2. REVIEW OF LITERATURE

2.1 CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) present the spectrum of diseases which involve heart, brain and peripheral circulation, therefore covering a wide range of disease phenotypes and severity. CVDs are major cause of mortality worldwide. In the year 2015, there were about 422.7 million prevalent cases of CVDs and about 17.92 million deaths accounting to the CVDs (Roth, Johnson et al. 2017) and the number of people affected from CVDs is anticipated to increase to 23.3 million by 2030 (Roth, Johnson et al. 2017). In India, three large prospective studies reported high mortality due to CVD (30–42%) and age-standardized CVD mortality rates to be 255–525/100,000 population in males and 225–299/100,000 population in females (Joshi, Islam et al. 2007; Pednekar, Gupta et al. 2011; Soman, Kutty et al. 2011). CVDs are amongst the top five reasons for mortality in the Indian population (Gupta, Guptha et al. 2012). Indians are affected a decade earlier in the most productive years with CVDs as compared to European descendants (Joshi, Islam et al. 2007; Xavier, Pais et al. 2008). In the Western populations, only 23% deaths in individuals <70 years of age are accountable to CVD whereas the number doubles to 52% for Indians (Harikrishnan, Leeder et al. 2014). India has witnessed a dramatic epidemiological transition in the last 2 decades which has led to a shift from the diseases of under nutrition, infectious diseases, and the maternal and childhood diseases to the non-communicable diseases (Gayathri, Ruchi et al. 2017).

The different CVDs are briefed below (Table 2-1):

Table 2-1: Different Cardiovascular diseases

| Aortic aneurysm/ Abdominal aortic aneurysm (AAA) | Large blood vessel supplying blood to the abdomen, pelvis and legs becomes unusually large/balloons outward. |
| Coronary artery disease (CAD) | The plaque buildup in coronary arteries leads to narrowing of the lumen and hence obliterating blood flow to heart. |
| Cardiomyopathy | Disease of myocardium that correlates with mechanical and/or electrical dysfunction usually exhibiting dilation or ventricular hypertrophy mainly due to genetic causes. They can be classified as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restricted cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC). |
Review of Literature

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital heart disease</td>
<td>Any defect of the heart or the central blood vessels which is present by birth such as abnormalities of heart valves, hole between the heart chambers, etc.</td>
</tr>
<tr>
<td>Peripheral arterial disease (PAD)</td>
<td>Arteries supplying blood to legs either become narrowed or get completely blocked off.</td>
</tr>
<tr>
<td>Rheumatic heart disease (RHD)</td>
<td>The heart valves are damaged by rheumatic fever caused by streptococcal infection.</td>
</tr>
<tr>
<td>Stroke</td>
<td>It refers to acute neurological injury when arterial blockage or rupture (haemorrhage) blocks blood supply to a part of brain, thus depriving it of oxygen and leading to cell death and impairing functioning of that particular part. If not quickly diagnosed and treated, can cause permanent neurological damage or death.</td>
</tr>
</tbody>
</table>

2.2 CORONARY ARTERY DISEASE

CAD or atherosclerotic heart disease or ischemic heart disease (IHD) or coronary heart disease (CHD) is major reason of deaths worldwide. CAD as the name indicates, is the disease of the arteries (both medium and large sized). The characteristic feature of CAD is atherosclerosis *i.e.* the buildup of plaques comprising of lipids, calcium, modified lipids, leukocytes, vascular smooth muscle cells (VSMCs) (Galkina and Ley 2009) known as “atheroma” covered by fibrous cap in arteries. Rupture of plaque leads to the blood clot formation on the surface. If a large clot forms, the complete occlusion of the coronary artery occurs and which leads to deficient supply of oxygen to myocardium.

2.2.1 Prevalence of CAD

“The 2017 Heart Disease and Stroke Statistics” update of the American Heart Association (AHA) has currently documented that about 16.5 million people aged ≥20 years in U.S. have CAD, whilst the prevalence have been reported to increase with increasing age for both the genders (Sanchis-Gomar, Perez-Quilis et al. 2016). In U.S., total CAD prevalence is about 6.3% in adults ≥20 years of age and further, 7.4% for men and 5.3% for women (Lloyd-Jones, Adams et al. 2010). It accounts for a third of adult deaths in individuals above 35 years in U.S. (Lloyd-Jones, Adams et al. 2010).

The regional diversity in India with diverse geographical and cultural conditions has led to diverse lifestyle habits leading to a diverse prevalence of CAD in India.
Moreover there is a huge difference in the urban and the rural populations hence, leading to a different prevalence of CAD according to the different regions. Prevalence rates of CAD in the urban areas of North Indian states viz. Delhi, Jammu and Kashmir and Uttar Pradesh and in the Western state such as Rajasthan is documented to be around 6–10% whereas in rural areas, it was reported to be 6–7% in Jammu and Kashmir, 3–5% in Himachal Pradesh, Punjab and in Rajasthan, it was 3–5% (Gupta, Joshi et al. 2008). In Delhi, CAD prevalence was 14.8% (urban) and 9.7% (rural) (Mahajan, Chaturvedi et al. 2012) whereas in Lucknow, it was reported to be 8.8% in urban and 3.8% in the rural areas (Joshi, Idris et al. 2013). In the South Indian state, Andhra Pradesh, overall prevalence of CAD was 5.4% (Murthy, Prasad et al. 2012). Studies conducted in the urbanized states like Mumbai and Kerala showed incredibly high mortality rates and approaching 500/100,000 for males and 250/100,000 for females (Pednekar, Gupta et al. 2011; Soman, Kutty et al. 2011). Recent data reports highest mortality in Punjab, Southern states, Eastern and North-Eastern states and lowest while mortality due to CVD is observed in Central states of Rajasthan and Bihar (Chauhan and Aeri 2015).

2.2.2 History of CAD

The immense field of the cardiovascular research dates back to 26th Century BC, when the Chinese Emperor Huang Ti stated that the blood flows in circulatory system inside the body and is controlled by heart. The onset of the knowledge about CAD is difficult to date precisely. Leonardo da Vinci (1452–1519) and Andreas Vesal (1514–1564) were the first to explain the coronary arteries and work on them also. Leonardo investigated and reported the coronary arteries and described “the coronary arteries are embedded in greasy material”. The key milestone was the discovery of closed blood circulation by William Harvey (1628) who is also known as “The father of Cardiology”. Following Harvey, the field of cardiology chased a new path in 17th and 18th centuries of descriptive anatomy and pathology, auscultation and its correlation in 19th century, the comprehension of the CVDs and its pathophysiology in later half of the 19th and the first half of 20th centuries and then to the major advancements in the field of diagnosis and treatment in 21st century (Willius and Dry 1948; Aciero 1994; Silverman 1999; Rolleston 2014).

2.2.3 Clinical forms of CAD

Several people live an asymptomatic life entirely despite of having plaque in the coronary arteries. However, some people develop the symptoms. The ischemic heart
problems of atherosclerotic CAD can be categorized in two: chronic stable angina and acute coronary syndromes (Kumar 2010).

2.2.3.1 Chronic stable angina (CSA)

CSA occurs when the coronary arteries have narrowed down and compromised the oxygen supply to the myocardial arteries to an extent that it is unable to cope up with the demands of the hard working heart. CSA is clinically characterized by retrosternal discomfort (heaviness) and pain presenting on exercise and subsiding when the patients stop the exercise. The pain is short-lived and has a predictable occurrence. It can be triggered by exercise, trauma, changes in weather or by strong emotions.

2.2.3.2 Acute coronary syndromes (ACS)

The sudden impulsive unpredictable episode of severe heart ischaemia is termed as ACS. It results from disruption of atherosclerotic plaque and causing severe ischaemia adequate enough to lead to destruction of the myocardial cells. It requires instant treatment in emergency room (Kim, Kini et al. 2011). The following are clinical presentations of ACS:

(a) Sudden cardiac death (SCD)

Most disastrous form of ACS is the SCD. It refers to the unanticipated death occurring from cardiac issues promptly within hour of first symptom presentation. In majority of the cases in adults (in 80-90% of patients), SCD is usually related with CAD (Kim, Kini et al. 2011).

(b) Myocardial infarction (MI)

MI is the most common type of ACS in which myocardial necrosis is caused by an unstable ischemic syndrome (Thygesen, Alpert et al. 2013). The plaque rupture and the blood clot formation leads to complete occlusion of the coronary arteries or its branches leading to complete blockage of the oxygen supply to that specific area. When the artery remains occluded for >30 minutes, cell damage will be irreparable. Therefore, prolonged ischaemia leading to myocardial cell death defined as MI and the damaged area is named as myocardial infarct. In epidemiological studies, MI incidence is used interchangeably with CAD prevalence (Thygesen, Alpert et al. 2013).
Acute MI is classified according to the presence or absence of ST-segment elevation on the ECG and can be clinically presented as ST elevated MI (STEMI) or Non-ST elevated MI (NSTEMI). In STEMI, an ST-segment elevation due to myocardial necrosis is observed in an ECG and if ST-segment depression or prominent T wave inversion is observed accompanied with positive biomarkers of necrosis in absence of ST-segment elevation is observed, that is termed as NSTEMI (Kim, Kini et al. 2011).

(c) Unstable angina (USA)

In an episode of USA, the ischaemic symptoms do not subside even after rest for 5-10 minutes. These episodes are witnessed in sudden disrupting plaques and the arterial occlusion might resolve spontaneously. A comparative less damage is observed in USA as compared to MI but it foreshadows the consequent MI event. With ECG, USA is defined as ST-segment depression or a prominent T wave inversion accompanied with non-elevation of the cardiac necrosis biomarkers.

2.2.4 Symptoms of CAD

As defined by World Health Organization (WHO) 2017 (http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)), the symptoms for CAD include:

- Chest pain, chest tightness, chest pressure and chest discomfort (angina)
- Shortness of breath
- Fainting (syncope) or near fainting
- Pain radiating to the neck, jaw, throat
- Pain or discomfort in the arms or shoulder
- Feeling weak, lightheaded, or nauseous

2.3 ATHEROSCLEROSIS

Atherosclerosis term was coined by Johann Lobstein and is a combination of two Greek words- “athero” meaning gruel and refers to necrotic core of an atherosclerotic plaque and “sclerosis” meaning induration or hardening and refers to fibrotic cap of plaque. It is a chronic condition in which artery narrows down as a consequence of fat deposition in its wall. Such bulges are referred to as atherosclerotic plaques.

2.3.1 Pathophysiology of atherosclerosis

The “response to injury” theory on pathophysiology of atherosclerosis is the most acceptable theory these days. Atherosclerosis is described as a complex interaction
between both the immune and non-immune mediated mechanisms, beginning from the intimal thickening to the complicated atheroma formation.

![Figure 2-1: Stages of the development of atherosclerosis. Adapted from (Nabel and Braunwald 2012).](image)

The lumen of the blood vessels is lined by endothelium that separates the vessel wall from the blood. The endothelial cells (ECs) of the wall of the arteries exhibit resistance towards adhesion and leukocyte aggregation and thereby, fibrinolysis. However, upon activation by triggers like oxidized lipoproteins, stress, smoking, inflammation, hypertension (HTN), obesity and unhealthy eating habits, pro-inflammatory cytokines and a number of adhesion molecules are expressed by ECs that further lead to the recruitment of other leucocytes.
The pathophysiology of CAD can be explained by the different stages of atherosclerosis:

**Stage I:** The first step towards atherogenesis involves the adhesion of the blood monocytes to the leucocyte adhesion molecules on the activated ECs (not expressed by normal ECs). This adhesion triggers the pro-inflammatory chemokines to release chemotactic stimuli that stimulates monocytes to enter the intima layer. Blood leukocytes migration is followed by maturation of monocytes into macrophages (which express low density lipoprotein receptor (LDLR) for engulfing modified low density lipoprotein (LDL)) which are accompanied by lipid uptake, transforming into foam cells, the key atherogenic entities.

**Stage II:** Vascular smooth muscle cells (VSMCs) migrate from intima to media. A necrotic core forms comprising of lipids, cholesterol, T lymphocytes, activated macrophages, SMCs and microvessels in middle region of plaque shielded by fibrous cap (Willerson and Ridker 2004).

**Stage III:** Thrombosis results in exposing blood coagulant components to the plaque tissue factors leading to formation of an occlusive thrombus eventually leading to an obstructed flow of blood (Libby, Ridker et al. 2011) (Figure 2-1).

Extensive widespread plaque build-up, inflammation and the vulnerable plaques determine an individual’s susceptibility towards CAD thereby contradicting the idea of terming atherosclerosis a segmental or localized disease.

The pathophysiology and the clinical presentation of CAD can be precisely summed up as following (Figure 2-2):

**Figure 2-2: Pathogenic spectrum of CAD**
2.3.2 LDL modification and oxidization in atherosclerosis

Elevated serum levels of LDL and triglycerides are primarily accountable for the atherosclerotic lesion formation (Albertini, Moratti et al. 2002) and thus, LDL modification and lipid metabolism have vital role in atherosclerosis development. LDL retention in arterial wall is regarded as initial step in the pathogenesis of atherosclerosis (Wiśniewska, Olszanecki et al. 2017). Under conditions of chronic stress like smoking, HTN, hyperlipidemia, hyperglycemia, etc. the production of free radicals and reactive oxygen species (ROS) (hydroxyl radicals, nitric oxide, carbon-center radicals, superoxide, and the thyl and perthyl radicals) from mitochondrial respiratory chain, NADPH oxidases, lipoxygenases, myeloperoxidase and endothelial nitric oxide synthase (eNOS) is remarkably enhanced which overcomes the body’s own endogenous antioxidant response. This heightened oxidative stress thereby leads to oxidation of LDL, acetylation, glycation, lipolysis and proteolysis and hence, the impaired endothelial function (FROSTEGÅRD, Ruihua et al. 2003; Bloomer 2007; Choi, Harkewicz et al. 2009; Zhou, Chadipiralla et al. 2013).

The oxidized LDL is broadly categorized as: “minimally modified LDL (mm-LDL)” and fully or extensively “oxidized LDL (oxLDL)”. The mm-LDL is chemically dissimilar to unmodified LDL but still identified by LDLR and not the scavenger receptors. Whereas oxLDL is recognized by all scavenger receptors and not the LDLR (Levitan, Volkov et al. 2010).

The polyunsaturated fatty acids in LDL, majorly arachidonic acid and linoleic acid are the prime targets of oxidizing agents (Levitan, Volkov et al. 2010). Arterial sub-endothelial space is the site for LDL oxidation. This phenomenon does not occur in the circulation due to the abundant antioxidants in circulation like tocopherol, ascorbate, urate, apolipoproteins and serum albumin and also the oxLDL will get rapidly cleared off by reticulo-endothelial system.

(Goldstein, Ho et al. 1979) first demonstrated the uptake of modified LDL in the macrophages. The macrophages express scavenger receptor A (SRA), cluster of differentiation 36 (CD36), lectin-like oxidized-LDL receptor 1 (LOX1) and toll-like receptor 4 (TLR4) which have the potential to bind to the modified LDL. The modifications on the Apo B protein are recognized by the SRA and LOX1, CD36 recognizes the oxidized phospholipids and the TLR4 recognizes the oxidized
cholesterylesters. In the ECs, the major oxLDL uptake pathway is the LOX1 receptor (Levitan, Volkov et al. 2010).

oxLDL has numerous potent atherogenic activities (Linton and Fazio 2003). It serves as a substrate for the unregulated cholesterol uptake on the macrophages and induces the expression and secretion of the inflammatory cytokines (Frostegard, Haegerstrand et al. 1991; Frostegård, Ulf gren et al. 1999; Boisvert, Curnss et al. 2000). It also stimulates the proliferation of SMCs. It also provokes the chemotaxis of monocytes and eosinophils (Han, Hong et al. 2004; Hashimoto, Kataoka et al. 2007), enhances platelet aggregation (Wraith, Magwenzi et al. 2013; Magwenzi, Woodward et al. 2015) and also activates the dendritic cells to release T cell stimulating cytokines (Seo, Yang et al. 2015). The chemotactic activity of oxLDL also encourages the binding of monocytes to ECs, inducing the over-expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Frostegard, Haegerstrand et al. 1991; Cominacini, Garbin et al. 1997).

2.3.3 Role of macrophages

Macrophages are considered as the central effectors of the innate immunity and also defined as patho-physiologic mediators in CAD (Moore, Sheedy et al. 2013). The activation of endothelium in response to stress, signals monocyte recruitment at the injury site and migration across endothelium to plaques and is mediated by numerous chemokines released by the ECs, SMCs and intimal macrophages (Moore, Sheedy et al. 2013).

In the sub-endothelial space, the monocytes differentiate into macrophages and uptake LDL and changes to cholesterol-loaded “foam cells” (Moore, Sheedy et al. 2013). This clearance of LDL by macrophages is considered to have advantageous role but a modest negative feedback exists for the LDL uptake thus making the macrophages highly lipid engorged (Gibson, Domingues et al. 2018). This resultant inappropriate regulation thereby leads to alteration in the macrophage phenotype thus compromising critical immune functions (Moore, Sheedy et al. 2013).

