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).
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 lifestyle (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 Humphries, 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 Committee 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>
<thead>
<tr>
<th>Age (years)</th>
<th>First-degree relative with FH†</th>
<th>Second-degree relative with FH</th>
<th>Third-degree relative with FH</th>
<th>General population</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>5.7</td>
<td>5.9</td>
<td>6.2</td>
<td>7.0</td>
</tr>
<tr>
<td>20–29</td>
<td>6.2</td>
<td>6.5</td>
<td>6.7</td>
<td>7.5</td>
</tr>
<tr>
<td>30–39</td>
<td>7.0</td>
<td>7.2</td>
<td>7.5</td>
<td>8.8</td>
</tr>
<tr>
<td>≥40</td>
<td>7.5</td>
<td>7.8</td>
<td>8.0</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Diagnosis (FH is diagnosed if total cholesterol levels exceed the cutpoint)

† 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>
<thead>
<tr>
<th>Table 2.3.2 Simon Broome Familial Hypercholesterolemia Register diagnostic criteria for familial hypercholesterolemia*</th>
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</thead>
<tbody>
<tr>
<td><strong>Criteria</strong></td>
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<tr>
<td>a</td>
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<td>b</td>
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<td>c</td>
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<td>d</td>
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<td>e</td>
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† 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.
2.3.3 Management and Treatment of FH

Once an individual is diagnosed with FH, the management and treatment is usually lifelong. 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: 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.](image)

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
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, splicing 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; Humphries 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 (Humphries 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°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™ from Transgenomic, USA. Figure 2.6.3 illustrates the melt curve profile at different temperatures.

![Melt curve profile at different temperatures on DHPLC](image)

**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).