Over the past two centuries, there has been a dramatic shift in the causes of illness and death. Before the advent of the 20$^{th}$ century, malnutrition and infectious diseases were the leading causes of mortality. Improvements in nutrition and public health measures accompanied the drastic developments in the industrial and technological sector reducing the prevalence of these medical conditions. Technological advancement, rapid urbanization and globalization have gradually changed the lifestyle of people in ways that are often associated with the development of many chronic non-communicable diseases (NCDs) (Abdel, 1971; Mackenbach, 1994). NCDs are diseases with usually slow progression and are not caused by infectious agents. Changes in daily habits including physical inactivity, unhealthy food habits, long working hours, frequent transcontinental travels, lack of proper sleep and, tobacco and alcohol abuse have been evidently identified as risk factors linked with development of NCDs (Wagner & Brath, 2012). Basically, a combination of genetic, physiological, behavioral and environmental factors result in NCDs.

Based on the global health scenario, World Health Organization (WHO) has identified NCDs as a major health concern owing to the number of deaths it has caused globally, in the past years. As of 2018, NCDs account for 41 million deaths every year corresponding to 71% of all global deaths. According to WHO projections, this number is estimated to increase up to 55 million till 2030, if timely measures for prevention and control of NCDs are not taken. Low- and middle-income countries are the worst affected by NCDs with 32 million deaths that represents more than three quarters of global mortality. According to the National Health Portal, India records nearly 5.8 million deaths caused by NCDs every year. This translates to a rise of 24% in NCD mortalities in 2016 from 37% in 1990, indicating towards a rapid shift of disease burden on NCDs.

The four major types of NCDs are:

- Cardiovascular diseases
- Cancer
- Chronic respiratory diseases
- Diabetes

Amongst these, cardiovascular diseases (CVDs) stands at the leading position killing 17.9 million people worldwide annually, representing 31% of the global statistics (Fig. 1) (WHO, 2017). This stands true even in India where the situation is adverse. About 52% of deaths from these disorders in India have been observed to occur in the most productive midlife, leading to a huge socio-economic loss to the country (Prabhakaran et al., 2016). The CVD epidemic in India is of major concern owing to the early age of onset of the disease, accelerated rise in the affected population and the high fatality rate.

**Cardiovascular diseases**

CVDs are group of disorders of the heart and the vascular system supplying the heart, brain, and other vital organs. It encompasses a wide array of disorders including, but not limited to, coronary heart disease (blood vessels supplying the cardiac muscles are affected), cerebrovascular disease (blood vessels supplying the brain are affected), peripheral arterial disease (blood vessels supplying the limbs are affected), rheumatic heart disease (streptococcal bacterial induced rheumatic fever causes injury to the cardiac muscles and valves), congenital heart conditions (malformations of heart structure exists at the time of birth), deep vein thrombosis and pulmonary embolism (blood clots in the vessels supplying to the legs can dislodge and move to the heart and lungs). Amongst all the CVDs, Ischemic heart disease (IHD) and stroke account for highest fatality (>80%) worldwide (Prabhakaran et al., 2016) as well as in India (~86%) (Indian Council of Medical Research, 2017).

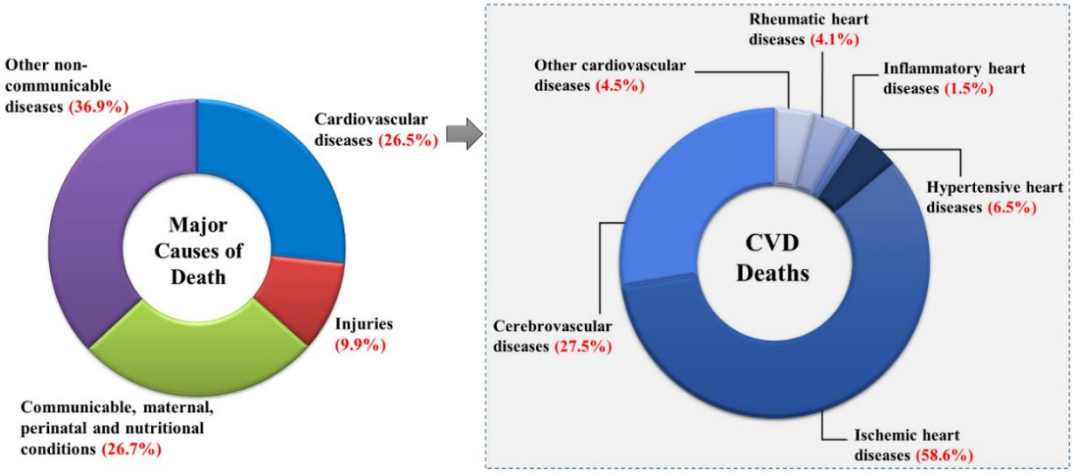


**Figure 1:** Causes of death in India (Indian Council of Medical Research, 2017)

Men are generally considered to be at a higher risk of CVDs compared to premenopausal women but, the postmenopausal statistics for women are similar to men. However, the mortality rate due to CVDs is higher in women despite the lower risk of its incidence (Botset et al., 2017). In fact, as of 2017, IHD remains to be the leading cause of mortality among both men and women (Heron, 2019). In younger women, estrogen imparts protection against incidence of CVDs. This often limits studies related to female-associated CVDs due to lack of relevant experimental models (Iorga et al., 2017). Testosterone is also believed to have a similar protective effect in men as deficiency of the same correlates with CVD complications (Kirby et al., 2019). However, the incidences of acute coronary syndrome in young women have been observed to be increasing, which has been attributed to the changing behavioral aspects among young adults (Spence & Pilote, 2015).

Although CVDs result from various underlying causes, atherosclerosis happens to be accepted as the most common cause. Basically, CVDs are acute disease outcomes resulting from the atherosclerotic transformation of the concerned arteries.

**Atherosclerosis- a chronic inflammatory disease**

Atherosclerosis is defined as a chronic disease of the arterial wall, where fatty deposition in the vessel wall leads to formation of plaque obstructing the blood flow. It develops in the sub-endothelial region of large and medium sized arteries and is more frequently observed at the branching points of the vessels. It is characterized by the excessive intimal recruitment of leukocytes and lipid accumulation, leading to a loss of normal vascular function and stenosis that interrupts the normal blood flow resulting in clinical consequences (Fig. 2) (Singh et al., 2002).