2.3.3.1 Macrophage cholesterol metabolism

Macrophages internalize the native LDL and oxidized lipoproteins via pinocytosis, phagocytosis, and the receptor-mediated uptake by SRA, LOX1, TLR4 and
CD36 and are trafficked to the lysosomes. Oxidation and/or glycation of the LDL promote the internalization via CD36, SRA, LOX1, and TLR4. Inside the lysosomes, the acid lipase hydrolyzes cholesterol ester (CE) and form free cholesterol (FC). This is proceeded by the esterification of FC by acyl CoA-cholesterol acyltransferase (ACAT) in endoplasmic reticulum (ER) and stored in cytoplasmic lipid droplets (Linton, Yancey et al. 2015). Elevated concentration of the FC in the ER regulatory pool triggers a signaling cascade which results in down-regulating the LDLR. However, the scavenger receptors evade this controlling mechanism and due to high affinity for oxLDL, immense intracellular lipid accumulation is witnessed resulting in the formation of foam cells (Mietus-Snyder, Friera et al. 1997; Barbieri, Cavalca et al. 2004; Hansson 2005). The interactions of apoE on the apoE remnants and VLDL with the apoE receptors (low density lipoprotein receptor-related protein 1 (LRP1) and the VLDL receptor) causes cholesterol accumulation also and is not regulated by the cellular cholesterol (Linton, Yancey et al. 2015).

Figure 2-3: Macrophage Cholesterol Metabolism. Adapted from (Linton, Yancey et al. 2015).
There are two major pathways for CE clearance. In one of it, FC removal from the plasma membrane (PM) cause FC transport to PM. On the other hand, the cytoplasmic CE can be packaged into the autophagosomes which fuses with lysosomes subjecting the CE to by acid lipase. The resultant FC is then trafficked to PM.

There are multiple mechanisms to efflux FC to lipid deficient HDL. The interaction of ABCA1 with the endogenous apoE and exogenous apoA-I stimulate FC efflux to form nascent HDL (e.g. apoA-I or apoE containing phospholipid discs) through scavenger receptor BI (SRBI), ABCG1 and aqueous diffusion. An overview of the whole process has been summarized in the following figure (Figure 2-3).

### 2.3.4 Atherosclerosis and inflammation

The main cause of CAD is atherosclerosis which is an outcome of the extensive interaction of the inflammatory entities interacting with each other (Hansson 2005). The damage to the endothelial wall and the activation of different inflammatory cell is the driving cause for arterial stenosis (Singh, Mengi et al. 2002).

![Figure 2-4: The role of inflammatory cells in atherosclerotic lesion. Adapted from (Wu, Li et al. 2017).](image)
The initial stage of atherosclerosis is basically considered as inflammatory response to oxLDL (Hansson 2005). During this phase, the prevalence of hypercholesterolemia conditions in the artery increases the LDL infiltration and its retention which causes activation of the endothelium and the release of pro-inflammatory chemokines and cytokines (Kume, Cybulsky et al. 1992). Furthermore, leukocyte adhesion molecules leads to the leukocyte infiltration followed by their adhesion (Dai, Kaazempur-Mofrad et al. 2004). In response to this, the leukocytes release chemo-attractant stimuli such as chemokines (Boring, Gosling et al. 1998; Gu, Okada et al. 1998). A brief overview of the immune cells along with their role in inflammation has been described in Figure 2-4.

2.4 RISK FACTORS FOR CAD

The huge burden of CAD worldwide is the result of high prevalence of cardiovascular risk factors. Exposure to the risk factors for all CVDs remains alarmingly common and presents one of the largest barriers to improve global health. Most of the deaths due to CAD (also other CVDs) are linked to these common risk factors. These diseases occur as a result of the combination of preventable behavioral and metabolic risk factors which can be modified so as to reduce the mortality and morbidity from CAD by 30-40% (Roberts and Stewart 2012; Lu, Hajifathalian et al. 2014). There is also another class of risk factors such as age, gender and ethnicity which cannot be modified. Therefore, the risk factors for CAD can be broadly categorized into two groups:

a) Modifiable risk factors
b) Non-Modifiable risk factors

**Modifiable Risk Factors:** These are those factors which, upon modification either by treatment or by control, would lead to a reduced CAD risk in an individual. The important modifiable risk factors are as follows:

2.4.1 Smoking

Smoking is the principal preventable cause of mortality in the U.S., killing >480,000 individuals every year. About 41,000 were accredited to second hand smoke exposure (Benjamin, Virani et al. 2018). It is found to increase the risk of developing almost every cardiovascular phenotype viz. CAD, IS, PAD, HTN, cardiac arrhythmia, MI
and HF. Smokers lose at least one decade of life expectancy as compared to never smokers and increase the risk of both CAD and stroke by two to four folds (Jha, Ramasundarahettige et al. 2013; Thun, Carter et al. 2013). Passive smoking/second-hand smoke is also found to display strong positive associations with CAD and also displays critical influence on CVD prognosis (Fischer and Kraemer 2015).

Smoking can instigate several immediate physiological changes within heart and its blood vessels which are marked by development of atherosclerotic and inflammatory events. The etiology involves initiation of immunogenic response to vascular injury having a multiple patho-physiological effects summarized in the figure below (Figure 2-5):

![Diagram of smoking effects](image)

**Figure 2-5**: Patho-physiological effects of cigarette smoking. Adapted from (Salahuddin, Prabhakaran et al. 2012).

Cigarette smoke delivers polycyclic aromatic hydrocarbons, such as benzopyrene, that are ligands for aryl hydrocarbon receptor (AhR). The extract from cigarette smoke was reported to upregulate the expression of pro-inflammatory genes and this
upregulation was shown to be inhibited by the chemical inhibitor of AhR (Wu, Nishimura et al. 2011). The fat cells (adipocytes) release a hormone, adiponectin which is considered to be insulin sensitizing and also have anti-atherogenic properties such as inhibiting the expression on adhesion molecules (Lihn, Pedersen et al. 2005). Also the expression of adiponectin mRNA in blood mononuclear cells is compromised in smokers and gradually decreases with the amount of smoking done daily but the levels increase after smoking is stopped (Tsai, Guo et al. 2011).

Moreover, smokers have increased levels of thrombopoietin as compared to non-smokers which boosts up the platelet activation under stress conditions (Lupia, Bosco et al. 2010). Also elevated levels of C-reactive protein (CRP), TNF-α, IL-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and ICAM-1 are seen in smokers (Levitzky, Guo et al. 2008; Petrescu, Voican et al. 2010; Barbieri, Zacchi et al. 2011).

2.4.2 Elevated blood pressure (BP)/Hypertension (HTN)

Elevated BP is estimated to be a major contributor towards CAD and is believed as one of the classical risk factor. According to the “2017 ACC/AHA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults”, 45.6% of adults in U.S. are suffering from HTN (Benjamin, Virani et al. 2018). Men are more prone to HTN than women until the 45 years of age, between 45 and 64 years of age, men and women have similar percentage of HTN and after 64 years of age, more women have been diagnosed with HTN than men. The HTN prevalence increases markedly with increasing age in all the races and ethnicities (Go, Mozaffarian et al. 2013). The threshold for the diagnosis of HTN traditionally is set as a systolic blood pressure (SBP) of 140mm Hg and diastolic blood pressure (DBP) of atleast 90mm Hg or above for both (Mancia, De Backer et al. 2007). Fatty deposition or plaque buildup in the arteries lead to reduction in diameter of the lumen, thereby restricting the flow of blood to heart and thereby increasing the BP subsequently leading to angina and ultimately HF.

HTN in classified as primary and secondary HTN. The former is found in 90-95% of total HTN cases. It affects the middle aged or the old people and arises due to genetic and lifestyle factors (Poulter et al. 2015). The latter is caused by other comorbidities like endocrine diseases or renal disease in addition to genetic and lifestyle factors. The
consequences of HTN are age dependent also. Each increment of 20 mm Hg in SBP or 10 mm Hg in DBP doubles the risk of CAD in individuals 40-70 years of age (Lewington, Clarke et al. 2003).

2.4.3 Dyslipidemia

Dyslipidemia is a condition of chronic increase or attenuation of serum lipoproteins and is an independent significant predictor of CAD. The atherosclerotic cascade of dyslipidemia includes endothelial dysfunction by damaging ECs, endothelial vasoconstriction thereby allowing penetration of lipid particles inside the endothelial layer and ultimately initiation of oxidative and immuno-inflammatory responses that ends up with plaque formation (Daoud, Scheede-Bergdahl et al. 2014). Clinically, it is defined as abnormal elevation in blood levels of different cholesterol viz. low density lipoprotein (LDL), total cholesterol (TC) and triglycerides (TG) and a decreased level of high density lipoprotein (HDL) (Choudhury, Mainuddin et al. 2014).

The circulating cholesterol is a combination of both lipids and proteins (apolipoproteins) to form lipoproteins. LDL cholesterol contains only one molecule of apolipoprotein namely apoB-100, constitutes 60-70% of TC and exerts atherogenic properties (Fedder, Koro et al. 2002). LDL is the major lipid-carrier protein. Higher levels lead to LDL accumulation resulting in oxidation and hence atherosclerosis. HDL cholesterol is anti-atherogenic, forms 20-30% serum cholesterol and contains apoliporotiens namely apoA-I and apoA-II (Hunt, Resendez et al. 2004). Each incremental increase in HDL cholesterol of 1mg/dl is associated with a 2-3% decrease in CAD risk (Khera, Cuchel et al. 2011). The third class of lipoproteins, VLDL is produced by liver, rich in triglycerides and constitutes remaining 10-15% of TC in the serum. They are precursors of LDL and comprises apoB-100, apoCs (C-I, C-II, and C-III), and apo E as key apolipoproteins. Usually they do not promote atherosclerosis but the VLDL remnants (partially degraded VLDL enriched in CE) participate in atherogenic activities (Hunt, Resendez et al. 2004). Chylomicrons are lipoproteins rich in triglycerides and synthesized in intestine. Chylomicrons have same apolipoproteins in VLDL except apo B-48 instead of apoB-100. Partially degraded chylomicrons, called chylomicron remnants also have atherogenic potential (Fedder, Koro et al. 2002). The properties of the major human plasma lipoprotein classes are summed up in the table below (Table 2-2):
Table 2-2: Properties of major human plasma lipoprotein classes. Adapted from (Jonas 2002).

<table>
<thead>
<tr>
<th></th>
<th>CHYLOMICRON</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/mL)</td>
<td>&lt;0.95-1.006</td>
<td>0.95-1.006</td>
<td>1.006-1.019</td>
<td>1.019-1.063</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>Diameter (Å)</td>
<td>103-104</td>
<td>300-800</td>
<td>250-350</td>
<td>180-250</td>
<td>50-120</td>
</tr>
<tr>
<td>Components (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>2</td>
<td>8</td>
<td>15</td>
<td>22</td>
<td>40-55</td>
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<tr>
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<tr>
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<td>7</td>
<td>7</td>
<td>8</td>
<td>4</td>
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<tr>
<td>Phospholipids</td>
<td>3</td>
<td>12</td>
<td>23</td>
<td>42</td>
<td>12-20</td>
</tr>
<tr>
<td>Apolipoprotein composition</td>
<td>A-I, A-II, A-IV, B-48, C-I, C-II, C-III, E</td>
<td>B-100, C-I, C-II, C-III, E</td>
<td>B-100, C-I, C-II, C-III, E</td>
<td>B-100, E</td>
<td>A-I, A-II, C-I, C-II, C-III, D, E</td>
</tr>
</tbody>
</table>

(a) Apolipoprotein B (ApoB)

ApoB is synthesized in the liver and one molecule of ApoB is present in the atherogenic VLDL, IDL and LDL whereas chylomicron consists of apoB-48 which is produced in the gut (Elovson, Chatterton et al. 1988; Marcovina and Packard 2006). ApoB-48 consists of 48% of the apoB-100 sequence and thus named like that. Therefore, total apoB gives us the clear idea about the whole atherogenic particles circulating in the blood. In majority of the cases, >90% of the total apoB in blood is present on the LDL. Modifications in these apoB are responsible for lipoprotein entrapment in the wall of the artery (Sniderman, Furberg et al. 2003; Walldius and Jungner 2004; Marcovina and Packard 2006; Sniderman, Barter et al. 2006).

(b) Apolipoprotein A-I (ApoA-I)

The chief constituent of HDL particles is apoA-I and believed to be anti-atherogenic. ApoA-I carries excessive cholesterol from peripheral cells and transports to
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ApoA-I also exhibits antioxidant and anti-inflammatory effects (Walldius and Jungner 2004; Schlitt, Blankenberg et al. 2005; Barter and Rye 2006; Marcovina and Packard 2006). In majority of the cases, apoA-I depicts athero-protective part and low values are considered risk factor for MI and CAD and this notion is supported by many studies (Francis and Frohlich 2001; Walldius, Jungner et al. 2001; Nissen, Tsunoda et al. 2003; Walldius and Jungner 2004; Schlitt, Blankenberg et al. 2005).

Some researchers rely on LDL and HDL values and their ratios while certain believe that apoB and apoA-I and their ratio is a better measure for risk prediction. The main reason for supporting this belief is that both these values can be directly measured by using standard internationally validated techniques (Marcovina, Albers et al. 1994; Marcovina and Packard 2006). Thus the values reflect two aspects of risk equation i.e. apo B “the atherogenic side” and apoA-I “anti-atherogenic side”. Therefore the ratio depicts the cholesterol transport balance. High value of apoB/apoA-I ratio indicates high levels of cholesterol circulating in plasma and it is likely that it will get deposited in arterial walls, thereby provoking atherogenesis. The low value on the other hand indicates low cholesterol in the plasma, more reverse cholesterol transport and thus lesser risk of CAD (Walldius, Jungner et al. 2001; Walldius and Jungner 2004; Yusuf, Hawken et al. 2004).

The “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 (ATP III))- 2017” serves as the reference standard for cholesterol goals and classifies the different cholesterol levels as follows:

Table 2-3: ATP III Classification- 2017 of LDL-Cholesterol, Total Cholesterol, HDL-Cholesterol and triglycerides.

<table>
<thead>
<tr>
<th>LDL-Cholesterol (mg/dl)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100</td>
<td>Optimal</td>
</tr>
<tr>
<td>100-129</td>
<td>Near optimal</td>
</tr>
<tr>
<td>130-159</td>
<td>Borderline high</td>
</tr>
<tr>
<td>160-189</td>
<td>High</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Total Cholesterol (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>≥190</td>
<td>Very high</td>
</tr>
<tr>
<td>&lt;200</td>
<td>Desirable</td>
</tr>
<tr>
<td>200-239</td>
<td>Borderline high</td>
</tr>
<tr>
<td>≥240</td>
<td>High</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HDL-Cholesterol (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;40</td>
<td>Low</td>
</tr>
<tr>
<td>≥60</td>
<td>High</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum Triglycerides (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;150</td>
<td>Normal</td>
</tr>
<tr>
<td>150-199</td>
<td>Borderline high</td>
</tr>
<tr>
<td>200-499</td>
<td>High</td>
</tr>
<tr>
<td>≥500</td>
<td>Very high</td>
</tr>
</tbody>
</table>

2.4.4 Physical inactivity

Lack of physical activity is a major contributor of developing CAD. Physical inactivity or sedentary lifestyle is ranked as the fourth major cardio risk factor followed by smoking, HTN and dyslipidemia (Mendis, Puska et al. 2011). Moreover, habit of daily physical activity is linked to overcome other established risk factors for CAD. Lack of regular physical activity leads to the deranged BP, HDL, LDL, blood glucose levels and increase in body weight (Perk, De Backer et al. 2012). The effects of regular physical activity are summed up in Figure 2-6.

2.4.5 Alcohol intake

Alcohol is ranked fifth among risk factors for fatality and disability accounting for 4% of life year’s loss due to disease (Holmes, Dale et al. 2014). Both positive and negative aspects of alcohol intake are observed in relation to cardiovascular health. Regular moderate alcohol intake shrinks the potential CVD risk in an individual (Cahill and Redmond 2012). The various cardio-protective effects of moderate drinking include elevation in serum HDL levels (Brien, Ronksley et al. 2011), improvement in arterial elasticity, reduction in chances of getting heart attacks (Biyik and Ergene 2007). The effects are summed up in the Figure 2-7.
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Figure 2-6: Potential mechanisms of physical activity that aids in CVD risk reduction. Adapted from (Cheng, Yu et al. 2013).

Figure 2-7: Schematic representation of the biological mechanisms of alcohol intake. Adapted from (Haseeb, Alexander et al. 2017).
As compared to all the different alcohol drinks available, red wine consumption is considered to be the most cardio-protective (Saleem and Basha 2010). If there is a light to moderate consumption of red wine, a number of beneficial effects on all phases of atherogenesis are observed (Lippi, Franchini et al. 2010). Especially the resveratrol and quercetin in red wine are reported to exhibit decreased oxidative stress, enhanced efflux of cholesterol from vessel walls, inhibition of oxidation of LDL and also the macrophage cholesterol accumulation thereby reducing the formation of the foam cells (Babal, Kristová et al. 2006; Li and Förstermann 2009). These components may also increase NO, thereby preventing the oxidative stress-induced endothelial dysfunction. The polyphenols in wine prevent the endothelin-1 induced vascular free radical production and consequent elevated activity of NADPH oxidase thus improving the endothelial function (López-Sepúlveda, Gómez-Guzmán et al. 2011). Also red wine decreases viscosity of blood, enhances insulin sensitivity, neutralizes platelet hyperactivity, hinders platelet adhesion and reduces von fibrinogen, Willebrand factor and coagulation factor VII levels in plasma (Bradamante, Baregghi et al. 2003; Novakovic, Gojkovic-Bukarica et al. 2006; Xi, Wang et al. 2009). Quercetin is reported to inhibit the thrombocyte aggregation (Formica and Regelson 1995) and has antihypertensive effect through vasodilator action on VSMCs (Inal, Altinişik et al. 2002).

