; Ward *et al*., 1996).

There are several approaches available for DNA-based mutation screening methods (Cotton, 1993; Grompe, 1993; Mashal & Sklar, 1996; Shi, 2001), although all have disadvantages either with regard to the use of toxic chemicals, or radioisotopes, or have issues with specificity and sensitivity (Humpries *et al*., 1997). The tools for mutation detection, in both scanning and diagnostic modes, are improving steadily (Cotton, 1997). While several useful technologies for mutation detection exist, no single method is applicable to all situations (Vago & Pena, 1997). Thus, a myriad of new techniques have been developed for the detection of mutations in DNA, most of them based on the polymerase chain reaction (PCR) (Vogel & Motulsky, 1986; Taylor, 1997).

Mutation detection techniques can be divided generally into techniques that test for known mutation (genotyping) and those that detect any unknown mutation in a particular target region (mutation scanning) (Taylor & Taylor, 2004; Cotton, 2000).

The ultimate technique for detection and confirmation of mutations is direct sequencing but it involves significant cost and labour (Sivakumaran *et al*., 2003).

The important considerations in any approach to mutation detection are sensitivity (the proportion of mutations that can be detected) and specificity (the proportion of false-positive) (Taylor & Taylor, 2004). With the increasing availability of primary sequence from genomes of human and other species, there is a need for high-throughput, high-accuracy, highly sensitive and specific mutation detection method for identifying disease- causing sequence variation (Xiao and Oefner, 2001).

The commonly used detection methods in FH cases are single strand conformation polymorphism (SSCP) (Humphries *et al*., 1997b; Sozen *et al*., 2004), denaturing gradient gel electrophoresis (DGGE) (Lombardi *et al*., 1995; Nissen *et al*., 1995), oligonucleotide ligation assay (Baron *et al*., 1996), denaturing high performance liquid chromatography (DHPLC) (Bodamer *et al*., 2002), DNA-array (Alonso *et al*., 2009), multiplex ligation-dependent probe amplification (MLPA) (Taylor *et al*., 2010), and direct sequencing (Romano *et al*., 2010; Blesa *et al*., 2006).

2.6 Denaturing High Performance Liquid Chromatography (DHPLC)

Since DHPLC was developed in 1995 (Oefner & Underhill, 1995; Oefner & Underhill, 1999; Xiao & Oefner, 2001), it has emerged as one of the most popular methods for the analysis of genetic variations. The DHPLC system has three modes of operations, depending on the temperature of the column (Xiao & Oefner, 2001). Depending on the mode of operation, chromatographic analysis using DHPLC can be used to detect single nucleotide substitution or small insertion or deletions in double-stranded DNA

fragments as well as to analyze and purify single stranded nucleic acids (Xiao & Oefner, 2001).

For most purposes, chromatographic analysis of nucleic acid is performed under partially denaturing conditions for screening of putative single nucleotide polymorphisms (SNPs) or detection of unknown mutations (Sivakumaran *et al*., 2003). Other modes of Wave System are performed under non-denaturing and completely denaturing conditions. Non-denaturing condition is used at column temperature of 50^{o}C and applied to accurately size and quantify PCR primers (Huber *et al*., 1995). Analysis in shorter DNA fragments of size 50-100 bp is performed under completely denaturing condition such as products of primers extensions and synthetic oligonucleotides, as well as RNA.

With the increasing number of genome sequences completed, DHPLC has established itself as one of the most powerful tools for DNA variation screening and allele discrimination (Xiao & Oefner, 2001; Frueh & Nover-Weidner, 2003). More than 350 human genes have been studied using DHPLC (http://insertion.stanford.edu/hplc_genes1.html) with the first gene subjected to DHPLC analysis was the calcium channel gene CACNL1A4 (Ophoff *et al*., 1996). Apart from its predominant application in identifying the mutations in human diseases (Pfeiffer *et al*, 2002; Brightwell *et al*., 2002), DHPLC has provided insight into the human evolution and prehistoric migration based on the screening of Y chromosomal and autosomal DNA sequences (Jin *et al*., 1999 ; Underhill *et al*., 2000). It has also been implemented in the quantitative measurement of gene expression (Doris *et al*., 1998) and the analysis of single nucleotide extension products (Hoogendoorn *et al*., 1999).