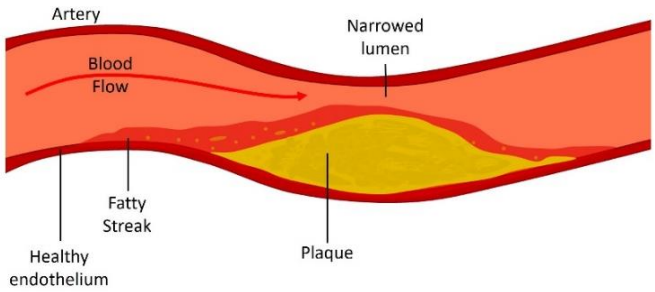


**Figure 2:** An artery showing progressive buildup of atherosclerotic plaque.

**Risk factors**

Although the exact causes of atherosclerotic disease development are not understood completely, certain conditions, traits, or habits that may increase the chance of developing the disease have been identified. Majority of risk factors including high blood cholesterol and low density lipoprotein (LDL) levels, lower high density lipoprotein (HDL) levels in blood, hypertension, smoking, diabetes mellitus, obesity associated with glucose tolerance, sedentary lifestyle, unhealthy eating habits, can be controlled and thus, the onset of atherosclerosis can be delayed or prevented. Other factors including age, gender and familial history of CVDs are classified as the non-modifiable risk factors. Further, high levels of C-reactive protein in blood, alcohol, sleep apnea, triglycerides and stress have

been classified as the emerging risk factors as the studies of their association with atherosclerosis is ongoing (Rafieian-Kopaei et al.,2014). Also, the likelihood of developing atherosclerosis increases in presence of multiple risk factors. The risk factors have also been classified as genetic and environmental based on the epidemiological evidences.

Table 1: Risk factors for atherosclerosis

| ***Factors with Genetic Component*** | ***Environmental factors*** |
|---|---|
| High LDL and cholesterol levels | Smoking |
| Low HDL levels | High fat/calorie diet |
| Elevated lipoprotein A | Sedentary lifestyle |
| Hypertension | Alcohol |
| Diabetes mellitus | Stress |
| Homocysteinemia | Infectious agents |
| Metabolic syndrome | |
| Insulin resistance | |
| Obesity | |
| Family history of CVDs | |

Amongst others, elevated levels of serum cholesterol and LDL stand out as being able to drive atherosclerotic development even in absence of any other known risk factor. The available insights into the cholesterol biosynthesis and LDL mediated cholesterol transport led to discovery of a class of cholesterol lowering drugs called 'statins' that have reportedly shown promising results of reduced CVD mortality in hypercholesterolemic patients (Gould et al., 1998) and is widely used in recent times (Davies et al., 2016). However, statins have also failed to completely prevent the progression of atherosclerosis in many susceptible individuals, suggesting towards the major contribution of other factors to disease development that needs to be scrutinized in order to develop novel preventive and therapeutic interventions (DuBroff & de Lorgeril, 2015).

**Origin of atherosclerosis**

With the increasing importance of modern lifestyle as a causal factor, it is interesting to note that the oldest evidence of atherosclerosis came from studies demonstrating its presence in specimens of ancient Egyptian mummies. Anatomists and physiologists performed autopsies of aorta from these specimens and observed the presence of 'calcareous plaques' and 'calcareous bone-like patches standing prominently from the walls of the vessels' (Bruetsch, 1959). However, before these studies, Fallopius gave the first formal description of atherosclerosis in 1575, where he described degeneration of arteries into bone (Acierno, 1994). In 1695, the aorta of physician Johann Jakob Wepfer was imaged and the description quoted "the internal coat in several places was ruptured, lacerated and rotten like fruit and hurt the fingers when thrust into it, from the roughness of the bone". During 1700s more mechanistic descriptions were mentioned but, it was not until 1815 that the chemical analysis by Joseph Hodgson revealed the arterial calcification to be different than that of a true bone. In 1829, the termed arteriosclerosis was coined by Jean-Fredrich Martin Lobstein and the most accepted term 'atherosclerosis' was proposed and justified in 1904 by Felix Marchand of Leipzig. Felix also suggested that atherosclerosis was responsible for almost all obstructive processes in the arteries (McMillan, 1995).

The first detailed microscopic studies of Virchow (1856) led him to believe that the plaque began 'in' rather than 'on' the intima as a proliferation of cells with formation of cell products that was followed by the fatty changes. It was also noted that the plaque had some elements of inflammation and that the lesions tended to locate where there was pressure from the blood stream. The presence of cholesterol was also documented but it was considered secondary. With the advances in technology and experimental methodologies,

the description of atherosclerosis became more precise. The major contribution came from the animal experiments conducted separately by Ignatowski, Metchnikov and Anitschkow during early 1900s (McMillan, 1995). A strong relationship between cholesterol-rich food and experimental atherosclerosis was drawn. Further, Adolf Windaus showed that atheromatous lesions contained higher amount of free and esterified cholesterol compared to normal arterial wall (Windaus, 1910). Anitschkow showed that cholesterol alone can caused the atheromatous changes in the vascular wall (Anitschkow, 1967). These discoveries introduced the starting of a new era in the studies of atherosclerosis. In the following years, many theories explaining the mechanism of atherosclerotic plaque formation came into existence but, the last theory given by Russell Ross termed as 'response-to-injury hypothesis' represents an amalgamation of the existing theories and remains to be the most accepted one.

**General Pathophysiology**

Atherosclerosis is a multifactorial chronic disease that begins early in life and slowly progresses towards plaque that projects as cardiovascular ailment. The pathophysiology of atherosclerosis can be roughly divided into the following stages:

1. Fatty streaks formation
2. Atheromatous plaque formation
3. Plaque rupture and thrombosis

1. Fatty streaks formation

Atherosclerotic lesion formation begins as subendothelial fatty streaks in arterial wall of larger arteries. Major events leading to fatty streaks formation includes oxidative modification of LDL and its uptake by macrophages to form foam cells.