However, long term heavy or binge drinking promotes atherosclerotic activities and is injurious to cardiovascular well-being. The adverse outcome of alcoholism on cardiac physiology includes the cardiomyopathy, HTN, arrhythmia and MI (Leong, Smyth et al. 2014). Even an event of a holiday binge drinking (episode of heavy drinking during leisure time or weekend) can bring abrupt, irregular heart beat with breathing difficulties, rise in BP and even unexpected death. This clinical appearance is termed as “Holiday Heart Syndrome” (Ettinger, Wu et al. 1978). The Coronary Artery Risk Development in Young Adults (CARDIA) study demonstrated that heavier alcohol consumption during young years of lifetime was associated with coronary calcification also (Pletcher, Varosy et al. 2005).

2.4.6 Diabetes

Diabetes is a chronic disease defined as an elevated state of blood glucose level (hyperglycemia). The etiology involves absolute deficiency of insulin due to autoimmune mediated destruction of β cells of pancreas known as Type 1 Diabetes Mellitus (T1DM)
or when beta-cells of pancreas fail to use insulin either due to defects in insulin receptor cells or sometimes due to insulin deficiency, termed as Type 2 Diabetes Mellitus (T2DM) (American Diabetes Association, 2011). In India, there are about >69 million T2DM affected people and numbers will drastically shoot up to about 140 million by the year 2040 (Shrivastava, Misra et al. 2017).

**Figure 2-8: Diabetes and susceptibility to atherosclerosis. Adapted from (Thomas and Foody 2007).**

![Diagram of diabetes and atherosclerosis](image)

Diabetes has been identified as an autonomous risk factor for progression of different CVDs like CAD, PAD and HTN (Vijayakumar, Vaduganathan et al. 2018). Hyperglycemia along with other co-existing conditions of HTN, dyslipidemia, obesity and smoking can lead to progressive damage to blood vessels and heart. In fact the process of atherosclerosis in diabetics tends to be accelerated, worse and widespread. Atheroma of a diabetic CAD individual has more lipid content, inflammatory changes and even thrombus formation than a non-diabetic CVD individual (Rydén, Grant et al. 2013). Multiple biochemical changes have been documented in the diabetics, most significantly, the protein glycosylation in the arterial wall which is considered to contribute substantially to the diabetic atherosclerosis (Chiha, Njeim et al. 2012). The non-enzymatic reaction between arterial wall proteins and glucose leads to formation of advanced glycation end products (AGEs) and this process is enhanced in the hyperglycemia condition. AGEs are considered to be directly involved in EC dysfunction and believed to accelerate the process of atherosclerosis (Chiha, Njeim et al. 2012). Also there is more production of ROS in hyperglycemia conditions which lead to inhibition of production of endothelial NO (vasodilator and regulates platelet activation) (D’Souza,
Hussain et al. 2009). Furthermore, these ROS destabilizes the plaque by inhibiting VSMC migration thus forming vulnerable plaques which are called as “diabetic coronary plaques” and are highly prone to rupture (Moreno, Purushothaman et al. 2004). When the plaque ruptures, enhanced platelet dysfunction and thrombogenesis in diabetics further deteriorate the clinical effects of rupture. Also the free circulating glucose molecules have the ability to enter the platelets. This raises the intracellular concentration of glucose concentration and activates protein kinase C, which decreases the platelet derived NO and further increases glycoprotein Ib expression and induces platelet aggregation (D’Souza, Hussain et al. 2009). This further explains the enhanced thrombosis in diabetic individuals. The various effects of diabetes are summed up in Figure 2-8.

2.4.7 Diet

Globally, the way one eats has changed greatly and the pace of change is quiet fast in both developing and the developed countries. From 1985-2005, an extensive increase in the added sugar intake in processed food and beverages has occurred (MONTEIRO 2010; Kleiman, Ng et al. 2012; Basu, Yoffe et al. 2013). The trans fat consumption is also seen to be very high in many low and middle income countries. In India, the vanaspati or the vegetable ghee is the prime source of trans fats and regularly exploited in the making of fried snacks, bakery products and foods sold by the street vendors (Anand, Hawkes et al. 2015). The consumption of snacks have also drastically increased in frequency as well as in number (Popkin and Duffey 2010; Ng, Zaghloul et al. 2011; Duffey, Rivera et al. 2014), eating frequency in restaurants and food outlets has increased dramatically, especially in low/middle income countries and majorly involves the fried and processed food (Monteiro, Gomes et al. 2010). Furthermore, there is an incorporation of processed food in the diet and a decreased consumption of fresh fruits and vegetables also witnessed (Monteiro, Moubarac et al. 2013; Poti, Mendez et al. 2015).

In the past few years, researchers are keen to decipher the role of dietary fats and various CVDs with a prime focus on CAD (Assmann and Schulte 1992; Kris-Etherton, Daniels et al. 2001). The effect has been thought to be mediated by certain indirect mechanisms like effect of dietary lipids on total energy, obesity, plasma lipid levels and on the glycemic control (Reddy and Katan 2004). Epidemiologically, the intake of high carbohydrate diet has been linked to low TC and TG levels (Truswell 1994). Additionally, the salt/sodium intake has direct correlation with mean SBP and DBP and HTN prevalence.
Also, it is worth mentioning that the food system has been affected by a number of environmental issues like emission of green house gases, sustained discharge of toxins in water bodies, progressive loss of biodiversity and excessive usage of fertilizers and pesticides have led to eutrophication (Hoekstra and Chapagain 2006; McMichael, Powles et al. 2007; Weis and Weis 2007; Hoekstra and Chapagain 2011). This all has led to a compromise in the quality of diet. Therefore, the additive effect of different aspects of modernization, industrialization and urbanization has led to a massive shift in the dietary patterns exposing the individuals to various disease and CAD is one among them.

2.4.8 Obesity

Obesity and obesity related complications are one of the major chronic health issues that 21\textsuperscript{st} century is facing. It has been acknowledged as an independent, vital risk factor for CAD which needs immediate attention (Wilson, D'agostino et al. 2002). In U.S. about 72 million individuals are reported to be obese (Jahangir, De Schutter et al. 2014). Even in a developing country like India, 23\% of the total burden of various CVDs is attributed by overweight and obesity and shown to affect >135 million individuals (Shrivastava, Misra et al. 2017).

Body Mass Index (BMI) has been extensively used as a surrogate to determine different categories of body weight. The BMI is defined as the body mass divided by the square of the body height, and is universally expressed in units of kg/m\textsuperscript{2}. In 1995, the World Health Organization (WHO) gave the classification of BMI which is as follows:

<table>
<thead>
<tr>
<th>Categories</th>
<th>BMI (kg/m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>≤18.5</td>
</tr>
<tr>
<td>Normal weight</td>
<td>18.5-24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25-29.9</td>
</tr>
<tr>
<td>Obese</td>
<td>≥30</td>
</tr>
</tbody>
</table>

In addition to BMI, measures of obesity can also be done on the basis of waist circumference (WC), waist-to-hip ratio (WHR), and more recently, waist to height ratio (WHtR) and it is found that they also play a vital role in determining CAD morbidity and mortality (Czernichow, Kengne et al. 2011).

The adipocytes are the key players for the detrimental effects of obesity (Lim, Quon et al. 2014). Adiponectin, secreted from adipocytes is found elevated in high levels
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in the blood of lean healthy individuals and these adipocytes release transforming growth factor-beta (TGF-β), IL-10, and NO, which all are anti-inflammatory and promote insulin-sensitivity and exert anti-atherogenic effects. But the levels are markedly reduced among individuals with T2DM, CAD or with obesity (Matsuzawa, Funahashi et al. 2004; Kim, Després et al. 2015). Moreover, elevated levels of fibrinogen, von Willebrand factor, factor VII, factor VIII, and plasminogen activator inhibitor-1 (PAI-1) and amplified platelet adhesion is observed in obese as compared to the lean individuals (López-Jiménez and Cortés-Bergoderi 2011). Obesity also promotes the clustering of other CAD risk factors like T2DM, HTN and hyperlipidemia (Wilson, D'agostino et al. 2002; Lu, Hajifathalian et al. 2014) and worsens the cardiovascular structure and function due to more cardiac workload (Alpert 2001; Lu, Hajifathalian et al. 2014).

2.4.9 Psychological factors

Of late, researchers and clinicians are focusing on the correlation between CAD outbreak and various psychological factors (Everson-Rose and Lewis 2005). Presence of any psychological disorder or its history could lead to a predisposition to CAD (Davidson 2012). Various psychological (emotional) and social (chronic stressors) factors like depression, anxiety, anger, sleep insomnia, nervousness, low socioeconomic status, work stress etc. have been regarded as persuasive risk factors for CVDs (Dogar, Khawaja et al. 2008; Low, Thurston et al. 2010; Khayyam-Nekouei, Neshatdoost et al. 2013).

One of the imperative quantifiable psychological factors for CAD is depression. It may be described as feeling of sadness, loss, unhappy, miserable, anger or frustration. Among CAD patients, depression has been reported to be quiet common and prevalence is reported to be 20% higher as compared to healthy individuals (Kuper, Marmot et al. 2002; Bunker, Colquhoun et al. 2003; Nekouei, Neshatdoost et al. 2013). Also, its 3 times more common in CAD patients after an MI event (Thombs, Bass et al. 2006) and considered to be responsible for high cardiac morbidity and mortality. The probable mechanism connecting depression to be involved in etiology of CVD includes: serotonergic dysregulation, activation of immuno-inflammatory response, autonomic nervous system dysfunction, hypothalamic-pituitary-adrenal (HPA) axis, endothelial and vascular changes, nutritional deficiencies, genetics and conventional CVD risk factors (Sher, Lolak et al. 2010). A meta-analysis by (Roest, Martens et al. 2010) revealed anxiety as independent risk factor for CAD.

One more undeniable psychological and social factor is the marital status. Although survival curves based on marital status have been documented in ACS patients,
only a few studies have aimed to evaluate the influence of marital status and adverse cardiovascular outcomes in CAD patients (Hadi Khafaji, Al Habib et al. 2012; King and Reis 2012; Barbash, Gaglia et al. 2013). (Barbash, Gaglia et al. 2013) in his study on patients with PCI, has reported high cardiovascular event rates in unmarried in comparison to married ones. In consensus with the above finding, (Schultz, Hayek et al. 2017) reported that that unmarried had 52% high rates of cardiovascular death and MI. Divorce prognosticates worse prognosis and diagnosis in comparison to married individuals (Manzoli, Villari et al. 2007; Sbarra, Law et al. 2011; Shor, Roelfs et al. 2012). Also the role of remarriage in attenuation of elevated risk after divorce has been clearly demonstrated pointing towards the fact that financial and emotional aspects of divorce play a minor role and can be attenuated with remarriage (Sbarra and Nietert 2009).

2.4.10 Drug abuse

There is a rapid increase in the intake of drugs and drug abuse has been identified as another major risk factor for the CAD. A study was conducted on 4800 drug users and revealed that about 223 suffered from CVD and also reported drug abuse to be the fourth most common reason after psychosis, schizophrenia and depression (Onyeka, Beynon et al. 2015). The prominent drugs associated with CVDs are the methamphetamine, opioid, cocaine and marijuana. These drugs disrupt the balance of catecholamines (neurotransmitters) leading alteration in BP, arrhythmia, increased tendency for blood clotting and thus more propedency for plaque formation (O'Connor, Rusyniak et al. 2005).

Methamphetamine is associated with many CVDs and adverse events such as cardiomyopathy, cerebral infarction, cardiac arrhythmia and MI (O'Connor, Rusyniak et al. 2005).Cocaine is also associated with maximum incidence of CVDs (O'Connor, Rusyniak et al. 2005). Cocaine has effects similar to the methamphetamine and causes sharp elevation in BP, heart rate and increased cardiac oxygen demand and increased risk of HTN, stroke, aneurysm and damage to cardiac tissue (Das 1993; Shih, Chu et al. 2014). Cocaine also influence the neurotransmitter signally, disturbs the calcium levels and hence has a role in cardiac cell damage and eventually death (Agrawal, Scarabelli et al. 2015). In adults 18-45 years of age, 25% of heart attacks and regular cocaine use is the reason behind that (Qureshi, Suri et al. 2001). Cocaine if combined with other forms of drug abuse such as alcohol lead to the formation of cocaethylene which is a highly cardiotoxic metabolite (Agrawal, Scarabelli et al. 2015).
Opioids mimic body’s natural endorphins and bind with the pain receptors thus inhibiting signaling to brain. Long term opioid aggravates the risk of CVDs by elevating LDL and TG levels in the body (Baldo, Giunco et al. 2007). A study conducted by (Aghadavoudi, Eizadi-Mood et al. 2015) compared 117 patients having history of opioid abuse with 208 similar patients who did not report any history of abuse reported that LDL and average TG levels were significantly higher in the former group. Another illicit drug, marijuana or cannabis is also linked with CAD risk(Casier, Vanduynhoven et al. 2014; Hall 2015). Also majority of people using cannabis have reported a history of tobacco smoking (Hall 2015).

Drug abuse negatively impacts the reproductive health in individuals of both the genders and lead to infertility, sexually transmitted diseases (STDs), irregular menstrual cycle, sexual dysfunction and cancer (McKay 2005; Treatment 2009). Drug abuse in pregnant women predispositions their unborn children at high risk during not only labor but influences their physical and mental health in the later years of life (Treatment 2009). In males, drug abuse leads to decrease in testosterone levels (Sansone, Di Dato et al. 2018), decreased sperm count (Gundersen, Jørgensen et al. 2015), impaired sperm morphology, motility and spermatogenesis (Whan, West et al. 2006; Amoako, Marczylo et al. 2013; Pacey, Povey et al. 2014). Another major consequence of drug abuse in Hepatitis C (Onyeka, Beynon et al. 2013).

### 2.4.11 Infections

CAD and chronic infections have been studied to elucidate the plausible role of latter in predisposition to CAD. Studies report the involvement of many infectious agents such as bacteria, viruses and parasites to be linked with CAD risk (Rezaee-Zavareh, Tohidi et al. 2016). Literature document strong associations for Helicobacter pylori (Zhang, Guo et al. 2008; Wang, Li et al. 2012), Chlamydia pneumonia (Chen, Zhu et al. 2013; Filardo, Di Pietro et al. 2015), Hepatitis C (Huang, Kang et al. 2014) and Cytomegalovirus (Ji, An et al. 2012). The role of influenza yet needs to be deciphered (Bouwman, Visseren et al. 2002). The infectious agents are thought to be involved in both direct and indirect mechanisms of inflammation effect on the process of atherosclerosis (Rezaee-Zavareh, Tohidi et al. 2016). Thus, managing all kinds of infections, mainly in people already having any of the traditional risk factor must be accounted for combating CAD risk and atherosclerosis.
2.4.12 High sensitivity C-reactive protein (hsCRP)

hsCRP is considered to be a marker as well as mediator of the process of atherosclerosis and CAD. CRP is basically a protein which is produced by the hepatocytes in response to IL-6 released from macrophages during inflammatory response (Shrivastava, Singh et al. 2015). It is sought to strongly predict adverse cardiovascular events, including MI, IS and SCD. It activates the complement pathway, lipid intake in macrophages and also stimulates the release of various pro-inflammatory cytokines. It is also involved in the promotion of endothelial dysfunction and NO production inhibition (Paffen and demaat 2006; Torpy, Burke et al. 2009; Pfützner, Schöndorf et al. 2010; Davis, Vidyasagar et al. 2012; Shrivastava, Singh et al. 2015). It is also involved in increasing IL-12 production by macrophages which subsequently induces IFN-γ production (Calabro, Golia et al. 2012).

There are many commercially available high sensitive CRP assays which are reliable, simpler and reproducible and have provided an easy way to diagnose and manage CAD (Mehta, Sukhija et al. 2007; Greenland, Alpert et al. 2010). The levels in healthy individual are found to be 0.8 mg/L and these values shoot up to 500 mg/L in case of infection (Pepys and Hirschfield 2003).

Non-modifiable risk factors:

These can be defined as those factors which could not be modified to reduce the CAD burden such as advance age, gender, and genetic risk factors.

2.4.13 Age

With advancing age, people swiftly become vulnerable to develop CAD and experience a sudden fatal heart attack. Above the age of 40 years, men have 49% risk to develop CAD whilst its 32% in case of women (Roger 2012). Investigators are conducting research on cardiac and arterial aging and trying to delineate how age affects the cardiovascular system and contributes towards CAD risk (Lakatta 2003; Lakatta and Levy 2003; Lakatta and Levy 2003). One proposed hypothesis states that with advancing age, there is a cumulative effect of the other traditional risk factors which intertwines with the age related structural and functional changes. With increasing age, there is increased risk for CAD in males and in post-menopausal females while in younger females, the estrogen-activity has a protective antioxidant effect (Pranavchand and Reddy 2013). To add on, early puberty age is associated with higher risks for CVDs in both men and
women and therefore represents a potential target for early preventive interventions and appears to have a profound impact on the health (Day, Elks et al. 2015).