However, DHPLC is not suitable for analysis of large gene deletions and multiple copy number aberrations (Marsh & Howell, 2010).

DHPLC analysis is an ion-pair reversed-phased high performance liquid chromatography for performing analytical separations of DNA based on temperature sufficient to partially denature DNA heteroduplexes (Lo *et al*., 2006; Sivakumaran *et al*., 2003; Oefner & Huber, 2002). Consideration that has to be taken into account in the DHPLC analysis is the selection of PCR reagents. PCR-associated materials such as Proteinase K, formamide or large concentration of high molecular weight carrier such as bovine serum albumin (BSA) should be avoided in order to prolong the life span of the column since these materials can damage and block the column (Mitchell & Cutler, 2011).

During DHPLC analysis, DNA fragments are carried by mobile phase consists of 0.1M triethylammonium acetate (TEAA) in Buffer A and 25 % acetonitrile in Buffer B. Separation of DNA fragments by size take place in the DNA Separation Column. The mobile phase is pumped by through the DNA Separation column and detector, and subsequently into the waste reservoir. Separation of DNA fragments are detected by a UV detector and the signal is converted into a digital value (Hannachi-M'Zali *et al*., 2002). The results are presented as chromatograms which are series of peaks corresponding to DNA fragments (Lam, 2006). Figure 2.6.1 represents the schematic of the DHPLC system.

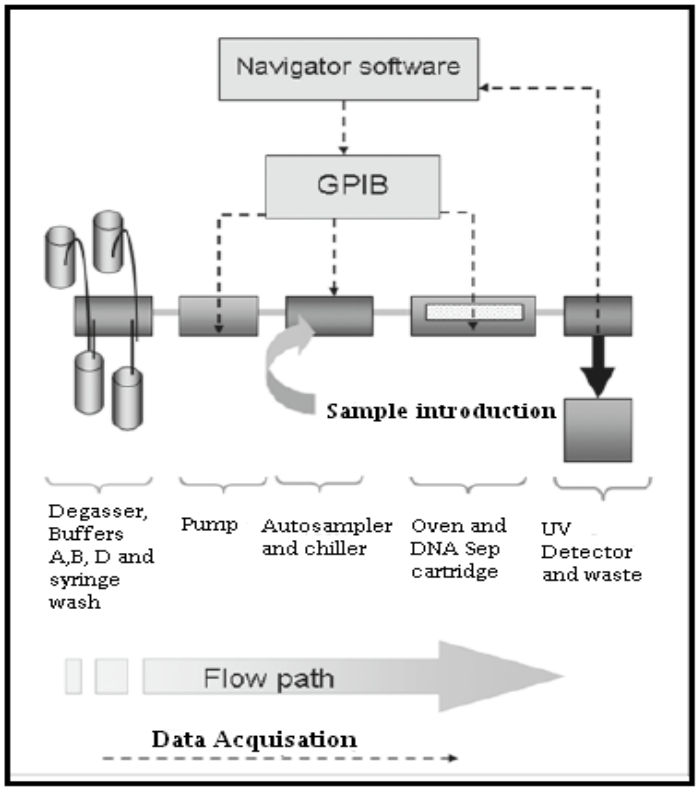


Figure 2.6.1: Schematic of DHPLC system showing the placement of hardware and the flow direction of the mobile phase. GPIB-general purpose interface board.

DHPLC detects sequence variation based on the different retention of homo- and heteroduplex DNA fragments in the separation column when wild-type and mutant DNA samples are denatured and re-annealed together (Donohoe, 2005; Rugg & Magee, 2005). The presence of ion pairing reagent TEAA and a linear gradient of acetonitrile have enabled the movement of the DNA fragments through the column (Marsh & Howell, 2010). At partial denaturing temperature of DHPLC, the less stable heteroduplexes are eluted faster than the homoduplex DNA (Harvey & Sampson, 2004).