*LDL modification*

Initial lesion generally result from focal intimal accumulation of lipoprotein molecules that are composed of proteins, phospholipids and other lipids such as triglycerides and cholesterol. LDL is a cholesterol rich lipoprotein (Fig. 3) that has been identified as a potent atherogen. The sub-intimal accumulation of LDL is accomplished due to its ability to infiltrate endothelium (Vasile et al., 1983) or due to its ability to bind extracellular matrix components such as proteoglycans (Hurt et al., 1990).

It is believed that LDL is well-protected from oxidation by robust anti-oxidant defenses when it is in circulation but, the same is rendered susceptible to enzymatic and non-enzymatic oxidation when it is trapped in the extracellular matrix of the arterial wall (Yoshida & Kisugi, 2010). The non-enzymatic oxidation is mediated via transition metal ions, hemin and other catalysts whereas, enzymatic oxidation involves lipoxygenase and myeloperoxidase (Daugherty et al., 1994; Heinecke et al., 1984; Parthasarathy & Steinberg, 1992; Ylä-Herttuala et al., 1989; Zhu et al., 2003). Further, nitric oxide (NO) secreted by endothelial cells inhibits oxidation of LDL (Hogg et al., 1993; Malo-Ranta et al., 1994), however, simultaneous production of NO and superoxide in endothelial cells results in formation of peroxynitrite that leads to lipid peroxidation (Beckman & Koppenol, 1996; Radi et al., 1991). Thus, both pro- and anti-oxidant activity of NO has been proposed and the balance between NO and superoxide decides the fate of lipid and LDL peroxidation.

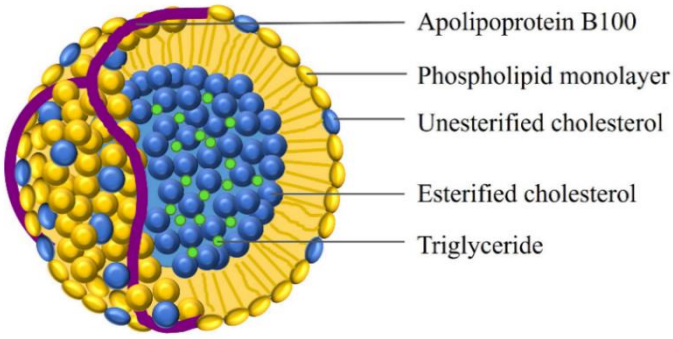


**Figure 3:** LDL particle mainly consists of the cholesterol ester, triglycerides, phospholipids, free cholesterol and ApoB-100.

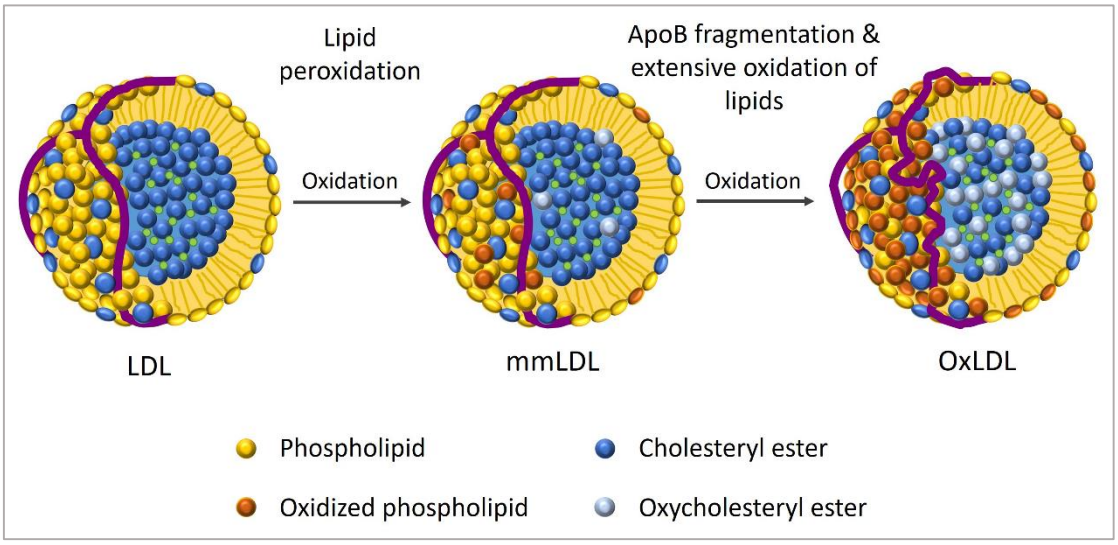


**Figure 4:** Stepwise oxidation of LDL to minimally oxidized LDL (mmLDL) and extensively oxidized LDL (OxLDL).

The oxidative modification of LDL involves modification of the lipid as well as apolipoprotein B (ApoB) components and the evidences in this regard have been obtained by studying *in vitro* oxidation of native LDL (Esterbauer et al., 1989; Laggner, 2012). The immunological properties of oxidized LDL (OxLDL) varies according to the degree of its oxidation ranging from 'minimal' modification (mmLDL) of lipids to extensively oxidized form (OxLDL) in which ApoB is highly fragmented with covalently modified lysine residues and reactive breakdown products of oxidized lipid components (Fig. 4). While

mmLDL can still be recognized by LDL receptors, OxLDL can be recognized by scavenger receptors (SRs) expressed on the surface of macrophages and SMC (Navab et al., 1996; Witztum, 1989).

*Activation of endothelial cells*

In a normal quiescent state, endothelium resists the binding of leukocytes but, under the influence of noxious stimuli, it expresses various cell adhesion molecules that facilitates the binding of leukocytes including monocytes. This pathophysiological condition associated with the increased expression of adhesion molecules on endothelial cell surface and secretion of chemokines contributing to recruitment of leukocytes in circulation is termed as endothelial activation. Cytokines and OxLDL have been recognized as crucial inducers of endothelial activation (Eiserich et al., 1998).