2.4.14 Gender

Prior to the 1990s, CAD has been considered predominantly affecting men (Roeters van Lennep, Westerveld et al. 2002). Therefore, major of the research studies of that time have been extensively conducted on male patients (Roeters van Lennep, Westerveld et al. 2002). Women present a lower perception of cardiovascular risk and consequently are less keen to seek medical care when experiencing symptoms; as a consequence, their prognosis is more often worse in women than men (Davis, Mishel et al. 2013). Awareness of CVD in women has improved in the last years due to education campaigns (Mosca, Barrett-Connor et al. 2011). Extensive research was later on initiated on female patients during the 1990s and majority proved greater susceptibility of females to CAD (Goldschmid, Barrett-Connor et al. 1994; Roeters van Lennep, Westerveld et al. 2002). The female hormone, oestrogen is largely considered to be cardio-protective and decline at menopause is associated with CAD risk. Certain factors like polycystic ovary syndrome or oral contraceptive usage are female specific. Studies document a 9 year delayed CAD onset as compared to men (Anand, Islam et al. 2008). However, this delay is narrowing and the adoption of western lifestyle and early menopause age are believed to be the driving factors (Vogel, Farhan et al. 2016).

Risk factors like low HDL levels, hypertriglyceridemia, drinking and smoking have profound effect in females (Roeters van Lennep, Westerveld et al. 2002). Diabetes increases the CAD risk by 3-7 folds in women (Seeman, de Leon et al. 1993; Goldschmid, Barrett-Connor et al. 1994), thus abolishing the premenopausal advantage (Barrett-Connor and Bush 1991). Also strong anatomical and cardiovascular function differences are there in both the sexes. Left ventricular mass, left ventricular end-diastolic dimension, left atrial dimension, ventricular wall thickness and the vessel size are found to be smaller in females (adjusted for age and race). Moreover, the menstruation in women has a strong influence on the hematologic and electrocardiographic indices (Huxley et al. 2007). Females already suffering from autoimmune diseases also show an inclination towards development of CVDs. Studies have reported that the microvasculature in women plays a pivotal role in predisposition of such affected females to have an accelerated disease development and progression of the disease (Gianturco, Bodini et al. 2015).
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(a) OESTROGEN

Oestrogen or estradiol is the key female hormone produced by fat tissue, the liver, the adrenal glands and majorly by the ovaries (Gopalakrishnan, Ragland et al. 2009). In premenopausal women, normal oestrogen levels range from 15-350 pg/ml and after menopause a sharp decline to <10 pg/ml is observed. Adult males also have oestrogen 10-40 pg/ml in their body (Traish, Miner et al. 2011). Studies report considerably low CAD incidence in pre-menopausal women as compared to same age group men and post-menopausal women signifying protective effect of endogenous oestrogen (Barrett-Connor and Bush 1991; van der Schouw, van der Graaf et al. 1996; Hu, Grodstein et al. 1999).

Oestrogen affects atherosclerosis through multiple mechanisms. It decreases LDL cholesterol levels owing to an upregulation of apolipoprotein B100 receptor (Evans 2018). It has the ability to protect LDL against oxidative damage (Løkkegaard, Jovanovic et al. 2006) and also increase HDL. It has the ability to inhibit activity of hepatic lipase and promotes hepatic apoA-I synthesis (Evans 2018). Also, oestrogen positively modulates the levels of endothelial nitric oxide synthase (eNOS) and subsequently, increased NO production (Evans 2018). This potent vasodilator regulates BP and platelet function and inhibits VSMC proliferation, inhibits expression of adhesion molecules and reduced release of endothelin-1, a potent vasoconstrictor. It also reduces angiotensin converting enzyme (ACE) activity, which benefits cardiovascular health (Evans 2018).

A sharp decline in the endogenous oestrogen is witnessed at menopause. Owing to the cardiovascular benefits of oestrogen, researchers have tried to augment this loss by providing exogenous oestrogens but the results are quiet controversial. A meta-analysis study reported reduction of CAD risk by 35-50% on administering exogenous oestrogen (Grady, Rubin et al. 1992). But many large-scale clinical trials like Heart and Oestrogen/progestin Replacement Study (HERS) (Hulley, Grady et al. 1998), Women’s Health Initiative (WHI) (Investigators 2002), and Women’s International Study of long Duration Oestrogen after Menopause (WISDOM) (Vickers, MacLennan et al. 2007) stated increased cardiovascular risk when treated with oestrogen analogues. The Early Versus Late Intervention Trial with Estradiol (ELITE) clinical trial utilized 17β-estradiol and revealed reduced atherosclerosis related cardiovascular events (Schierbeck, Rejnmark et al. 2012; Hodis, Mack et al. 2016; Solomon 2016). The WHI study reported that starting the oestrogen therapy at a relatively younger age leads to reduced cardiovascular events in future (McSweeney, Rosenfeld et al.; Harman, Vittinghoff et al. 2011). The
discordance in different studies might have arisen due to different population characteristics being studied and variability in the study design.

(b) TESTOSTERONE

Testosterone is male specific sex hormone derived from cholesterol (WAYNE HOU, COLLINS et al. 1990; Zouboulis and Degitz 2004) and synthesized in zona reticularis of adrenal cortex and to a lesser extent in skin (Zouboulis and Degitz 2004; Xing, Edwards et al. 2011). In females, testosterone is also produced by the theca-interna cells of the graafian follicle but majority is converted to estradiol by the enzyme aromatase (Young and McNeilly 2010; Finkelstein, Lee et al. 2013).

In healthy males, testosterone ranges between 270-1070 ng/dL having an average level of 679 ng/dL (Traish, Miner et al. 2011). Reduced levels have been associated with increased CVD risk (Khaw, Dowsett et al. 2007; Haring, Völzke et al. 2010; Oskui, French et al. 2013). The association of testosterone levels with augmented CVD risk even persists when corrected for age, because its levels peak at age 30 approximately, and then decreases with increasing age (Jones 2010; Kelly and Jones 2013). (Khaw, Dowsett et al. 2007) in their 7-year follow-up study comprising of 2314 males documented that every 173 ng/dL (6 nmol/L) increase in serum testosterone was linked with 21% lower risk of all-cause mortality after correcting for other parameters.

With advancing age, both serum and total testosterone are reported to decline and associated with many disease conditions (Feldman, Longcope et al. 2002; Stanworth and Jones 2008). Low testosterone promotes pro-atherosclerotic environment (Nettleship, Jones et al. 2007; Tirabassi, Gioia et al. 2013). Testosterone has been identified as a vasodilator and an endothelium-repairing hormone in many body parts including coronary arteries (Corona, Monami et al. 2011). It is also depicted to decrease pro-inflammatory cytokine production such as IL-6, IL-1β and TNF-α which are influential in atherosclerotic profiles (Corrales, Almeida et al. 2006; Corona, Monami et al. 2011). The beneficial effect of testosterone in elevating HDL levels is attributable to the formation of estradiol by the action of aromatase (Corona, Monami et al. 2011).

Randomized controlled trials employing testosterone treatment have reported inconsistent results. The “Testosterone Treatment in Older Men with Mobility Limitations (TOM)” trial of men, aged ≥65 years, was terminated due to occurrence of CV events in the
testosterone treated group (Basaria, Coviello et al. 2010). Other trials were too small to illustrate conclusive results on exogenous testosterone treatment even though adverse event rates were similar in the treated group versus placebo (Basaria, Coviello et al. 2010; Basaria, Harman et al. 2015; Budoff, Ellenberg et al. 2017). Unambiguous and undisputable in clinically-relevant population are required to test the safety and efficacy of this treatment (Webb and Collins; Onasanya, Iyer et al. 2016).

Figure 2-9 (a): Biosynthesis of sex hormones. Adapted from (Handa, Sharma et al. 2011) and (b) Aromatase action on testosterone to form estradiol (oestrogen). Adapted from (https://en.wikipedia.org/wiki/Aromatase).
2.4.15 Genetic risk factors

The involvement of lifestyle and environmental components in CAD cannot be ignored but one more vital factor that has received attention is the role of genetic factors. There are multiple lines of evidences which support the role of genetics in the development of CAD which are given below:

(a) *The spectrum of CAD prevalence in different ethnic groups*

Racial-ethnic minorities are indicated to be at high risk of CVDs through statistical studies. Mexican-Americans, African-Americans, American-Indians, native Hawaiians and some Asian-Americans have been reported to have more predisposition towards CAD than Caucasians (Leigh, Alvarez et al. 2016). The reason behind this increased prevalence is that non-whites especially the African-Americans have many risk factors like, they have high prevalence of diabetes, obesity and HTN, that are the major risk factors for CAD also (Thom, Haase et al. 2006; Roger 2012).

While talking about the Indian subcontinent, it comprises of one-sixth of the total world population and huge diversity is seen in the ethnic, cultural and linguistic groups. Hence, this population is a hub for genetic studies. There are a number of genetic association studies conducted in the past few decades and have replicated the association patterns of a plethora of candidate genes involved in atherosclerosis and CAD (Pranavchand and Reddy 2013). Studies have been performed to investigate genetic basis for CAD in Indian population. The Indian Atherosclerotic Research Study (IARS) had been carried out in clinics in Mumbai and Bangalore and have identified the *APOC3-Sac1* SNP as a crucial variant linked with plasma lipics and hence, CAD (Shanker, Perumal et al. 2008). Researchers from the Indian Statistical Institute, Kolkata, have also screened 144 nuclear families of a homogenous Marwari population from Kolkata for 209 SNP markers in 31 genes of ten quantitative traits. They revealed nine SNPs of four genes *viz. fibrinogen, vascular endothelial growth factor-A, selectin-E and nuclear factor kappa-B1 (NFκB1)* to have significant impact on quantitative precursors of CAD (Malllik and Majumder 2011). Individuals from Indian descent have unfavorable risk factor profile (*e.g.* higher prevalence of dyslipidemia and diabetes (Klomp, Damman et al. 2012) and moreover WHO reports higher prevalence of CAD in Indians as compared to the
Caucasian population (Alwan 2011). WHO projects that by the year 2030, bulk of the global CAD population will be of Asian descent (Alwan 2011).

**(b) Twin studies**

Genetics came into limelight when various twin studies were conducted and revealed that the relative death hazard from CAD when one’s twin died before 55 was found out to be more for monozygotic twins (8.1) than dizygotic twins (3.8) (Marenberg, Risch et al. 1994). Family and twin studies have approximated the CAD inheritability to be 40-50% (LeBlanc, Zuber et al. 2015). Recently, 45 risk alleles have been recognized that confer CAD risk (Deloukas, Kanoni et al. 2013). Also, in Framingham study, it was observed that participants with at least one parent with CAD had greater risk of CAD as compared with the individuals having no parental history of CAD (Lloyd-Jones, Adams et al. 2010).

**(c) Family History**

A positive family history is the total of multiple low effect genetic variants and familial environmental risk factors. Gender related imprinting contributes to family related risk: the paternal history of MI can simply replicate more MI prevalence in males (Nielsen, Andersson et al. 2013). Family history is also an independent risk factor for CAD there being a 2.2 and 2.4 fold increased risk for CAD in females and males, respectively (Schildkraut, Myers et al. 1989), 15 first-degree relatives have a 2-3-folds increased risk (Hopkins, Williams et al. 1988; Colditz, Rimm et al. 1991) while another estimate is of 1.5-folds (Yusuf, Hawken et al. 2004).

### 2.5 Diagnostic Techniques for CAD

Preliminary evaluation of patients with chest pain or angina involves a comprehensive examination of clinical history, physical evaluation so as to exclude non-cardiac reasons of chest pain followed by tests and procedures needed for clear diagnosis and assessment of CAD severity.

#### 2.5.1 Electrocardiography (ECG)

ECG refers to documenting electrical activity of heart over a period of time using electrodes placed on the skin. Einthoven was the pioneer to develop it and awarded Nobel Prize in Medicine for this discovery in 1924 (Batohi and Sidhu 2014). Resting ECG can
provide baseline status, and dynamic ECG alteration associated with symptoms is helpful for diagnosis. Twenty-four hours dynamic ECG can help improve detection rate of myocardial ischaemia (Li, Xiao et al. 2016).

2.5.2 Cardiac ultrasound (echocardiography)

Images of heart are generated using standard two-dimensional, three-dimensional and Doppler ultrasound. Swedish physician Inge Edler is recognized as the “Father of Echocardiography” (Batohi and Sidhu 2014). It is routinely used in diagnosis, management and the follow-up of patients with suspected or known heart diseases. It also gives the estimates of heart function, such as a calculation of the ejection fraction, cardiac output and the diastolic function. Segmental wall motion abnormality increases the chance of diagnosis of CAD and the overall left ventricular (LV) contractile function is meaningful for prognostic risk assessment of CAD patients.

2.5.3 Stress echocardiography

It is an extensively used technique for assessing extent and CAD and provides dynamic assessment of myocardial function and structure physiological stress (exercise) or pharmacological stress (inotrope, vasodilator) (Lancellotti, Pellikka et al. 2016). It is recommended in all major cardiology guidelines (Sicari and Cortigiani 2017). Transient worsening of the regional function during stress is hallmark of inducible ischaemia (Sicari and Cortigiani 2017). The patient undergoes stress either in the form of exercise (treadmill test) or with chemically (generally dobutamine) (Sicari, Nihoyannopoulos et al. 2008). On achieving the targeted heart rate, the stress echocardiogram images are recorded. The two echocardiogram images (prior to the test and another obtained after the test) are compared to evaluate any abnormalities in the wall motion of heart (Varga, Garcia et al. 2006; Lowenstein, Caniggia et al. 2014). This is an economical, simple, convenient, practical, relatively safe, non-invasive with high sensitivity and specificity for CAD diagnosis (Belardinelli, Lacalaprice et al. 2003). However, it cannot further characterize specific coronary arteries, and the influence of less exercise duration and impaired exercise tolerance should be taken into consideration.

2.5.4 Coronary computed tomographic angiography (CCTA)

CCTA employs injecting iodine-rich contrast material and CT scanning to inspect arteries supplying blood to heart and detect plaque buildup (Zhou, Yang et al. 2016). The
generated images are reformatted to create a three-dimensional image that can be printed on a film, viewed on monitor, or can be transferred to the electronic media.

This technique has best spatial and temporal resolution to assess anatomical structures of coronary artery, and mainly used to diagnose epicardial coronary stenosis and CCTA is characterized by high sensitivity (Mark, Berman et al. 2010; Zhou, Yang et al. 2016). CCTA can be used not only to evaluate the narrowing of vessels, but also to quantitatively evaluate plaques to make preliminary diagnosis of plaque vulnerability. Therefore, it has important clinical diagnostic values for suspected CAD (Vanhoenacker, Heijenbrok-Kal et al. 2007; Motoyama, Sarai et al. 2009). Some patients who cannot tolerate stress cardiac imaging may also select CCTA as an alternative option (Mark, Berman et al. 2010; Montalescot, Sechtem et al. 2013). CCTA examination is characterized by convenience, rapid acquisition and high spatial resolution. It can collect data of pulmonary vasculatures, coronary artery, the heart and ascending and descending aorta but has certain limitations also. Firstly ionizing radiation is used. Secondly, the iodinated contrast agent in CCTA may result in hypersensitivity, shock, or adverse reaction such as worsened renal function. However, the dosage of contrast agents has been reduced significantly. Moreover it is not recommended to conduct skin tests for iodinated contrast agent (Chen, Chen et al. 2014).

2.5.5 Coronary Angiography

Angiography is an imaging technique that visualizes the lumen of arteries, veins and the heart chambers by injecting a radio-opaque contrast agent followed by imaging using X-ray based technique (fluoroscopy). It was developed by Portuguese neuorologist and physician at the University of Lisbon in 1927 (Wake, Yoshiyama et al. 2011). Usually it is performed through femoral artery using guide wires and catheters. The contrast agent/dye absorbs the X-rays and in the areas of artery narrowing and blockage, reduced flow of dye is observed. It stands as a gold standard test for identification of the presence and severity of CAD. It is generally considered a safe procedure. However, allergy to the contrast dye, bleeding under the skin at the wound site (haematoma) can be observed which subsides after a few days (Zhou, Yang et al. 2016).

2.5.6 Radionuclide myocardial perfusion imaging (RMPI)

It includes the use of positron emission tomography (PET) or single-photon emission computed tomography (SPECT) to detect and evaluate myocardial perfusion status and myocardial viability to provide functional information of the coronary arteries.
MPI has been extensively utilized in the detection of CAD, including diagnosis of myocardial ischaemia and infarct, evaluation of viability of the myocardium, ECG gated imaging can also provide information of myocardial perfusion, segmental wall motion, mechanical synchronization and cardiac function parameters at the same time (Bateman, Heller et al. 2006). SPECT has shown the average sensitivity for detecting more than 50% angiography stenosis to be 97%, whereas the average specificity is 87% (Di Carli, Dorbala et al. 2007).

It has higher spatial resolution and better diagnostic accuracy than SPECT. It can quantify absolute blood flow to detect microvascular diseases and determine the myocardial blood flow reserve, early detection of CAD, especially for the patients with microvascular disease, balanced multi-vessel CAD and obesities (Di Carli and Hachamovitch 2007). Although majority of technical advantages of PET have been recognized since long, access for routine detection of CAD remains somewhat limited due to the high costs. However, clinical applications are increasing gradually and it is useful for the comprehensive assessment of coronary artery function and myocardial perfusion.

### 2.5.7 Cardiac magnetic resonance imaging (CMRI)

CMRI acquires two/three dimensional images of the heart employing the use of contrast agent which identifies areas of ischaemia, ventricular dysfunction, MI and proximal coronary artery anatomy. CMRI serves as a gold standard for evaluating cardiac structure and function. It can also evaluate tissue characteristics, such as edema, fibrosis, and hemorrhage (Coelho-Filho, Rickers et al. 2013). For CMRI, the sensitivity was found to be 86.5% and specificity of 83.4% and CMRI is reported to be superior than SPECT (Greenwood, Maredia et al. 2012). CMRI has no ionizing radiation and may image in any orientation. The imaging takes longer time than CCTA and the images are not as high resolution as CCTA in clinical practice. It cannot be used in claustrophobic patients and patients implanted with ferromagnetic metals/devices. Some objects with low or non-ferromagnetic object (e.g. stent implantation) may generate artifact.