When non-mutant samples are analysed, a single peak represents homoduplex is observed in the chromatogram; whereas, when mutant samples are injected, two or more peaks represent heteroduplex will be detected (Sivakumaran *et al*., 2003; Mitchell

& Cutler, 2011). Figure 2.6.2 illustrates the separation of heteroduplex and homoduplex on DHPLC.

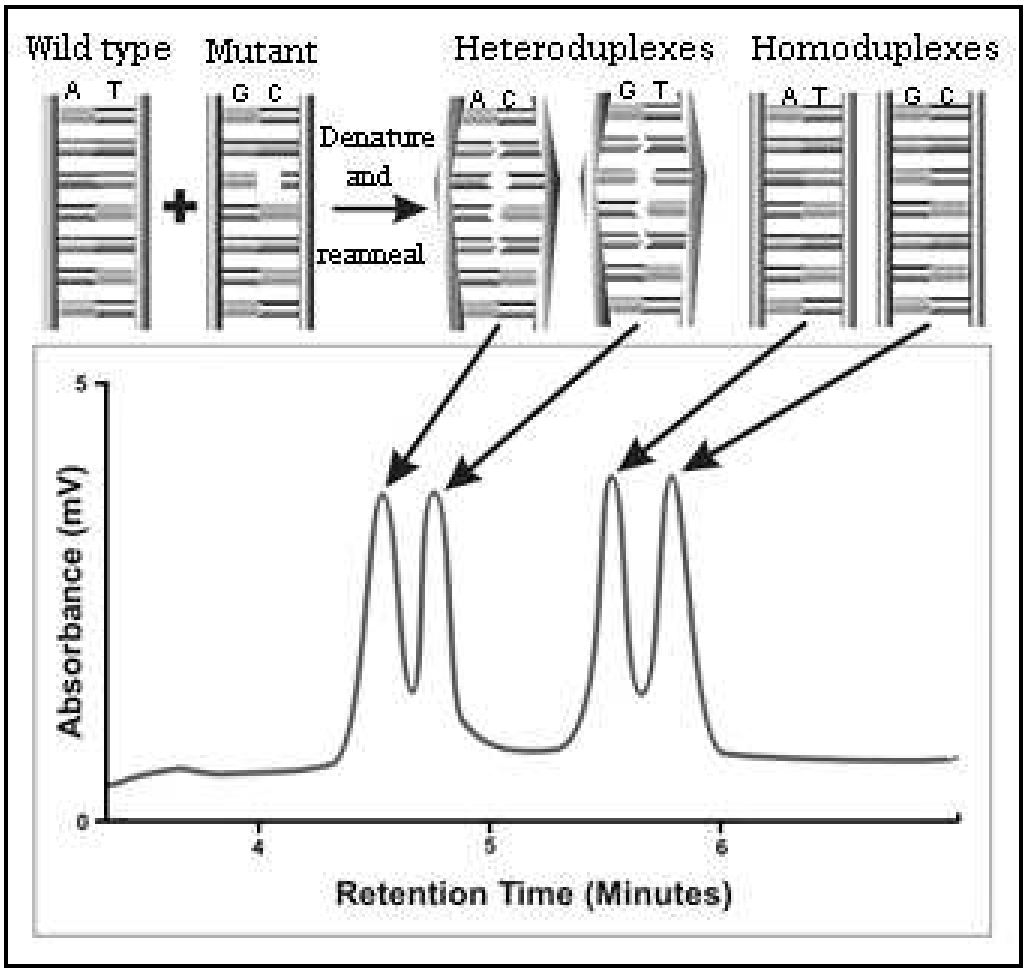


Figure 2.6.2: Use of DHPLC to separate homoduplexes and heteroduplexes. The illustration shows the complete resolution of homo- and heteroduplexes formation following heating and controlled-rate cooling cycle.