Several adhesion molecules have been suggested to play role in monocyte recruitment but, vascular cell adhesion molecule 1 (VCAM-1) was the first to be implicated (Cybulsky & Gimbrone, 1991). This was followed gene deletion studies in $ApoE^{-/-}$ mice that acknowledged the role of E-selectin, P-selectin and intercellular adhesion molecule 1 (ICAM-1) as crucial mediators of monocyte recruitment (Collins et al., 2000; Dong et al., 1998). These adhesion molecules ensure leukocyte entrapment by interacting with their counterparts on the surface of the leukocytes. In particular, the binding of CC-chemokine ligand 5 (CCL5) and CXC-chemokine ligand 1 (CXCL1) expressed on leukocytes with P-selectin expressed on the luminal side of endothelial cells, contributes to their immobilization on the endothelium. Further, VCAM-1 and ICAM-1 bind to integrins very late antigen 4 (VLA4; also known as α4β1 integrin) and lymphocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin), respectively, contributing majorly to the 'firm adhesion' of monocytes to the luminal surface of endothelium (Ley et al., 2007). In

addition, endothelial secretion of monocyte chemoattractant protein 1 (MCP-1; also known as chemokine ligand 2 (CCL2)), along with CCL5 and $CX_3C$-chemokine ligand 1 ($CX_3CL1$) is also responsible for monocytes transmigration through endothelium into the sub-intimal space (Tacke et al., 2007).

Thus, the overall recruitment of monocytes at lesion-prone sites is mediated via integration of three distinct processes namely, capture, rolling and transmigration (Moore et al., 2013).

*Foam cell formation*

The development of macrophage foam cells is a hallmark of both early and late stage atherosclerosis. Although macrophage clearance of modified lipids serves to be a beneficial immune response in the initial phase, the continued accumulation of these lipids with little negative feedback after uptake renders the macrophages engorged with excessive lipids. The resulting imbalance of lipid metabolism changes macrophage phenotype by compromising the crucial immune functions (Chistiakov et al., 2016). Lipid-laden macrophages exhibit diminished capability to migrate that explains their accumulation in the lesion contributing to their failure to resolve inflammation and thereby to the development of complex, advanced plaques wherein other immune cells and SMC participate (Huang et al., 2014).

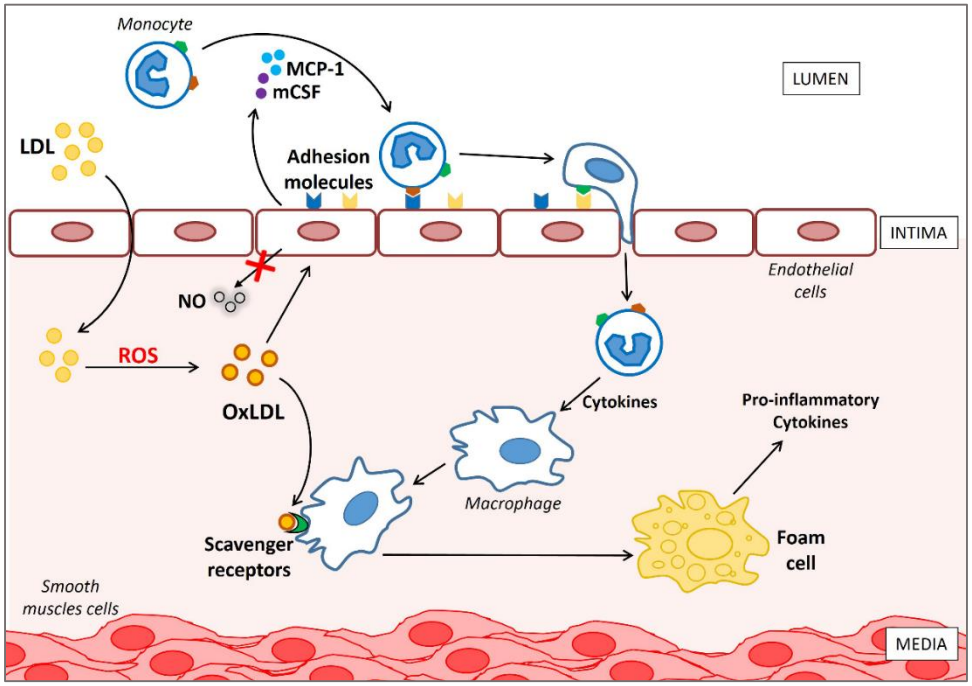


**Figure 5:** Formation of fatty streak in arterial intima involves LDL accumulation and oxidation, endothelial activation and foam cell formation.

Cholesterol accumulation in macrophages is primarily mediated via scavenger receptors (SRs) that recognize the oxidized phospholipids and covalent adducts on ApoB (Zhang et al., 1993). Several members of the SR family including scavenger receptor A1 (SR-A1), macrophage receptor with collagenous structure (MARCO; also known as SR-A2), CD36 (also known as platelet glycoprotein 4), scavenger receptor B1 (SR-B1), lectin-like oxidized LDL receptor 1 (LOX1), scavenger receptor expressed by endothelial cells 1 (SREC1) and scavenger receptor for phosphatidylserine and oxidized LDL (SR-PSOX; also known as CXCL16), can bind to oxidized LDL and can promote foam cell formation (Kzhyshkowska et al., 2012). Of these, SR-A1 and CD36 contributes 75-90% of OxLDL uptake in macrophages *in vitro* (Kunjathoor et al., 2002). Further, studies have shown that native LDL in arterial intima, undergoes pinocytosis in macrophages when its concentrations are similar to those of hyperlipidemic conditions (Kruth, 2011).

Apart from uptake, the degree of lipid accumulation is also regulated by the extent of lipid efflux. In foam cells, cholesterol efflux is mediated via transporters such as ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1) and SR-B1. Besides passive diffusion from plasma membrane has also been reported. ABCA1 promotes the transfer of cholesterol to lipid-poor APOA1, which is the building block of HDL, whereas ABCG1 promotes efflux to mature HDL particles (Yvan-Charvet et al., 2010). SR-B1 selectively transfers cholesterol to HDL for its transport to liver for elimination in bile and feces (Shen et al., 2018). The physiological importance of SR-B1 is suggested by studies that reported reduced atherosclerosis in mice overexpressing SR-B1 (Kozarsky et al., 1997). However, in atherogenic conditions, the inhibition of ABCA1 and ABCG1 by pro-inflammatory environment fails to regulate intracellular lipid homeostasis leading to higher accumulation (Khovidhunkit et al., 2003).