### 2.5.8 Fractional flow reserve-computed tomography (FFR-CT)

It is high performance diagnostic technique capable of identifying patients having haemodynamically significant obstructions with a high sensitivity (86%) and specificity (79%) (Nørgaard, Leipsic et al. 2014; Douglas, Pontone et al. 2015). It employs merging both anatomical and functional information to assist suitable therapeutic decision making by the clinicians.
Recently, advancement to FFR has been developed that is known as QFR-CT (quantitative flow ratio computed tomography) (QFR by Medis medical imaging) which evaluates the functional implication of coronary stenosis relying on the computer calculation of FFR value. These calculations are executed by exploring the coronary angiogram and therefore this reduces or eliminates the requirement of measuring FFR by pressure wires. This method combines 3D reconstructions of target vessels based on the two angiographic projections and contrast flow velocity in order to compute the "FFR value" (https://clinicaltrials.gov/ct2/show/NCT02811796).

2.5.9 Intravascular ultrasound (IVUS)

IVUS allows differentiating among different plaque phenotypes and is appropriate for high risk plaque detection (Calvert, Obaid et al. 2011; Cheng, Garcia-Garcia et al. 2013). Histo-pathologically, the thin cap fibro-atheroma (TCFA) is illustrated by a necrotic core having a thin fibrous cap containing few VSMCs and abundant macrophages. The TCFA evaluated by IVUS-virtual histology in the coronary region is an independent predictor of incidence of an adverse cardiac event (Stone, Maehara et al. 2011).

2.5.10 Optical coherence tomography (OCT)

Of lately, OCT has emerged as a novel intra-coronary imaging tool providing with high resolution (10 mm) and provides with a thorough comprehensive assessment of the surface components including the fibrous lipid cap thickness measurements of atherosclerotic plaque (Barlis and Schmitt 2009). OCT presents an unsurpassed spatial resolution with the ability to differentiate different lipid content, calcium buildup, intimal hyperplasia and allows visualization of the fine structures in coronary arterial wall (Yabushita, Bouma et al. 2002; Kume, Akasaka et al. 2006). The easy image acquisition in OCT permits the optimized expansion and the placement of stent and also permits the evaluation of its long-term endothelialization (Barlis and Schmitt 2009).

2.5.11 Near-infrared (NIR) spectroscopy

NIR is used normally in physical sciences to elucidate chemical composition of the substances. This technique has been employed for identification of chemical components of coronary plaques and hence a proper evaluation of risk. The imaging requires tissue irradiation with NIR light source. Aortic and coronary artery autopsy specimen studies have led to identification of lipid components specifically the TCFA but
failed to provide data about lumen and plaque anatomy and morphology (Caplan, Waxman et al. 2006). However, this issue resolved upon developing a hybrid catheter that combined both NIR spectroscopy and IVUS imaging (Schultz, Serruys et al. 2010). Also, combining NIR with OCT promises biological and morphological imaging of atheromas in coronary tree (Yoo, Kim et al. 2011; Verjans, Osborn et al. 2016).

2.6 MANAGEMENT OF CAD

It is crucial to implement aggressive strategies in order to curb the risks associated with CAD and prevent its further effect on an individual’s health. Due to diverse distribution of the risk factors across different geographical locations in India, it is mandatory to focus on the management strategies which include lifestyle changes, pharmacological management and revascularization procedures.

2.6.1 Lifestyle management and control of risk factors

The very first approach that one can adopt to reduce the risk for CAD is to incorporate healthy lifestyle habits in daily routine. Both direct and passive smoking contribute robustly to CAD, thus establishing abstinence from such practice is an essential part of a healthy lifestyle management (Boffetta and Straif 2009; Ramakrishnan, Bhatt et al. 2013). While up to 50% reduction in the risk of coronary event has been noticed within the first two years after cessation, the benefits are clearly noticeable in the first few months (Jha, Ramasundarahettige et al. 2013; Fischer and Kraemer 2015). Moreover, the positive effect of quitting even in the later stages of life strengthens the notion that it is never too late to quit. Potential treatments such as sustained release bupropion and nicotine are being reported to have beneficial effects (McRobbie and Thornley 2008).

A Mediterranean diet, Dietary Approach to Stop Hypertension (DASH) diet and the AHA II diet are highly recommended for preventing CVDs. These diets comprise of fruits, vegetables, low-fat dairy foods along with reduced saturated and total fat and have been shown to modulate the BP. They provide an effective nutritional strategy for preventing the CVDs (Siervo, Lara et al. 2015). Meta-analysis studies reported low-carbohydrate diet associated with considerable weight loss (Sackner-Bernstein, Kanter et al. 2015). In the Indian context, studies have demonstrated a significant protective effect of fruit and vegetable intake against CAD risk (Shridhar, Dhillon et al. 2014). High water consumption and reduction of sweetened beverages such as juices and soda can further help in reducing excessive weight considerably (Malik, Schulze et al. 2006; Vij and Joshi...
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2014). Also, it is advisable to a CAD patient to restrict his alcohol consumption to 2 glasses daily for men and 1 glass for non-pregnant women (Perk, De Backer et al. 2012). Moreover, omega-3 polyunsaturated fatty acids demonstrate an encouraging role in prevention of all the CVDs (Lee, O’keefe et al. 2009; Perk, De Backer et al. 2012).

Reliance on technology and urbanization has made the world lethargic with little or no scope of any physical activity. Physical activity or in other words, regular exercise is known to increase one’s lifespan and offer a much healthier lifestyle irrespective of adverse inherited factors. Physical activity of any sort, at all ages, protects against a multitude of chronic health problems and CVDs. Being consistent with structured physical activities and aerobic exercises can significantly reduce the risk of all types of coronary accidents (Löllgen, Böckenhoff et al. 2009; Warren, Barry et al. 2010). Thus, it is recommended that a significant amount of time should be dedicated to physical activity or exercise daily to prevent the onset of CAD (Wendel-Vos, Schuit et al. 2004). Physical exercise will help in reduction of the weight which is directly proportional to the risk of onset of CAD, hypertension, dyslipidemia and glucose metabolism. Hence, in order to significantly reduce such episodes, it is necessary to be actively engaged in routinely physical activities and exercise so as to control the obesity (Perk, De Backer et al. 2012).

One of the major risk factor for cardiac diseases is dyslipidemia (Nelson 2013) and need to be managed according to lipid management guidelines incorporating both the pharmacological and lifestyle interventions (Reiner, Catapano et al. 2011). Furthermore, management of diabetes also have a comprehensive risk reduction towards CAD risk (Montalescot, Sechtem et al. 2013). Hypertension is another major risk factors for CAD. Major studies and trials continue to demonstrate the effect of blood pressure reductions up to 3 to 5mm of Hg results in considerable reductions in risk for CVDs (Chobanian 2003). Moreover, diabetic patients are cautioned regarding their blood pressure levels and recommended to lower it to at least 140/85 mm of Hg (Lewington, Clarke et al. 2003).

Depression and anxiety also need to be combated in CAD patients. Thus it becomes paramount to assess such cases for psychological distress and intervene by providing appropriate care. When the symptoms of depression anxiety and hostility are clinically significant, psychotherapy can help a great deal. Medication and collaborative care for high risk patients can help to cope with these symptoms although there is still a lack of conclusive evidence of such measures on cardiac endpoints (Perk, De Backer et al. 2012).
2.6.2 Medical management of CAD

The prime objective of the pharmacological management of CAD is to obtain relief from anginal symptoms and cardiovascular event prevention. The optimal medical therapy for CAD has been summarized in the table below (Table 2-4):

Table 2-4: Drug management in CAD

<table>
<thead>
<tr>
<th>DRUGS</th>
<th>MECHANISM OF ACTION</th>
<th>SIDE-EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin converting enzyme (ACE) inhibitors (Ramipril, Enalopril, Captopril, Perindopril)</td>
<td>Vasodilation and decreasing the BP by preventing angiotensin I to angiotensin II conversion.</td>
<td>Tiredness and dizziness</td>
</tr>
<tr>
<td>Angiotensin II receptor blockers (Telmisartan, Olmisartan, Losartan)</td>
<td>Vasodilation by blocking angiotensin II receptor activation and decreased secretion of vasopressin</td>
<td>Sinus pain, tiredness and dizziness</td>
</tr>
<tr>
<td>Anti-platelets (Aspirin, Clopidogrel, Prasugrel, Ticagrelor)</td>
<td>Reduces platelet accumulation by irreversibly inhibiting platelet cyclooxygenase-1 and thus thromboxane production. Some inhibit platelet aggregation by acting antagonist to platelet adenosine diphosphate receptor</td>
<td>Bleeding risk</td>
</tr>
<tr>
<td>β -blockers (Metoprolol, Bisoprolol, Atenolol, Carvedilol, Nebivolol)</td>
<td>Reduction in heart rate by blocking adrenaline binding to G-protein coupled β-adrenoceptors</td>
<td>Fatigue, peripheral vasoconstriction, block of atrioventricular conduction</td>
</tr>
<tr>
<td>Calcium channel blockers (Nifedipine, Amlodipine, Verapamil, Diltiazem)</td>
<td>Lowers BP by non-competitive inhibition of calcium influx via the voltage-dependent L-type calcium channels.</td>
<td>Drowsiness, hot flashes, constipation, fluid retention in legs</td>
</tr>
<tr>
<td>Statins (Atorvastatin, Rosuvastatin, Fluvastatin, Lovastatin, Pravastatin, Simvastatin)</td>
<td>Lowers cholesterol by competitively inhibiting HMG-CoA Reductase</td>
<td>Muscle pain, increase blood glucose levels</td>
</tr>
</tbody>
</table>
DRUGS | MECHANISM OF ACTION | SIDE-EFFECTS
---|---|---
**Ezetimibe** | Impairs cholesterol absorption by inhibiting ACAT in intestinal villi | Numbness, dizziness, depressed mood

**Nitrates** *(Nitroglycerin, Isosorbidedinitrate)* | Vasodilator. NO activates guanylyl cyclase thus increasing cyclic guanosine monophosphate. Also inhibits potassium channels and hyperpolarise the membrane. | Headache, flushing, hypotension eventually with syncope.

**Nicolandil** | Activates ATP sensitive potassium channels, thus causing vasodilatation | Oral, intestinal and perianal ulceration, headaches

**Trimetazidine** | Inhibits intracellular ATP reduction by conserving cellular metabolism in ischaemic regions. | Contraindicated in patients with Parkinson’s disease and motion disorders

**Ranolazine** | Inhibits influx of sodium in myocardium in frequency and voltage dependant manner. | Compromised clearance in renal and hepatic disorders, nausea, dizziness, headache

**Ivabradine** | Reduces heart rate by blocking F channel in the sino-atrial node and inhibiting IF current. | Visual disturbances, headache, dizziness, bradycardia, atrial fibrillation, heart block

### 2.6.2.1 Monoclonal antibodies as a novel therapeutic approach

Apart from the standard medical drug management, antibody based therapy is one of the newest and fastest growing field *(Fiedler 2017)*. By January 2017, about 68 monoclonal antibodies (mAbs) had been licensed for clinical use *(Cai 2016)*. However, only 48 of these have cardiovascular applications and mainly represent different studies for the same few drugs – thus accounting to a total of 10 mAbs *(Fiedler 2017)*. Three of the current ten are now approved for CAD *(Alirocumab, Evolocumab and Abciximab)*. Targets, indication and status of all current and withdrawn mAbs are summarised in Table 2-5.
Table 2-5: mAbs to treat CVDs- its targets, trade name, company and status. Adapted from (Fiedler 2017).

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Trade Name, Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abciximab</td>
<td>Glycoprotein IIb/IIIa receptor antagonist, inhibits platelet aggregation</td>
<td>Reopro, Janssen Biologics/Eli Lilly</td>
<td>Phase II to IV</td>
</tr>
<tr>
<td>Alirocumab</td>
<td>Blocks PCSK9, lowers LDL</td>
<td>Praluent, Regeneron/Sanofi</td>
<td>Phase III to IV</td>
</tr>
<tr>
<td>Canakinumab</td>
<td>Neutralises IL-1β</td>
<td>Ilaris, Novartis</td>
<td>Phase I to III</td>
</tr>
<tr>
<td>Eculizumab</td>
<td>C5 complement inhibitor</td>
<td>Soliris, Alexion Pharmaceuticals</td>
<td>Phase IV</td>
</tr>
<tr>
<td>Evinacumab</td>
<td>Blocks angiopoietin-like 4 (Angptl3), lowers LDL</td>
<td>Regeneron</td>
<td>Phase I</td>
</tr>
<tr>
<td>Evolocumab</td>
<td>Blocks PCSK9 and lowers LDL</td>
<td>Repatha, AMG 145, Amgen</td>
<td>Phase I to III</td>
</tr>
<tr>
<td>Inclacumab</td>
<td>P-selectin on ECs, blocking inflammatory cell extravasation</td>
<td>RO4905417, Genentech/Roche</td>
<td>Phase II</td>
</tr>
<tr>
<td>Tadocizumab</td>
<td>Glycoprotein IIb/IIIa integrin on platelets, blocking interaction with fibrinogen and fibronectin</td>
<td>Yamanōchi Pharma America</td>
<td>Phase II</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Blocks IL-6 receptor</td>
<td>Actemra, Hoffman-LaRoche</td>
<td>Phase II</td>
</tr>
<tr>
<td>TS23</td>
<td>Inhibits α-2 antiplasmin</td>
<td>Daiichi Sankyo</td>
<td>Phase I</td>
</tr>
</tbody>
</table>

The use of mAbs in treating CVDs is a newly emerging field having high therapeutic potential. But proper efficacy assessment requires long term follow-up. Many trials are ongoing and results will be available in few years and the findings of such studies will determine the future of this mAb treatment for various CVDs.

2.6.3 Revascularization

Coronary revascularization is defined as the practice of reinstating perfusion to the ischaemic myocardium. Revascularization is necessitated when the symptoms cannot be relieved by the medical treatment (Montalescot, Sechtem et al. 2013). The necessity for revascularization in CAD patient relies on the presence of significant obstructive coronary artery stenosis and degree of related ischaemia. Also, the decision to
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revascularize depends on the individual’s risk of the respective procedure, severity of the disease, financial status and other body parameters. Revascularization can also be opted at an early stage when the high risk is diagnosed in the non-invasive test findings (Mancini, Gosselin et al. 2014). The two major revascularization procedures adopted are as follow:

(a) Percutaneous coronary intervention (PCI)

First PCI was performed on Sept 16, 1977 by Grüntzig in Zurich, Switzerland and since then the field of PCI has evolved rapidly (Rösch, Keller et al. 2003). The goal is to open the clogged arteries, allowing blood to flow freely through them. Once real-time imaging determines the catheter is in correct place, balloon is inflated, which compress the plaque and opens up the blocked artery. The catheter is inserted either through femoral or radial artery and then guided up to heart to the blocked artery. A stent is inserted at the time of ballooning to ensure the vessel remains open, and balloon is then deflated and withdrawn. The stent, a mesh-like tube of thin wire, is inserted after the balloon is inflated (Boden, O'rourke et al. 2007).

There are two types of stents: bare metal and drug eluting stents. However, bare-metal stents undergo restenosis due to tissue growth either inside or around the edges of stent that was placed. The next innovation is the drug-eluting stents, which are coated with medicine known to suppress restenosis. These drug-eluting stents are thinner, more flexible, and coated with improved polymers to release the medicine that prevented tissue growth; and showed improved results for patients compared to bare-metal stents. These days, drug eluting stents are routinely used in angioplasty procedures, and significantly reduce the likelihood of the artery becoming obstructed again (Boden, O'rourke et al. 2007).

(b) Coronary artery bypass graft (CABG)

Although these days, PCI is the most frequently used modality for myocardial revascularization, the CABG continues to play imperative role in managing patients suffering from highly advanced obstructive CAD. CABG surgery is carried out to treat CAD by bypassing blocked arteries with a section of healthy blood vessel from another part of the body(GLANCE 2017). CABG is a choice of option for patients having multi-vessel CAD, advanced T2DM, patients allergic to the dye used in PCI (Investigators 2002; Serruys, Morice et al. 2009; GLANCE 2017).
2.7 GENETIC POLYMORPHISMS

The field of genetics has seen a revolution with a special focus on the arena of the personalized medicine. The Human Genome Project led to the identification of these subtle variations among the populations (Consortium 2004; Consortium 2007). The SNPs refer to the variation in a single point change in the genome and reported to have a frequency of more than 1% of the population (Sachidanandam, Weissman et al. 2001). About >9 million SNPs are accounted in the databases. The SNPs can be biallelic, while very rare tri- or tetra-allelic forms are also found. Average frequency of SNPs is approximately 1 in 1000 bp in human genome (Sachidanandam, Weissman et al. 2001). These SNPs are considered to be primarily responsible for the phenotypic differences between individuals and also have an effect on the disease development, response to the drug treatment and environmental stress. However, the SNPs, have an uneven distribution in the genome. Due to the natural selection pressure, SNPs are sparse in the coding regions of genome and most prevalent in non-coding regions. Although the SNPs in the non-coding will not lead to an altered encoded protein but can serve as physical or genetic markers in comparative and evolutionary genomic studies. SNPs in regulatory regions modulate transcription rates thereby leading to altered levels of encoded proteins. Coding region SNPs can alter the protein structure, hence its function thereby causing the disease or altered drug response (Craddock, Hurles et al. 2010).