Every test samples are assayed with the presence of at least one homozygous wild type sample that acts as a comparator sequence (Leung and Yip, 2008). Any difference in the elution profile is indicative of the presence of sequence variation (Leung and Yip, 2008; Sivakumaran *et al*., 2003).The analysis temperature is critical for the success of mutation detection by DHPLC, and it is necessary to run samples at more than one temperature during optimisation process (Sivakumaran *et al*., 2003). Retention times at different temperatures are determined and a melt curve is plotted to ensure the appropriate temperature for analysis of an individual DNA fragment (Oefner & Underhill, 1995; Oefner, 2000). The melting temperature (*T*m) of the DNA fragment may also be determined from the melting curve calculated by the melting temperature software such as WAVEMaker^{TM} from Transgenomic, USA. Figure 2.6.3 illustrates the melt curve profile at different temperatures.

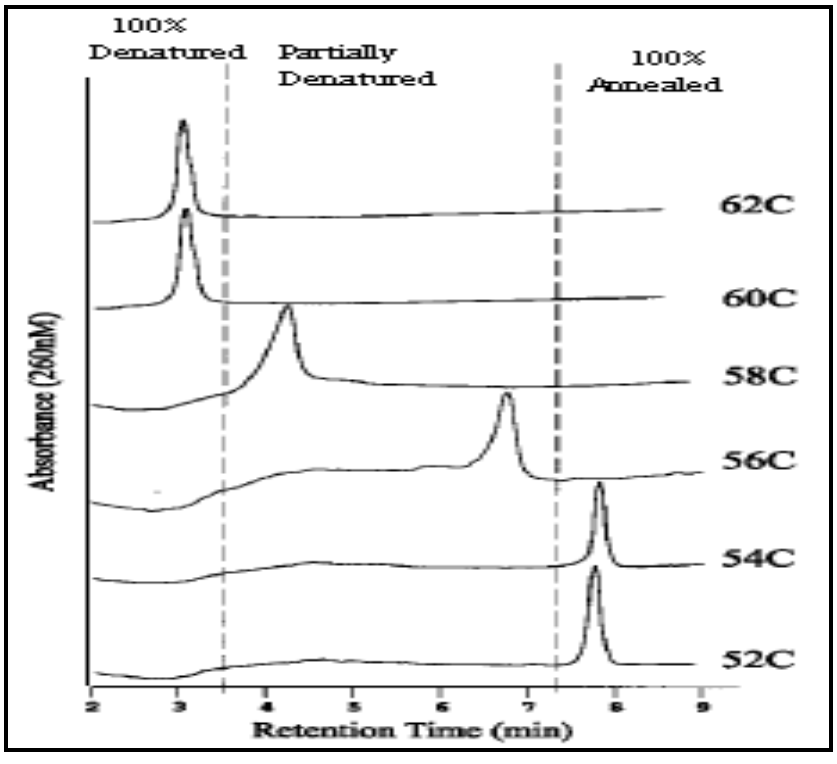


Figure 2.6.3: Melting curve of a DNA fragment at different temperature showing peak at different retention time on DHPLC.

Many studies have been done to compare DHPLC with other mutation scanning techniques including SSCP (Bunn *et al*., 2002; Yamanoshita *et al*., 2005) and DGGE (Breton *et al*., 2003), conformation sensitive gel electrophoresis (CSGE) (Khorram Khorshid & Dalgleish, 2008), protein truncation test (PTT) (Andrulis *et al*., 2002), and two dimensional gene scanning (TDGS) (Eng *et al*., 2001). The sensitivity of DHPLC has been widely studied and is estimated at 97 % or greater (Jones *et al*., 1999a; Ellis *et al*., 2000). Jones and colleagues reported that DHPLC detected 96 % of all mutations in PCR products varying in sizes from 173 bp to 690 bp in comparison to SSCP that detected only 85 % of the mutations (Jones *et al*., 1999b; Jones *et al*., 2001). Another study on BRCA1 gene, the sensitivity and specificity of DHPLC and SSCP were reported to be 100 and 94 % respectively (Gross *et al*., 1999).