Cholesterol crystals present in the macrophages and the surrounding extracellular matrix activates NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome, which leads to processing and secretion of IL-1β. In addition to preformed cholesterol crystals, it has been suggested that cholesterol accumulation in macrophages can lead to de novo formation of cholesterol crystals that can induce NLRP3 pathway (Duewell et al., 2010). In this regard, CD36 has been found to play crucial role in nucleation of cholesterol crystals, its lysosomal disruption and NLRP3 activation in macrophages exposed to OxLDL (Sheedy et al., 2013). Further, OxLDL binding to CD36 has been reported to activate toll-like receptor 4 (TLR4) mediated inflammatory pathway (Stewart et al., 2010). mmLDL has also been identified as a ligand for TLR2 and TLR4 and can directly activate the downstream inflammatory pathway representing pro-atherogenic modulations (Chávez-Sánchez et al., 2010).

2. Atheroma plaque formation

The transition of a relatively simple fatty streak to more advanced lesion involves the migration of SMC from medial layer of the arterial wall to the sub-intimal space by crossing the internal elastic lamina. The SMC migration is triggered by the cytokines and growth factors secreted by endothelial cells and macrophages in the arterial wall. This severely damages the arterial wall as the intimal SMC may proliferate and accumulate OxLDL to form foam cells. Further, SMC foam cells also synthesize extracellular matrix proteins like collagen that leads to development of fibrous cap. At this stage, a complex interplay of different cellular components including, SMC, endothelial cells, macrophages and T-lymphocytes has been observed (Ross, 1999).

The interactions of macrophages and T-cells resulting into a range of humoral and cellular immune responses culminating in a chronic inflammatory state. Dendritic cells (DCs) are also present in the plaque and the interaction between T-cells and DCs is speculated to induce immune responses against the endogenous and exogenous antigens with activated T-cells expressing both Th1 and Th2 cytokines (Hansson, 1997). In addition, macrophages, endothelial cells and SMCs also secrete pro-inflammatory cytokines such as TNF-α, IL-6 and MCP-1 adding to the inflammatory cascade. The observed immune responses have been reported to be of dual nature viz. pro-atherogenic and anti-atherogenic, the net effect being pro-atherogenic, giving rise to a more stable plaque (Glass & Witztum, 2001). The continuous growth of plaque hinders the diffusion of oxygen to the plaque core, creating a hypoxic zone in the intima (Bjornheden & Bondjers, 1987). The immune responses together with hypoxic condition contribute to the increasing cell death in the deeper region of the plaque leading to formation of necrotic core that is rich in non-degradable cholesterol crystals (Bennett, 1999).

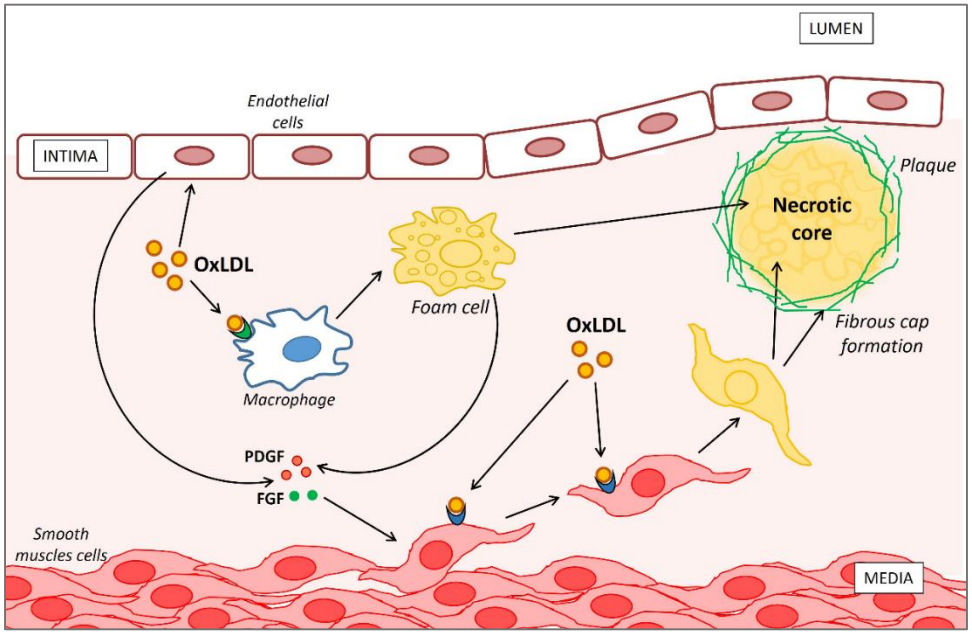


**Figure 6:** Development of atheromatous plaque involves major contribution of VSMCs.

3. Plaque rupture and thrombosis

The narrowing of the blood vessel caused by advanced plaques may result into ischemic symptoms like hypertension but, the acute cardiovascular events like stroke and myocardial infarction are generally thought to be the outcome of plaque rupture and thrombosis. Plaque rupture results into exposure of plaque core tissue to components of blood into lumen triggering the recruitment of platelets initiating a clotting cascade and thrombosis (Davies et al., 1993; Lee & Libby, 1997). The thick fibrous cap provides a structural stability to the plaque and preventing contact of lipids of the necrotic core with the blood. A thin fibrous cap may however, rupture on experiencing tensile stress. Internal constituents of the plaques may also initiate events resulting into plaque rupture (Hatsukami et al., 2000). The stability of plaque depends upon the reparative ability of SMCs that continually helps the plaque to evolve. As time passes SMCs lose their reparative capacity making the plaque vulnerable (Labropoulos et al., 1998; Shanahan & Weissberg, 1998). Inflammatory cells synthesize and activate matrix metalloproteinases (MMPs) that are basically proteolytic

enzymes that erode the fibrous cap. Further, IFN-γ secreted by T-cells have been found to inhibit SMC mediated matrix collagen synthesis that predisposes the plaque to rupture (Ovchinnikova et al., 2009). Platelet aggregation and intraluminal thrombosis drives a fresh wave of SMC proliferation and repair. There occurs a complex interplay of pro-thrombotic and anti-thrombotic reactions and the net effect decides the occurrence of acute cardiovascular event (Raja B Singh et al., 2002).