2.7.1 SNPs in CAD

CAD has imperative genetic underpinnings considered to be equivalent to those of environmental factors. Heritability of CAD is estimated between 40-60% by the family and the twin studies (Vinkhuyzen, Wray et al. 2013). Multiple studies have indicated that genetic influences contribute majorly to the early-onset of CAD events (Zdravkovic, Wienke et al. 2002; Lloyd-Jones, Nam et al. 2004; Schunkert, König et al. 2011).

The completion of the Human Genome Project, the sustainable sequencing of large panels of genes or whole exome/genome sequencing, has promised the progressing individual genotyping and genetic characterization of individuals at risk (Delude 2015). A recent GWA study meta-analysis comprising 185,000 individuals confirmed a number of known CAD linked loci. This led to identification of ten new loci and thus revealing candidate genes implicated in various biological processes in the vessel walls (Nikpay, Goel et al. 2015). This study also revealed that the genetic susceptibility is greatly influenced by SNPs having a little effect size (Nikpay, Goel et al. 2015).
GWA studies conducted worldwide have led to the discovery of common SNPs at 45 loci which correlated to MI/CAD risk (McPherson, Pertsemidis et al. 2007; Schunkert, König et al. 2011; Deloukas, Kanoni et al. 2013; Won, Natarajan et al. 2015). Even though these newly identified loci have offered vital and novel biological insights (Kathiresan and Srivastava 2012), still they are able to explain only 15% estimated heritability (Deloukas, Kanoni et al. 2013). This reveals that the majority of genetic factors are still unknown. Thus, the most profounding task which is faced by the biomedical researchers is to unravel the complete genetic basis of the cardio-metabolic disorders like diabetes, CAD, obesity and HTN.

SNP studies aim to identify SNPs causing changes in the cellular biological processes (Levy, Sutton et al. 2007; Manolio, Collins et al. 2009). The classic approach is the “case-control study” that employs SNP genotyping in patient and healthy control population to compare the genotypic differences for all the selected phenotypic characteristics so as to characterize the susceptibility genes linked with the disease.

2.7.2 Pharmacogenomics

Pharmacogenomic studies aim to reveal the effect of SNPs on response to drug (Giacomini, Brett et al. 2007). Consequently, only the patients taking specific drug can participate in such studies, thus clinical trials are the sole source of sampling. Despite the obstacles, the application of pharmacogenomics has become progressively popular in diseases like asthma, heart failure, endometrial cancer, diabetes, rheumatoid arthritis, acute lymphoblastic leukemia (Rost, Fregin et al. 2004; Hughes, Beasley et al. 2006; Giacomini, Brett et al. 2007; Ingelman-Sundberg, Sim et al. 2007; Donnelly, Doney et al. 2008; Yu and Bukaveckas 2008; Azuma and Nonen 2009; Bland, Calingaert et al. 2009; Stocco, Cheok et al. 2009). The major driving reason for this increasing popularity is that personalized medication will help in overcoming the adverse drug reactions which is a regular problem witnessed by the clinicians and the patients and in some cases, it might prove fatal.

Pharmacogenomics approach has been utilized in Indian population for cardiovascular drugs such as clopidogrel (Kar, Meena et al. 2013; Shalia, Shah et al. 2013; Subraja, Dkhar et al. 2013), warfarin (Pavani, Naushad et al. 2012; Pavani, Naushad et al. 2012; Gaikwad, Ghosh et al. 2013; Kumar, Shewade et al. 2014), acenocoumarol (Rathore, Agarwal et al. 2012; Kaur, Khan et al. 2013) and statins
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(Poduri, Khullar et al. 2010; Kadam, Ashavaid et al. 2016; Rafeeq, Ahmad et al. 2016). Many companies in India has also come up in this field such as Xcode Lifesciences (https://xcode.in/), NutraGene (http://www.nutragene.com/), Acton Biotech, Pune, OncQuest Laboratories, Mumbai (www.oncquest.net/), TCG Life Sciences, Advinus Therapeutics, Avesthagen, Bangalore and Jubilant Biosys, Bangalore.

The fundamental requirement of all the disease genetic studies and the pharmacogenomic studies is the need to genotype multiple SNPs in equally large sample populations (Pulley, Denny et al. 2012). Therefore achieving high throughput has been the major indispensable factor in developing the genotyping assays (Lamb, Crawford et al. 2006; Thorn, Whirl-Carrillo et al. 2007; Gamazon, Huang et al. 2010; Tatonetti, Dudley et al. 2010). This has led to the advent of high-throughput techniques for SNP genotyping.

2.7.3 High-throughput technique for SNP genotyping

With the introduction of high-throughput techniques for SNP genotyping, researchers have been able to genotype multiple SNPs in large sample sizes which has aided in comprehensive genome analysis. This has helped in uncovering numerous variations in genes rather than focusing only on one or few variants. Chip-based genotyping assays alongside the data on patterns of co-inheritance of the markers (through linkage disequilibrium) has been developed using HapMap Project has encouraged the GWA studies of complex diseases. However, the major issue is the selection of the appropriate high-throughput genotyping method for our goal and also on the stage of the experiment.

2.7.3.1 SNP TaqMan Light Typer Pyrosequencing

The KTH Royal Institute of Technology in Stockholm, Sweden (Europe) were the pioneers to develop the technique of pyrosequencing in 1988 (Hyman 1988). It employs the luminometric detection of the pyrophosphate (PPI) which is released during elongation step by incorporating the nucleotide with help of DNA polymerase (Fakruddin, Chowdhury et al. 2012). As the released PPI is detected for the genome sequence analysis, thus it is named as “pyrosequencing”.

In the elongation step, the incorporation of a nucleotide is accompanied with the PPI release. This PPI needs adenosine 5’-phosphosulfate (APS) which quickly convert
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adenosine triphosphate (ATP) employing sulfurylase. Luciferase is basically an ATPase utilizing the released ATP so as to convert luciferin to oxyluciferin along with light production in few milliseconds. A charge coupled device camera records the produced light thus quantitatively measuring nucleotides incorporated in the form of a pyrogram(Royo and Galán 2009). In the SNP detection studies using this technique, the 3’-end of the primer is designed in such a manner that it hybridizes to the bases before polymorphic position. Pyrosequencing allows the clear detection of all the genotypes and allele combination (homozygous or heterozygous) in a single tube with a clear distinction (Ekström, Önnerfjord et al. 2000; Nordström, Nourizad et al. 2000). This method has been employed for detecting SNPs in β-fibrinogen (-455G/A and -854G/A), coagulation factor V Leiden (Arg506Gln), coagulation factor VII (-401G/T and -402G/A), coagulation factor XIII (G163T; Val34Leu), plasminogen activator inhibitor-1 (−675 4G/5G), methylenetetrahydrofolate reductase (C677T; Ala222Val), glycoprotein IIIa (C1565T; Leu33Pro) and endothelial nitric oxide synthase (G894T; Glu298Asp) (Holmberg, Persson et al. 2005).

2.7.3.2 SNaPshot Multiplex-PCR

Applied Biosystems (ABI) developed high-throughput technique for genotyping that employs the primer extension method using a single-tube reaction. The technique has the ability to recognize at least 10 SNPs irrespective of chromosomal position and remoteness of neighboring loci SNPs (Di Cristofaro, Silvy et al. 2010). SNPs are identified depending on the fluorescence of the labeled dideoxy NTPs (ddNTPs) incorporated at 3’ end of each of the probe primer to template. In the first step, multiplex-PCR reaction generates amplicons of target SNPs. Then the multiplex-PCR single-base extension assay incorporates complementary fluorescently labeled ddNTPs at the 3’ end of each probe primer to the template near to the SNP loci. Then the capillary electrophoresis (CE) determines the extended probe primers size and hence the fluorescence colour readout (Di Cristofaro, Silvy et al. 2010). The method has been employed to detect KRAS and BRAF mutation activating Ras/Raf/mitogen-activated protein kinase in a study conducted on French population for colorectal cancers (Magnin, Viel et al. 2011). Recently, study conducted by (Coutinho, Valverde et al. 2014) in pre-Columbian Native American populations genotyped 26 mitochondrial SNPs characterizing Native American sub-haplogroups from pre-Columbian.
2.7.3.3 SNplex Genotyping System

SNplex is a cost friendly technique from ABI, USA having the capability to genotype simultaneously about 48 SNPs in one oligonucleotide ligation (OLA) reaction (Tobler, Short et al. 2005). The OLA-PCR reaction is done by having a specific Zipcode sequence ligated to allele-specific oligos at 5’ end and a locus specific oligos having a primer binding site at 3’ end. This step is followed by the purification step in which the exonucleolytic digestion of the product is done to get rid of excess probes and linkers. Then the PCR is done so as to amplify the ligation products obtained with biotinylated primers. Then the streptavidin coated microtiter plates capture the amplified PCR products. ZipChute probes connected with mobility modifiers bind with single-strand PCR products which are already bound on the microtiter plates and the hybridized ZipChute probes are eluted out (Tobler, Short et al. 2005). SNplex has been used to identify 563 new SNP-based markers in Grapevine (*Vitisvinifera* L.) with 80% efficiency (Pindo, Vezzulli et al. 2008) thus pointing towards its use in future research programs. In 2009, two software tools viz. GMFilter and SXTestPlate have been developed by (Teuber, Wenz et al. 2009) that enhance analysis of SNplex™ plates with GeneMapper software.

2.7.3.4 Sequenom Mass ARRAY platform

Sequenom was developed by an American company but sold to Agena Bioscience in June 2014. This platform utilizes matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) coupled with single-base extension PCR to have a high-throughput multiplex SNP detection. Using it, one can multiplex 40 SNPs per reaction (Gabriel, Ziaugra et al. 2009; Oeth, del Mistro et al. 2009). The technique involves the locus-specific primer extension that adds and anneals the primer immediately upstream of polymorphic site and incubation with the mass-modified ddNTPs terminator which is done by iPLEX assay (Storm and Darnhofer-Patel 2003).

(Karas and Hillenkamp 1988) were the pioneer to introduce MALDI-TOF MS. In it, ddNTPs DNA molecule are desorbed, ionized and then exposed to intense electric field which accelerates the fragments with common kinetic energy which in turn depends on relative mass/charge ratio of the fragments. The time required by each fragment to collide is detected by the ion to electron conversion detector. Thus, mass of primer is indicative of the alleles present at polymorphic site (Gabriel, Ziaugra et al. 2009). This technique has been employed to genotype 69 SNPs in CYP2D6 during pharmacological treatment in
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250 healthy individuals belonging to Sardinian population (Falzoi, Mossa et al. 2010; Falzoi, Pira et al. 2012) and for genotyping methicillin resistant *Staphylococcus aureus* (Syrmis, Moser et al. 2011).

2.7.3.5 Illumina Golden Gate Custom SNP Chips

A South Korean biotechnology company, Macrogen Inc. offers Illumina Golden Gate genotyping arrays. Three oligonucleotides are needed, two specific for allele specific oligos and third is locus specific oligos, that are tagged to the nucleic acid barcode in order to recognize the reaction (Fan, Oliphant et al. 2003; Fan, Gunderson et al. 2006). Hybridization of oligos to genomic DNA is followed by linking using DNA polymerase and ligase. The amplification is carried out by fluorescently labeled oligos binding to the beads. Each one of the bead is complementary only to one of barcodes in locus specific oligos (Perkel 2008). (Song, Ramus et al. 2009) used this technique in his ovarian cancer study and successfully genotyped 93.2% of SNPs for whole genome amplification samples.

2.7.3.6 Affymetrix’s Genome-Wide Human SNP Array 6.0

Affymetrix, Inc., an American company based in California manufactures the DNA microarrays. (Wang, Fan et al. 1998) were the first to design this assay and successfully genotyped 1,494 SNPs on one chip. This led to a revolution in the high throughput genotyping techniques and led to a stepwise increase from 10,000 to 1,00,000 to 5,00,000, and ultimately to nearly one million SNPs (Lamy, Grove et al. 2011) with >99.5% accuracy. The probe is designed such that it is complementary or almost complementary to SNP site. The technique relies on the signal strength depending on DNA concentration in the sample. It also includes the copy number probes that find mutation in the DNA sequence (LaFramboise 2009). The technique has been used by (Walter, Payton et al. 2009) to see acquired copy number variations in the genome of acute myeloid leukemia. These chips were used to look out for Autism Spectrum Disorder candidate genes and highlighted many novel genes responsible for it (Liu, Shimada et al. 2016).

2.7.3.7 Illumina’s High Density Human 1M-Duo chip

Illumina, Inc. was established in April 1998 and is an American company dealing with genetic variation analysis systems. The Illumina Bead Array, just like Affymetrix augmented its capacity progressively from 1,00,000 SNPs (Human-1) to 2,40,000 to
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3,17,000 to 5,50,000 and then ultimately to 6,50,000 (Perkel 2008). It is based on the same principle as Affymetrix but it uses 50-mer oligos (1 per SNP) in comparison to the Affymetrix which requires 25-mers. Also, it relies on the base extension methods but Affymetrix uses the method of probe hybridization. However, the discrete difference between them is that the probes for Illumina are based completely on the haplotype tagging ‘tag SNPs’ which have been identified by International HapMap Consortium while Affymetrix array has half unbiased SNPs and half tag SNPs (Perkel 2008).

2.7.3.8 The Qbead system

The method was first developed and used by (Xu, Sha et al. 2003) and an accurate, cost-effective technique for the SNP genotyping. It employs quantum dots (fluorescent Qdot semiconductor nanocrystals (Bruchez, Moronne et al. 1998)) to encode latex beads. They basically are nanoscopic inorganic crystallites exhibiting the properties on their size and composition (Xu, Sha et al. 2003). One example can be the Cadmium Selenide (CdSe) nanocrystals that can be tuned to have particles 1-7 nm diameter.

Green light will be emitted by CdSe nanocrystals with a 3nm core whereas emission of red light will be seen in the CdSe nanocrystals having 6 nm core (Xu, Sha et al. 2003). Variable emission intensity can be observed using nanocrystals of two emission colors viz. 530 and 565 nm at different concentrations. The biggest advantage that this technique offers is that modifying the probes conjugated to the microspheres, it can be applied for gene expression analysis and for the analysis of protein–protein interactions (Xu, Sha et al. 2003).

2.8 CANDIDATE GENE POLYMORPHISMS AND RISK OF CAD

2.8.1 Genes involved in atherosclerosis

2.8.1.1 Arachidonate 15-lipoxygenase (ALOX15) rs2619112 and rs7217186

Oxidative hypothesis of atherogenesis states that the crucial step in atherosclerosis is the formation of oxidised LDL from LDL which is pro-atherogenic and regulates the lipid uptake in macrophages (Parthasarathy, Steinberg et al. 1992; Yu, Fu et al. 2013) and this step is mediated by lipoxygenase enzymes which act on arachidonic acid (AA) and linoleic acid to form oxygenated fatty acid derivatives (oxylipins) having a number of pathological and physiological implications (Haeggström and Wetterholm 2002; Kuhn, Walther et al. 2002; Kuhn 2005; Kühn and O’Donnell 2006; Wittwer, Bayer et al. 2007;
Wittwer and Hersberger 2007; Bäck 2009; Obrosova, Stavniichuk et al. 2010; Dobrian, Lieb et al. 2011). Naming of ALOX enzymes is accordance with specific carbon atoms of the AA being oxidized. The 12/15-ALOX is involved in conversion of AA to hydroxyeicosatetraenoic acid (12(S)-HETE and 15(S)–HETE) (Chen, Kurre et al. 1994). In humans, six functional ALOX genes have been reported (Haeggström and Funk 2011) out of which ALOX15 is principally found in macrophages and responsible for the maximum lipoxygenase activity in the process of atherosclerosis (Wuest, Crucet et al. 2012). Yokohama 1986, first isolated ALOX15 from porcine leukocytes but it is abundantly found in blood vessels, kidney and brain (Kühn and O’Donnell 2006).

ALOX15 expression has been reported in the human myocardium and linked to the history of atherothrombotic events (Gertow, Nobili et al. 2011; Magnusson, Lundqvist et al. 2012). Prominent expression of ALOX has been observed in the ischemic heart tissue, atherosclerotic plaques and human macrophages (Hulten, Olson et al. 2010; Magnusson, Lundqvist et al. 2012). Studies have documented ALOX15 in inflammation, oxidative stress (Suzuki, Kayama et al. 2014), atherosclerosis, neurodegenerative disorders and diabetes (Natarajan and Nadler 2004; Kühn and O’Donnell 2006). Silencing of ALOX15 in human macrophages led to a decreased lipid buildup and lowered pro-inflammatory cytokines secretion(Magnusson, Lundqvist et al. 2012) whereas its over-expression caused heightened chemokine secretion (Danielsson et al. 2008; Heller et al. 2006). Studies on mouse models and rabbits also support the above studies (Kühn and O’Donnell 2006; Shen, Shi et al. 2015). (Gertow, Nobili et al. 2011) revealed an abundant expression of ALOX15 in human carotid lesions and in macrophage rich areas and specifically in symptomatic lesions. Also ALOX15 enhances VSMC proliferation, migration and hypertrophy and also an elevated expression of adhesion molecules (Taylor, Hanchett et al. 2005). These all evidences point towards the role of ALOX15 in atherogenesis. However, certain studies have a contradicting view.

However, ALOX15 is considered to exert anti-atherogenic effects also such as inhibiting oxidative stress produced by metabolites of ALOX15, decreased formation of the anti-inflammatory lipids (lipoxins, protectins and resolvins) which have a vasodilatory activity. Whether ALOX15 upregulates or reduces adhesion molecules expression and VSMCs proliferation is yet debatable (Reilly, Srinivasan et al. 2004; Bolick, Orr et al. 2005; Taylor, Hanchett et al. 2005; Dobrian, Lieb et al. 2011).
Population genetics association studies examining the association of ALOX15 with CAD also point towards neutral, pro- and anti-atherogenic effect of ALOX15 (Wittwer, Bayer et al. 2007; Assimes, Knowles et al. 2008; McCaskie, Beilby et al. 2008; Hersberger, Müller et al. 2009; Hersberger 2010). The human 12/15-LOX is encoded by ALOX15 that is located on chromosome 17p13.3. ALOX15 has 14 exons and 13 introns (Kuhn and Thiele 1999). The ALOX15 rs7217186 and rs2619112 genotypes in the regulatory region were studied. None of the studies pertaining to ALOX15 have been done on the Indian population and no data with respect to the allelic or genotypic frequency is available for these SNPs in Indian context. The present study has been conducted in the North Indian population which aimed to elucidate the allelic and genotypic frequency of ALOX15 polymorphisms in CAD pathogenesis and correlation with other selected parameters.

2.8.1.2 Lectin-like oxidized-LDL receptor 1 (LOX1) rs11053646 (G501C) and rs1050283 (C188T)

LOX1 was first cloned in 1997 by (Sawamura, Kume et al. 1997) as a mammalian endothelial receptor for oxLDL. It is a 50 kDa transmembrane receptor expressed on monocytes, macrophages, VSMCs, platelets and fibroblasts (Kataoka, Kume et al. 2001; Mingyi, NARUMIYA et al. 2001; Vecchione, Gargiul et al. 2007). LOX belongs to C-type lectin family and has four domains: short cytoplasmic N-terminal domain, transmembrane domain, connecting neck domain and long extracellular C-type lectin like domain (Chen, Masaki et al. 2002; Chen and Sawamura 2005; Chen and Du 2007; Tate 2007; Vecchione, Gargiul et al. 2007) (Figure 2-10 (a)). The C-type lectin like domain, also called the carbohydrate recognition domain is the functional domain for binding of oxLDL(Mehta, Chen et al. 2006) and found to be highly conserved among species (Murphy, Tedbury et al. 2005; Chen and Du 2007; Vecchione, Gargiul et al. 2007). Lectin-like domain is made up of positively charged amino acids which recognize the negative charges on oxLDL (the modifications on the apo B moiety) (Mehta, Chen et al. 2006; Tate 2007).

LOX1 binds with a greater affinity to oxLDL as compared to unoxidized LDL and is a key molecule accountable for the binding, internalization and degradation of oxLDL in the endothelial cells (Vecchione, Gargiul et al. 2007). This binding triggers a cascade of events leading to formation of foam cells, VSMC proliferation, platelet activation,
ROS generation, and collagen degradation (Ellis 2006). Therefore LOX1 plays eminent role in growth of atherosclerosis (Figure 2-10 (b)). The soluble LOX1 has been shown to be increased in vascular carotid plaque in patients with IS (Skarpengland, Skjelland et al. 2018).

Figure 2-10 (a): Structure of human LOX1. Adapted from (Hofmann, Brunssen et al. 2017) and (b): Proposed mechanisms of LOX1 biology in atherogenesis. Adapted from (Pothineni, Karathanasis et al. 2017).
Seven genetic polymorphisms have been reported in the *LOX1* gene. One of these polymorphisms viz. the G to C change at position 501 in exon 4 of *LOX1* gene results in (Lys/Asn) in codon 167 (Ellis 2006; Hattori, Sonoda et al. 2006). The amino acid at 167 resides in C-terminal lectin-like domain and mediates oxLDL binding. Presence of basic residues in this domain strengthens the ligand binding and if these residues get substituted, this will lead to a decreased binding and thus, internalization of oxLDL (Ellis 2006). Multiple studies report this polymorphic change to be associated with MI and CAD (Mango, Clementi et al. 2003; Tatsuguchi, Furutani et al. 2003; Ohmori, Momiyama et al. 2004; Wang, Yanuck et al. 2011).

One more polymorphism is reported in 3′ untranslated region (UTR), 188 bp downstream of stop codon in *LOX1* gene resulting into a C to T change (Chen, Reis et al. 2003; Mango, Clementi et al. 2003; Hattori, Sonoda et al. 2006). This polymorphic change influences the binding of nuclear proteins (NF-κB) and the polymorphic allele is known to be linked with heightened risk of CAD and MI (Chen, Reis et al. 2003; Mango, Clementi et al. 2003; Ohmori, Momiyama et al. 2004; Novelli, Borgiani et al. 2007).

Therefore, this study has attempted to explore the potential role of *LOX1* rs11053646 and rs1050283 SNPs in CAD in a North Indian population and its correlation with other selected selected parameters.

### 2.8.1.3 Antisense non-coding RNA in the INK4 locus (ANRIL) rs1333049 C/G

Lately, a number of GWA studies have discovered a locus on chromosome 9p21 which spans 58 kb and linked to CAD and MI (Jarinova, Stewart et al. 2009; Cunnington, Koref et al. 2010; Ahmed, Ali et al. 2013). Though this locus lacks the atherosclerosis associated genes, but an *antisense non-coding RNA in the INK4 locus* (ANRIL) gene dwells within the vicinity of cell cycle regulating genes found in this region. It is reported to be in strong linkage disequilibrium with cell cycle proliferatory genes such as *cyclin dependant kinase inhibitors 2A and 2B (CDKN2A and CDKN2B)* (Cunnington, Koref et al. 2010). The *CDKN2A* is basically a tumor suppressor gene and encodes two proteins viz. p14ARF and p16. p16 controls G1 to S transition in the cell cycle and p14ARF stimulates cell cycle arrest in G2 phase subsequently leading to apoptosis. The *CDKN2B* lies adjoining the *CDKN2A* and encodes proteins that inhibit the cell cycle G1 progression (Cunnington, Koref et al. 2010).
CDKN2B anti-sense RNA (CDKN2B-AS1) spans about 126.3 kb and overlaps with the CDKN2B (p15) at the 5' end and comprises of 20 exons that are prone to the phenomenon of alternative splicing (Jarinova, Stewart et al. 2009). Differential expression of CDKN2B-AS1 is observed in variety of tissues like ECs and VSMCs in the coronary arteries (Jarinova, Stewart et al. 2009). CDKN2B-AS or CDKN2B-AS1 or INK4 are used as synonyms for ANRIL. CDKN2B-AS1 has been found to associate with the risk of CAD (Dehghan, Bis et al. 2016), MI (Matsuoka, Abe et al. 2015), HTN (Bayoglu, Yuksel et al. 2016) and stroke (Bai, Nie et al. 2014) and polymorphisms in this gene have been recognized as predictors of CVDs (Huang, Ye et al. 2014), cerebrovascular disease (Kremer, Koeleman et al. 2014) and also brain tumors (Adel Fahmideh, Lavebratt et al. 2015). ANRIL locus is reported to alter neighbouring gene’s expression by apparently acting either by RNA interference, gene silencing, chromatin remodeling or DNA methylation (Jarinova, Stewart et al. 2009).

The SNP rs1333049 C/G is positioned in the 3'UTR of CDKN2B-AS1 and considered to play pivotal role in advancement of the cardio and cerebro-vascular disease by modifying dynamics of VSMC proliferation (Cunnington, Koref et al. 2010). Wellcome Trust Case Control consortium study have documented rs1333049 displaying powerful association (Consortium 2007). The association of rs1333049 is studied in many diseases like CAD (Dechamethakun, Ikeda et al. 2014), atherosclerosis (Bochenek, Häsler et al. 2013), MI (Haslacher, Perkmann et al. 2016), metabolic disease (Hannou, Wouters et al. 2015) and Alzheimer’s disease (Popov and Gil 2010). Several GWA studies (Helgadottir, Thorleifsson et al. 2007; McPherson, Pertsemlidis et al. 2007; Samani, Erdmann et al. 2007) and meta-analysis studies (Palomaki, Melillo et al. 2010; Preuss, König et al. 2010) implicate the relationship of this specific region with CAD and MI.

Therefore, the present study is conducted with aim of determining allelic and genotypic frequencies of ANRIL rs1333049 and risk association with CAD and other selected parameters in a North Indian population.

2.8.1.4 Proprotein convertase subtilisin/kexin type 9 (PCSK9) rs505151 A/G (E670G)

PCSK9 or neural apoptosis regulated convertase 1 (NARC1) belongs to proprotein convertase family (Seidah, Benjannet et al. 2003), locates to chromosome 1p32.3 and comprises of 12 exons encoding for 692 amino acid long glycoprotein (Lambert, Charlton
et al. 2009). It is synthesized by the liver in an inactive zymogen form called as pro-PCSK9 (73 kDa). It contains a signal peptide, prodomain (residues 31–152) and catalytic domain (residues 153–451) which is followed by the C-terminal domain (residues 452–692) (Lambert, Charlton et al. 2009). In the endoplasmic reticulum (ER), the pro-PCSK9 undergoes intra-molecular autocatalytic processing at the FAQ152↓SIP site to form mature PCSK9 having a 14-kDa pro-domain and 63-kDa mature PCSK9 (Seidah, Benjannet et al. 2003; Hampton, Knuth et al. 2007; Kwon, Lagace et al. 2008; Seidah 2011). This autocatalytic cleavage is very important and necessitated for the trafficking of PCSK9 from ER to the lysosomal pathway (Lagace, Curtis et al. 2006; Cunningham, Danley et al. 2007). It is chiefly synthesized and secreted from the liver and small expression is also seen in kidney, intestine and brain (Seidah, Benjannet et al. 2003). It is also sought to be secreted by VSMCs in the atherosclerotic plaques possibly triggering lipid accumulation and modification (Ferri, Tibolla et al. 2012).

PCSK9 works as a molecular chaperone and binds to epidermal growth factor–like domain A of the LDLR. The PCSK9/LDLR complex is internalized into the cell (Lagace, Curtis et al. 2006). The C-terminal domain not needed for LDLR binding but obligatory for the complex internalization (Ni, Condra et al. 2010). Interestingly, the PCSK9 also has the ability to bind to LDLR intracellularly and regulate its expression on the hepatic cells (Chan 2011). The PCSK9/LDLR complex is internalized by clathrin mediated endocytosis and targeted to lysosomes for LDLR degradation (Seidah, Benjannet et al. 2003; Lagace, Curtis et al. 2006; Rousselet, Marcinkiewicz et al. 2011; Zhang, Song et al. 2016). Therefore, PCSK9 activation will lead to the down regulation of the expression of LDLR which will hinder the uptake of LDL, leading to LDL level elevation and thus ultimately hypercholesterolemia, CAD or IS (Aung, Yin et al. 2011; Zhang, Song et al. 2016) (Figure 2-11). There are two possible spots to execute this effect. The first being the post-ER compartment, like Golgi apparatus, where the PCSK9 binds to LDLR and targets to lysosome for degradation. A second option is that the secreted PCSK9 binds to LDLR on hepatic cell surface. Upon internalization, the recycling of the LDLR is prevented from endosome to cell surface and thus, re-routing it to lysosome. But till now it is not clear whether PCSK9 directly cleaves the LDLR (Scartezini, Hubbart et al. 2007).
Figure 2-11: PCSK9 regulates the LDLR expression. Adapted from (Mombelli, Castelnuovo et al. 2015).

PCSK9 expression is chiefly controlled by the cholesterol levels and the transcription factor “sterol-responsive element-binding protein (SREBP)-2” (Maxwell, Soccio et al. 2003; Jeong, Lee et al. 2008). The latter coordinates numerous genes engaged in the cholesterol homeostasis like 3-hydroxy-3-methyl glutaryl-coenzyme A reductase and the LDLR expression also (Horton, Shah et al. 2003; Maxwell, Soccio et al. 2003). Animal studies on mice have revealed that the one lacking SREBP-2 have decreased levels of PCSK9 mRNA (Horton, Shah et al. 2003). Low dietary intake of saturated fats or cholesterol reduction by statins will lead to a decrease in the free cholesterol available in the hepatocytes. This signals the SREBP activation which is a key transcription factor for the LDLR leading to elevated expression of LDLR mRNA and thus the protein and also the PCSK9. Therefore, latter acts as counter-regulatory mechanism to avert the excessive cholesterol uptake into the cells. As PCSK9 is involved in LDLR degradation, the expression of both the genes is co-regulated (Dubuc, Chamberland et al. 2004; Ding and Kullo 2008).

Literature reports that mutations of PCSK9 alters its activity towards LDLR and causes hypercholesterolemia or hypocholesterolemia (Abifadel, Rabès et al. 2009). Gain-of-function (GOF) mutations reduce the expression of LDLR on cell surface, thus
inhibiting cellular internalization of LDL. This leads to elevated LDL levels leading to autosomal dominant hypercholesterolemia and premature atherosclerosis (Chan and Watts 2012). On the other hand, the loss of function (LOF) mutation promotes LDLR recycling which leads to decreased serum LDL levels and consequently conferring protection against CAD (Abifadel, Varret et al. 2003; Cohen, Pertsemlidis et al. 2005).

PCSK9 is greatly polymorphic having over 40 non-synonymous, exonic SNPs reported in the humans (Abifadel et al. 2007). Current studies reveal multiple variations in PCSK9 that modulates plasma LDL levels, either negatively or positively and thus influence CAD risk. An SNP in exon 12 of PCSK9 (rs505151 A/G), results in substitution of glutamate for glycine at position 670 in PCSK9 protein i.e. E670G (Aung, Yin et al. 2011). This variant resides in cysteine-rich C-terminal domain that is involved in regulating auto-processing, as deleting this domain led to the accumulation of processed PCSK9 (Naureckiene, Ma et al. 2003).

Previous studies have been done to determine the role of rs505151 and severity of atherosclerosis but results are inconclusive (Chen, Ballantyne et al. 2005; Evans and Beil 2006; Kotowski, Pertsemlidis et al. 2006; Abboud, Karhunen et al. 2007; Scartezini, Hubbart et al. 2007; Polisecki, Peter et al. 2008; Norata, Garlaschelli et al. 2010; Aung, Yin et al. 2011; Slimani, Harira et al. 2014).

Till date, none of the studies has been done on the North Indian population with respect to PCSK9 and CAD. So keeping the above said observations in mind, the present study aims to determine allelic and genotypic frequencies of PCSK9 rs505151 A/G polymorphism in a North Indian population and to ascertain its relationship with CAD.

2.8.2 GENES INVOLVED IN INFLAMMATION

2.8.2.1 Interleukin 8 (IL-8) rs4073 (-251A/T)

IL-8 gene belongs to the CXC (small basic heparin-binding proteins) chemokines superfamily and maps to chromosome 4q13-21 (Gerszten, Garcia-Zepeda et al. 1999; Rigamonti, Fontaine et al. 2008) and codes for the IL-8 protein which is a non-glycosylated protein having 72 amino acids produced by monocytes, tissue macrophages, neutrophils, mast cells, vascular ECs, VSMCs, T lymphocytes, etc. (Hay and Sarau, 2001; Zhang et al., 2011). It was detected by somatic cell hybridization and in situ hybridization method (Modi et al., 1989; Modi et al., 1990). Data reports the role of IL-8 in innate and
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acquired immune responses and as an inflammatory molecule in atherosclerotic plaque development and has shown the IL-8 to behave as a chemoattractant for neutrophils and macrophages (Morris, Nelson et al. 1992; Boisvert, Curnss et al. 2000; Simonini, Mosucci et al. 2000; Vogiatzi, Apostolakis et al. 2008).

Minimally oxidized LDL, oxidized LDL, CD40 ligand, and certain intra-lesional cytokines viz. TNF-α, TNF-β, IL-1β, MCP-1 and IL-6 are found to be upregulated in the endothelium at the early stage of plaque formation which then stimulates the fibroblasts, endothelial cells, monocyte and macrophages to synthesis and secrete IL-8 (Hebert, Luscinskas et al. 1990; Mielke, Bauman et al. 1990; Strieter, Chensue et al. 1990; Boisvert, Curnss et al. 2000). IL-8 displays high affinity towards CXCR1 and CXCR2 receptor on neutrophils, monocytes, macrophages, T lymphocytes, mast cells, and NK cells (Zhang, Zhang et al. 2011). In addition, it mediates monocytes adhesion to the endothelium and modulates the advanced plaques formation by recruiting T lymphocytes and by stimulating angiogenesis. IL-8 acts as a mitogen and chemo-attractant for VSMCs by signaling the VSMCs to release prostaglandins that modulate both VSMC proliferation and migration (Li, Dubey et al. 2003). Also the VSMC migration is induced via increasing the production of lipoxygenase metabolites by shifting eicosanoid metabolism from the cyclooxygenase pathway to the lipoxygenase pathway (lipoxygenase pathway produces 12-hydroxyeicosatetraenoic acid and leukotriene B4 which aid in migration). It also induces enhanced Bcl-2 and decreased Bax expression in endothelial cells, leaning the balance towards the anti-apoptotic effect of IL-8 (Li, Dubey et al. 2003). Also it rapidly and transiently induces proto-oncogene c-fos and zinc finger protein 268 (zif268) expression and activates MAP kinase in VSMCs (Yue, Wang et al. 1994). IL-8 is also sought to be involved in the upregulation of matrix metalloproteinases (MMP2 and MMP9) which degrade the extracellular matrix and thus leads to endothelial cell migration, invasion and also the capillary tube organization and therefore promoting angiogenesis. In the later stages of atherosclerosis, this increased level of MMPs weaken the plaques through the degradation of the fibrous cap which ultimately leads to the rupture of the plaque (Li, Dubey et al. 2003). Studies report that the IL-8 levels remain elevated in the blood for a longer period as compared to other inflammatory cytokines which tend to get cleared off within few hours and hence, can be considered as a marker for detecting USA and MI (DeForge et al., 1992; Shebuski and Kilgore, 2002)(Kanda, Hirao et al. 1996). Polymorphisms in the
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*IL-8* gene might cause an altered expression and thus lead to either a positive or negative contribution towards CAD. Numerous polymorphisms have been identified in this gene. A promoter polymorphism at -251 position results into A/T change (rs4073) and the minor allele correlates with amplified IL-8 expression (Hacking, Knight et al. 2004; Ohyauchi, Imatani et al. 2005; Hildebrand, Stuhrmann et al. 2007). The association of IL-8 serum levels with the genotype and CAD risk has been investigated in few studies but with contradictory findings. The IL-8 levels were found to be higher in MI and USA patients in comparison to controls (Riesenber, Levy et al. 1997; Zhou, Shi et al. 2001; Romuk, Skrzep-Poloczek et al. 2002). However (Zhang, Zhang et al. 2011) reports increased IL-8 plasma levels and ACS risk in a Chinese Han population whereas (Vogiatzi, Apostolakis et al. 2008) report a reduced risk of MI among CAD patients in a Caucasian population with IL-8 levels.