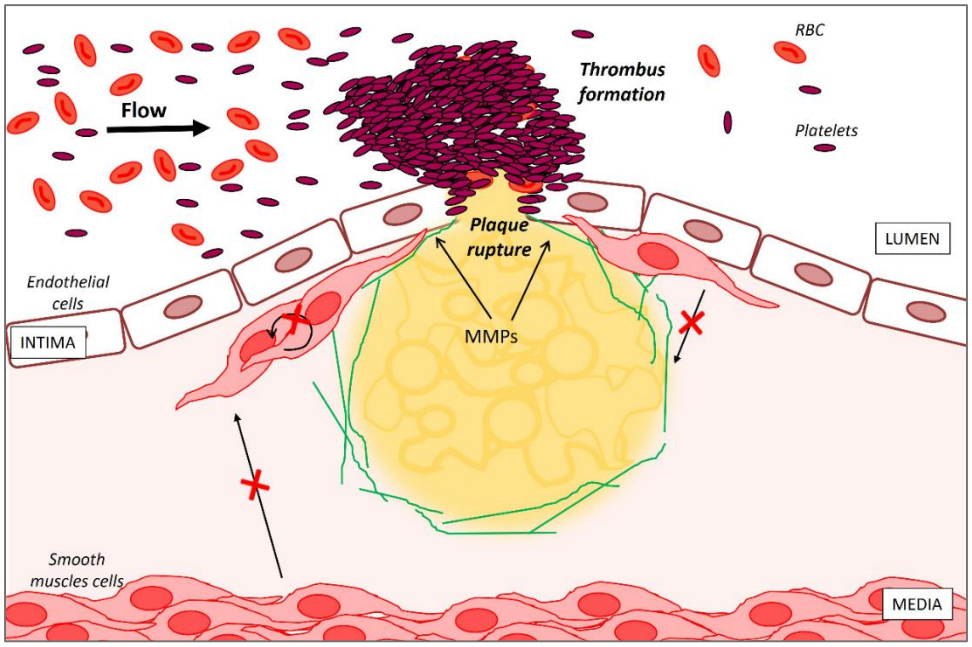


**Figure 7:** Destabilization of plaque leading to rupture and thrombosis.

**Competing hypotheses of atherogenic initiation**

The pathophysiology of atherosclerosis clearly states that the disease development involves multiple processes including- endothelial injury or activation, lipoprotein accumulation and oxidation, macrophage chemotaxis and foam cell formation, alterations in SMCs and local platelet aggregation. Yet, an intriguing question that remains unanswered is that which of these events is of key pathological relevance in early stage of atherosclerosis that is absolutely required as well as self-sufficient in triggering the pathological cascade in an

otherwise normal artery? A few hypotheses explaining the atherogenic initiation have been put forth by various researchers.

*Response-to-injury hypothesis*

The response-to-injury hypothesis is the widely accepted mechanistic basis of pathogenesis of atherosclerosis and the basic idea behind the hypothesis traces back to theories developed by Rudolph Virchow in 1856 (Mayerl et al., 2006). It took more than a century for the acquisition of molecular and cellular details based on which Russell Ross formulated the response-to-injury hypothesis with his colleague John Glomset in 1973 that was published as a review article in 1977. The hypothesis states that '*atherosclerosis begins with injury to the endothelial lining of arteries*' (Ross et al., 1977). Initially, the endothelial injury was thought to be denudation at the site of lesion. However, study of whole mount aortic intima from very early lesions of cholesterol fed rabbits showed that the endothelium had a normal appearance thus, disregarding the idea of endothelial denudation as injury (Duff et al., 1957).

With the development of electron microscopy, it was found that endothelial cells in cholesterol fed rabbit were altered morphologically in a way that facilitated the transfer of lipids and migration of SMCs into the growing lesions (Parker, 1960; Parker & Odland, 1966a, 1966b). The development of immunocytochemical analysis recorded the presence of macrophages and T-lymphocytes in the early lesions and that fat accumulation preceded that of monocytes (Gown et al., 1986; Guyton & Klemp, 1992). In 1993, Russell Ross came up with another review article discussing the response-to-injury hypothesis, which largely represents the present day view of pathogenesis of atherosclerosis. As quoted by Dr. Ross, the hypothesis explains that 'the lesions of atherosclerosis represent a specialized form of a protective, inflammatory-proliferative response to various forms of insult to the

artery wall. Depending upon the nature and duration of the insult, the protective response may become excessive and over many years in its excess become a disease state' (Ross, 1993).

Overall, atherosclerosis begins as an endothelial injury, which was initially presumed to be endothelial denudation but, with subsequent experimental evidences, was found to be a damage that can be very subtle, resulting in only dysfunction of the endothelium. Such altered endothelium favors the recruitment of mononuclear cells in the arterial wall, where the uptake of lipoproteins triggers an immune response that lays foundation for progression into chronic inflammatory stage.

*Response-to-retention hypothesis*

Many of the researchers working in the field of atherosclerosis strongly support subendothelial retention of atherogenic lipoproteins as the central pathogenic process in atherogenesis. The response-to-injury hypothesis identified subtle endothelial injury as the initiating event in atherogenic lesion formation and one of the major functional alterations hypothesized in the endothelium is of enhanced permeability, particularly to lipoproteins (Nielsen et al., 1992). This idea formed the basis of the 'lipid infiltration hypothesis', which traces its origin to Anichkov and Khalatow (Anitschkow, 1913). A healthy endothelium is known to normally transport or leak many molecules including lipoprotein (Lin et al., 1989; Vasile et al., 1983). Thus, alterations in endothelial permeability or microscopic endothelial losses exceeding the normal turnover of endothelial cells did not seem to be requisite for atherosclerosis initiation.

*In vivo* studies evidently showed that atherogenic LDL accumulation occurs at certain focal sites known to be susceptible to atherosclerosis (Schwenke & Carew, 1989a). Further, it

was also observed that the rate of LDL entry into susceptible and resistant sites do not differ (Schwenke & Carew, 1989b) suggesting that retention, but not enhanced permeability to lipoprotein, is essential for lesion initiation. However, it has been proposed that endothelial permeability, though not essential, might be a contributing factor in certain cases such as smoking, dyslipidemia and possibly hypertension but, only if lipoprotein is retained in certain amount (Lin et al., 1992; Nordestgaard & Nielsen, 1994; Schwenke & St Clair, 1993). Also, lipoproteins have been observed to induce expression of adhesion molecules in endothelium suggesting that these endothelial changes are a consequence of the lipoprotein retention rather than being a cause of atherogenic initiation (Chisolm, 1993). Therefore, as per the response-to-retention hypothesis, atherogenic initiation is solely caused by lipoprotein retention in the arterial wall.