Therefore, in this study, a possible influence of *IL-8* rs4073 A/T polymorphism towards the CAD susceptibility in a North Indian population has been investigated.

**2.8.2.2 Interleukin 10 (IL-10) rs1800872 (-592C/A)**

Inflammation is a key player in the atherosclerotic plaque formation and progression and both the pro- and anti-inflammatory molecules are involved whose upregulation and downregulation is observed in the disease pathogenesis (Virmani, Kolodgie et al. 2005; Libby, Ridker et al. 2009). IL-10 is an eminent anti-inflammatory cytokine secreted by B cells, Th2 lymphocytes and monocytes (Madeshiya, Singh et al. 2017).

The adhesion of the monocyte to vascular endothelium is believed to be the primary step in the vascular wall invasion. This anti-inflammatory IL-10, an inhibitor of this step, as it down-regulates the expression of adhesion molecules viz. CD18 and CD62-L on the immune competent cells (Mostafa Mtairag, Chollet-Martin et al. 2001). Moreover, IL-10 restrains adhesion of LDL to the endothelium and also down regulates the biosynthesis of fibrinogen thereby demoting the monocyte adhesion to the endothelial wall (Tedgui and Mallat 2006). The MMPs are involved in destabilizing the atherosclerotic plaque. IL-10 also has the capability to inhibit the synthesis of the MMP9 by inducing the production of tissue inhibitor of metalloproteinases-1 (TIMP-1) (it inhibits MMP synthesis) (Lacraz, Nicod et al. 1995). IL-10 hinders the transcription factor NF-κB and encourages the phenotypic switch of the lymphocytes into Th2
phenotype by inhibiting the synthesis of Th1 producing cytokines such as IFN-γ, IL-1, IL-2, IL-6, TNF-α and TNF-β), therefore modulating the vascular inflammation process and influencing the plaque stability (Heeschen, Dimmeler et al. 2003; Nishihira, Imamura et al. 2006; Guo, He et al. 2012) and thus regulates equilibrium between the cell and the humoral mediated immune responses (Madeshiya, Singh et al. 2017).

The athero-protective role of IL-10 has been well documented (Mallat, Besnard et al. 1999; Smith, Irving et al. 2001; Fichtlscherer, Breuer et al. 2004). Animal experiments have revealed amazing anti-atherosclerotic effects of IL-10. In LDLR knockout mice, progression of atherosclerosis can be avoided by the over-expression of the human *IL-10* (VON DER THÜSEN, Kuiper et al. 2001). Epidemiologic studies illustrate lower levels of the plasma IL-10 correlating with elevated risk of ACS and IS (Anguera, Miranda-Guardiola et al. 2002; Heeschen, Dimmeler et al. 2003; Seljeflot, Hurlen et al. 2004; Wojakowski, Maslankiewicz et al. 2004; Xie, Myint et al. 2013).

![Diagram](image)

**Figure 2-12:** Potential inhibitory effect of IL-10 on atherosclerotic mechanisms. Adapted from (Girndt and Kohler 2003).

*IL-10* polymorphisms have been shown to be related with T2DM (Mohebbatikaljahi, Menevse et al. 2009; Saxena, Agrawal et al. 2012), diabetic
nephropathy (Kung, Lin et al. 2010; Erdogan, Cetinkalp et al. 2012), ACS (Srikanth Babu, Pulla Reddy et al. 2012), obesity and insulin resistance (Scarpelli, Cardellini et al. 2006), arterial thrombotic diseases (Meuwissen, Van der Wal et al. 2004; Marousi, Antonacopoulou et al. 2011).

**IL-10** is situated on chromosome 1 and has 5 exons and maps to the junction between 1q31 and 1q32 (Koch, Kastrati et al. 2001). The **IL-10 rs1800872 C/A** lies in the putative transcription factor binding site which decreases the expression of IL-10 when mutant allele A is present (Bidwell, Keen et al. 1999; Trompet, Pons et al. 2007). In this study, the role of **IL-10 rs1800872 C/A** in correlation with CAD and other associated parameters in a North Indian population have been explored.

### 2.8.2.3 Interferon gamma (IFN-γ) rs2430561 (+874T/A)

IFN-γ appears to serve as a paramount contender in CAD pathogenesis. It is a pleiotropic, soluble, antiviral and anti-tumourous cytokine (McLaren and Ramji 2009; Voloshyna, Littlefield et al. 2014). IFN-γ is basically a T cell-derived cytokine, serves as marker for the Th1 activation, which promotes and leads to an amplified inflammatory response. The antigen presentation is stimulated by IFN-γ as it induces the expression of Class I and Class II major histocompatibility complex (MHC) molecules on T-lymphocytes and macrophages and stimulates ROS, proteases, TNF-α and production of NO from macrophages and thereby resulting in tissue damage (Firestein 2005). IFN-γ producing cells include the Th1 subpopulation of helper T cells, cytotoxic T cells and NK cells and also dendritic cells and the B cells(Schroder, Hertzog et al. 2004; McLaren and Ramji 2009).

During inflammation, IL-12 and IL-18 secreted by APCs, chiefly by monocytes, macrophages and the dendritic cells, controls the **IFN-γ** production (Schroder, Hertzog et al. 2004). This cytokine has a multitude of functions. It stimulates monocytes differentiation into macrophages (Boehm, Klamp et al. 1997; Schroder, Hertzog et al. 2004). It upregulates the expression of many chemokines *i.e.* MCP1, IFN-inducible protein of 10 kDa, macrophage inflammatory protein-1α and β, CXC-chemokine ligands and IL-12 (Boisvert 2004; Charo and Taubman 2004).

IFN-γ induces the expression of VCAM-1 and ICAM-1 on the ECs and SMCs and also involved in VSMC migration and adoption of a proliferative phenotype (Li, Cybulsky et al. 1993; Chung, Lee et al. 2002; Blankenberg, Barbaux et al. 2003). Also it
may play a role in calcification via increased expression of 1-α-hydroxylase (it catalyzes 25-hydroxyvitamin D conversion to 1-α, 25-dihydroxyvitamin D, which is active metabolite of vitamin D contributes to the process of calcification (Shioi, Mori et al. 2000; Esteban, Vidal et al. 2004).

It also has the capability to encourage the foam cell apoptosis through the apoptotic genes \textit{viz. fas, TRAIL, caspase 4} and 8 (Inagaki, Yamagishi et al. 2002; Ramana, Gil et al. 2002). IFN-\textgamma participates in plaque destabilization by restricting expression of collagen 1 and 3, inhibits VSMC proliferation and matrix synthesis and augments production of MMP1, 2, 3 and 9 (Amento, Ehsani et al. 1991; Schönbeck, Mach et al. 1997; Yuan, Yufit et al. 1999). The MMPs are reported to localize particularly to the shoulder region of plaque where it is most susceptible to rupture (Galis, Sukhova et al. 1994). A brief representation of the multiple effects of IFN-\textgamma is given in Figure 2-13.

![Figure 2-13: Role of INF-\textgamma in atherosclerosis. Adapted from (Harvey and Ramji 2005).](image)

Considering the effect of IFN-\textgamma in regulation of proliferation, differentiation and apoptosis, it seems that it affects plaque formation in the arteries (Harvey and Ramji
2005). IFN-γ is prominently expressed in the atherosclerotic lesions and therefore, considered a significant factor in CAD development and progression (Schroecksnadel, Frick et al. 2006). Studies document IFN-γ as a major trigger for production and release of the ROS in endothelium (Schroecksnadel, Frick et al. 2006). Expression of IFN-γ from the atherosclerotic lesions in both rodent models and clinical samples has been established (Hansson, Holm et al. 1989; Frostegård, Ulfgren et al. 1999).

The IFN-γ gene is situated on 12q15 chromosome, contains four exons and three introns and was first detected by fluorescence in situ hybridization (Zimonjic et al., 1995). Within this region, a functional SNP at +874 A/T in the first intron of IFN-γ gene (rs2430561) maps to putative NF-κB binding site. The efficiency of NF-κB binding is enhanced by the presence of T allele and this leads to an increased IFN-γ expression in vitro (García-Bermúdez, López-Mejías et al. 2012; Manne, Gunde et al. 2012; Sun, Lu et al. 2015). AA is linked to low production, AT to intermediate, TT to high production of IFN-γ respectively (Pravica, Perrey et al. 2000) and hence, associated with disease severity in different inflammatory and autoimmune diseases, USA and MI (Chong, Ip et al. 2006; Dai, Chuang et al. 2006; Pasqui, Di Renzo et al. 2006; Pacheco, Cardoso et al. 2008; Khor, Gardet et al. 2011).

Therefore, keeping the aforesaid associations in mind, the present study has been designed to look for the genotypic and allelic frequency of IFN-γ rs2430561 A/T polymorphism in a North Indian population and its association with CAD and also, to ascertain the relationship with various risk factors of CAD.

2.8.2.4 Toll-like Receptor 4 (TLR4) rs4986790 (896A/G or Arg299Gly) and rs4986791 (1196C/T or Thr399Ile)

Toll-like receptors (TLRs) are key molecules involved in innate immunity and pathogen recognition (O'neill 2008). TLRs are highly evolutionarily conserved and were initially identified as “Toll proteins” in Drosophila in 1984 (Steward, McNally et al. 1984; Lemaitre, Nicolas et al. 1996; Akira and Takeda 2004).

One of the best-studied TLR is TLR4. It is expressed in cardiomyocytes, monocytes and ECs (Zarembber and Godowski 2002; Kielian 2006; Mitchell, Ryffel et al. 2007). Apart from binding to the lipopolysaccharide component of gram negative bacteria, mycobacteria, fungi, and malaria parasites, TLR can also bind to endogenous
molecules such as “damage-associated molecular pattern molecules (DAMPs)” (Poltorak, He et al. 1998; Means, Wang et al. 1999; Netea, Van der Graaf et al. 2002; Mockenhaupt, Cramer et al. 2006). These DAMPs share structural similarity with microbial “pathogen-associated molecular patterns (PAMPs)” and lead to activation of “pattern recognition receptors (PRRs)” on immune and vascular cells (Binder, Papac-Milicevic et al. 2016; Miller and Shyy 2017). This leads to activation of inflammatory responses in myocardial cells. This symbolizes first line of innate host defense and further leads to modulation of the adaptive immune responses. Multiple signaling cascades are activated which boost the pro-inflammatory cytokine release, oxLDL uptake and formation of foam cells (Cole, Georgiou et al. 2010; Lundberg and Yan 2011; Molteni, Gemma et al. 2016). There are two pathways for the TLR4- myeloid differentiation primary-response protein 88 (MyD88) dependent and independent pathway (Figure 2-14). The MyD88-dependent pathway involves rapid activation of NF-κB (Kagan and Medzhitov 2006), while interferon regulatory factor-(IRF3) activation occurs in MyD88-independent pathway activates (Halaas, Husebye et al. 2007; Kagan, Su et al. 2008).

Figure 2-14: TLR4 signalling pathway. Adapted from (Omrane, Baroudi et al. 2018).
Structural analysis of the TLR4 revealed that it comprises of three domains- an extracellular leucine rich repeat domain, a transmembrane domain and an intracellular Toll/interleukin-1 receptor domain (Gay and Gangloff 2007; Roach, Racioppi et al. 2013). The oxidized cholesteryl esters (oxCEs) of mmLDL bind to TLR4 (Miller, Choi et al. 2012). CD14 receptor recognizes the LPS/cholesterol and presents to TLR4-MD2 complex. OxCE of mmLDL also induces recruitment of MD-2 to TLR4 and further aids in TLR4 dimerization (Choi, Yin et al. 2013). MD2 is an LPS-binding receptor, which provides a hydrophobic pocket for five of the six saturated fatty acyl chains of LPS (Miller and Shyy 2017). TLR4 has the ability to activate transcription factors like NF-κB and interferon regulatory factors (IRF3) (Davoodi, Hashemi et al. 2012) that controls the expression of pro-inflammatory cytokines, chemokines and interferons. It also stimulates IL-12 production (Romagnani 2004) which further differentiates naive T cells to Th1 cells and therefore disturbs the Th1/Th2 balance (O’neill 2008).

Literature reveals that markedly enhanced TLR4 expression in human atherosclerotic plaques (Edfeldt, Swedenborg et al. 2002; Vink, Schoneveld et al. 2002; Otsui, Inoue et al. 2007). The same has been replicated in the animal study thus revealing the vital role of TLR4 in CAD (Takeishi and Kubota 2009). TLR4 gene is located on chromosome 9 (9q32-q33) and consists of four exons and three introns (Arbour, Lorenz et al. 2000; Opal and Esmon 2002). In the fourth exon, two co-segregating SNPs have been reported: rs4986790 (Asp299Gly or A896G) and rs4986791 (Thr399Ile or C1196T) that modify the amino acid sequence in extracellular leucine rich repeat domain of the TLR4 protein impairing its recognition ability (Douville, Lissitsyn et al. 2010).

TLR4 has been reported to perform diverse functions in many pathological conditions including CVDs (Liu, Wang et al. 2015), allergic diseases (Fairweather and Frisancho-Kiss 2008), obesity-associated metabolic diseases (De Laat, Clement et al. 2014), neuronal degeneration (de Laat, Gruntmeir et al. 2014; Gawri, Rosenzweig et al. 2014), apoptosis (Gay, Symmons et al. 2014) and infectious diseases (Li, Huang et al. 2013). Many genetic association studies have been performed to elucidate role TLR4 polymorphisms with CAD but results are inconsistent (Balistreri, Candore et al. 2004; Kolek, Carlquist et al. 2004; Zee, Hegener et al. 2005; Koch, Hoppmann et al. 2006; Nebel, Flachsbart et al. 2007; Incalcaterra, Caruso et al. 2010; Džumhur, Zibar et al. 2012). Therefore, present study aims to explore the plausible association of TLR4 rs4986790 A/G and rs4986791 C/T and CAD in a North Indian population.
2.9 RATIONALE OF THE STUDY

India is a vast country with different ethnic groups inhabiting different geographical regions of the country (Thangaraj et al., 2006). Lifestyle diseases like CAD are strongly influenced by both genetic and environmental factors and the ethnic heterogeneity also plays a crucial role in determining the genetic susceptibility to CAD. Indians are racially more predisposed to CAD owing to the Asian Indian phenotype, an increased susceptibility to T2DM and greater abdominal obesity (Mohan et al., 2010). The urbanization, industrialization and rural to urban migration has led to the adoption of sedentary lifestyle, lack of routine exercise and alteration in the eating habits (Kaur et al., 2010; Kaur et al., 2013). It is also well known that higher average calorie consumption in the North Indian state of Punjab on account of fewer cereals, more fat and sugar intake.

The majority of genetic studies for the search of genetic variants in CAD are on the Western populations and there is a complete lacuna for the genetic studies in CAD patients from Indian populations, especially the North Indian population groups. Predicting high risk individuals will aid in early stage screening. Also this will help in pharmacogenomics for the personalized medicine approach. Thus, it becomes imperative to study the role of genetic factors along with other demographic and environmental factors for predisposition to CAD as well as their existence in the population of North India.

CAD basically involves atherosclerosis in the myocardial arteries and is accompanied with inflammation. A number of different immune molecules are involved in each and every step of atherosclerosis creating a highly inflammatory environment in the vessel walls. Any genetic polymorphisms occurring in the genes involved in the atherosclerosis and inflammation will alter the predisposition towards CAD.

Therefore, the present study aims to genotype the genes of the entities involved in atherosclerosis and the genes of inflammatory and anti-inflammatory molecules with a hypothesis that the polymorphisms in ALOX15, LOX1, ANRIL, PCSK9, IL-8, IL-10, INF-γ and TLR4 are among the genetic factors predisposing population of North India to CAD. These genes were chosen based on their functions in atherosclerosis and inflammation regulation that may be involved in CAD pathogenesis and their polymorphisms based on their potential regulation of gene expression and modification of risk towards the disease.