*Atherogenic initiation: A Mystery*

Although the response-to-injury hypothesis represents the currently accepted theory of atherogenic initiation, the response-to-retention hypothesis is not entirely untrue. In the present situation, neither of these hypotheses can individually stand out as the best explanation. No definitive *in vivo* evidence exists that suggests that endothelial injury is either necessary or sufficient for lesion formation. Further, the LDL retention fails to completely explain the atherogenic initiation in certain susceptible regions only. In fact, shear stress induced endothelial injury appears to explain the increased susceptibility in certain regions because most of the atheromatous plaques develop in curvatures and branching points of the arteries where shear stress is maximal (DePaola et al., 1992; Zand et al., 1991). Overall, both the hypotheses have overlapping components suggesting a rather complex process underlying the atherogenic initiation.

**Autoimmune concept of atherosclerosis**

The autoimmune hypothesis of atherosclerosis was formulated by Georg Wick and his colleagues in 1992, according to which the earliest stage of atherosclerosis results due to autoimmune reaction against heat shock protein 60 (HSP60) (Wick et al., 1992). At that time, it was just a hypothesis based on the experimental and clinical findings but, with time many evidences in support of the hypothesis have accumulated and the hypothesis is rightly termed as 'Autoimmune *concept* of atherosclerosis'.

Inflammatory processes have long been identified as key components of atherogenesis, however, most of the related studies were based on late advanced stages of the disease, creating an ambiguity of inflammation being a primary or secondary atherogenic event. Studies with the early lesions showed the intimal infiltration T-cells to be the earliest event that surprisingly precedes monocyte infiltration, suggesting that inflammatory processes serve as the primary event in initiation of atherosclerosis lesions (Kleindienst et al., 1993; Xu et al., 1990). At this stage, intima is already populated with vascular associated DCs (VADCs) that has a surveillance role and functions to identify and present exogenous and endogenous antigens to T-cells, thereby activating T-cells (Millonig et al., 2001; Packard et al., 2008).

OxLDL also qualifies to be a potent auto-antigen as OxLDL-reactive T-cells have been found to be localized in plaques, lymph nodes and plasma of atherosclerosis patients and experimental animals (Ghio et al., 2013; Grundtman & Wick, 2011; Stemme et al., 1995). Also, OxLDL formation in intima have been observed to precede monocyte recruitment and formation of fatty streaks (Napoli et al., 1997). However, if OxLDL was to be considered as a primary risk factor, it does not explain the cholesterol controversy wherein

people having normal levels of cholesterol have shown formation of atherosclerosis lesions.

*The Cholesterol Controversy*

The levels of cholesterol have been recorded to be within the normal range in more than 60% of atherosclerosis patients. This level corresponds to 200mg/dL or less for total cholesterol and 100mg/dL or less for LDL cholesterol. Higher circulating levels of LDL cholesterol have often been correlated with events of atherosclerosis and cases of heterozygous or homozygous familial hypercholesterolemia implicate towards primary atherogenic role of high LDL cholesterol levels. However, this scenario fails to provide an explanation for the so-called 'poor man's atherosclerosis' (Grundtman & Wick, 2011).

Adaptation to westernized life-style has always been linked to incidences of cardiovascular ailments with association to relatively higher cholesterol/LDL levels. However, atherosclerosis is known to be an old disease since the earliest evidence of came from analysis of nearly 4000 years old Egyptian mummies. But, it cannot be denied that the mummification of dead bodies and preservation of organs was a privilege of the affluent parts of the society and thus, even the oldest evidence does not represent the situation of majority population that must be malnourished, hardworking and probably having low cholesterol/LDL levels (Charlier & Huynh, 2010; David et al., 2010). Furthermore, statins, although being a class of lipid lowering drugs, have showed promising results in cases of CVDs in patients without hypercholesterolemia but with high levels of C-reactive protein, a marker of systemic inflammation (Ridker et al., 2008). Statins and aspirin have also been reported to have anti-inflammatory activity emphasizing towards the primary role of inflammation in atherogenesis (Baigent et al., 2009).

Thus, it has been hypothesized that the classical atherogenic risk factors act as endothelial stressors leading to the first inflammatory immunological stage of the disease. In normocholesterolomic individuals, the endothelial stress creates a condition that facilitates LDL accumulation in the intima (Grundtman & Wick, 2011).

*HSPs in atherosclerosis*

HSPs are a group of proteins whose expression increases in cells under conditions of elevated temperature and other stress. In normal resting state, HSPs function as molecular chaperones assisting in folding of native polypeptide chains to their functional three-dimensional structure using ATP. Certain HSPs also assists in transporting newly synthesized proteins to various cellular locations. HSPs also prevent the stress induced aggregation of proteins in the cells. Further, they unfold misfolded proteins in order to refold them and in cases where refolding fails, they guide the misfolded proteins to degradation (Craig et al., 1993). Besides, HSPs have also been implicated as primary pathogenic agents and in secondary events of various diseases including autoimmune diseases and CVDs in particular (Van Eden et al., 2007).

Soluble forms of HSP10 have been detected in serum of individuals suffering from fulminant type I diabetes and autoimmune pancreatitis (Takizawa et al., 2009). Also, HSP10 upregulation has been recorded in B-cell lymphoma (Shan et al., 2003). However, no specific correlation of HSP10 with CVDs have been found till date. HSP27, another small HSP known to have anti-apoptotic functions, has been found to be downregulated in plaque core and upregulated in the less-affected area surrounding the core (Mehlen et al., 1996). Extracellular expression of HSP27 has also been found to inhibit foam cell formation suggesting a protective role in atherosclerosis (Rayner et al., 2008). HSP70 is

known to have pro-inflammatory properties that has been implicated in pathophysiology of heart failure wherein higher levels of soluble HSP70 have been detected (Li et al., 2013). However, its role in atherosclerosis lacks definitive evidence. Elevated expression of HSP90 have been observed in late stage atheromatous plaques, especially in shoulder regions of vulnerable plaque (Madrigal-Matute et al., 2010). The anti-HSP90 antibodies have been detected in patients with atherosclerosis that suggests its pathogenic role in atherosclerosis but, the idea lacks further evidences (Businaro et al., 2009). Till date, HSP60 stands as the only HSP for which the primary pathogenic role in atherogenesis has been proven through experimental and clinical studies that formed a basis for the 'Autoimmune Concept of Atherosclerosis'.

**HSP60 as auto-antigen**

HSP60 is a mitochondrial chaperone that is encoded in the nucleus. It acts in concert with co-chaperone HSP10 to assist folding of mitochondrial matrix proteins including those constituting the electron transport chain (Dubaquié et al., 1997; Nisemblat et al., 2015). The expression of HSP60 is generally upregulated in cells as a protective response in conditions of stress (Pellegrino et al., 2013). Of note, HSP60 is highly conserved evolutionarily with >55% sequence homology between the bacterial and mammalian counterparts (Grundtman et al., 2011). This homology even exceeds 70% in certain molecular domains (Craig et al., 1993). All humans are exposed to bacterial HSP60 either by infection or through vaccination leading to development of cellular and humoral immune response. Both antibodies and T-cells reactive against bacterial HSP65 are known to cross-react with human HSP60 owing to the structural homology. Hence, when autologous HSP60 is ectopically expressed in stressed endothelial cells, it tends to trigger autoimmune response that initiates atherogenesis (Wick et al., 2014).

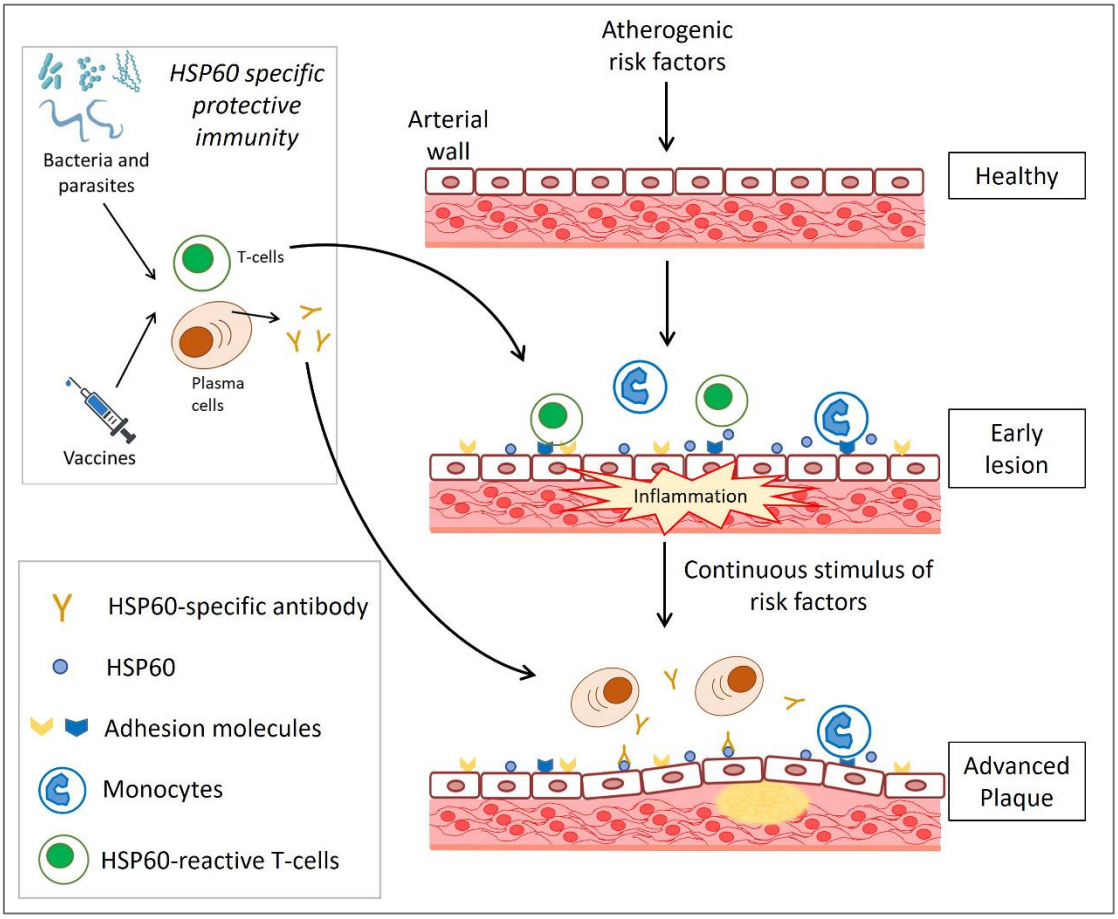


**Figure 8:** HSP60 induced autoimmune reactions in atherosclerosis.

*Surface expression and secretion of HSP60*

HSP60 is known to be a mitochondrial protein but, its surface expression has been reported in stressed endothelial cells. Majority of the classical atherogenic risk factors are now known to induce simultaneous surface expression of HSP60 and adhesion molecules in endothelial cells (Table 2), thus fulfilling the pre-requisite for recruitment of mononuclear cells including T-lymphocytes and monocytes. In fact, various atherogenic risk factors that qualify as endothelial stressor have been ranked based on their HSP60 inducing capability wherein, OxLDL stands at the lowest third position emphasizing that it is not the primary determinant of atherogenic initiation (Grundtman & Wick, 2011). Furthermore, stressed endothelial cells have been observed to secrete soluble HSP60 that can act as danger

associated molecular pattern (DAMP) to activate inflammatory immune reactions. In addition, soluble HSP60 has been observed to be biochemically altered leading to autoimmune reactions (Kreutmayer et al., 2011). In this context, soluble HSP60 is has been found to activate human monocytes and macrophages through CD14/TLR-4 and p38 mitogen activated protein kinase (MAPK) pathways that may be relevant in atherogenic progression (Kol et al., 2000). Soluble HSP60 mediated B-cell activation has also been reported that is crucial in late stage of atherosclerosis (Cohen-Sfady et al., 2005). Further, soluble HSP60 induces migration of vascular SMCs (VSMCs) that contributes to the development of advanced, stable plaque (Zhao et al., 2015).

Epidemiological studies have shown a strong correlation of soluble HSP60 levels with severity of atherosclerosis as well as with presence of classic atherogenic risk factors like hypertension, hypercholesterolemia and pro-inflammatory cytokines like TNF-α (Xu et al., 2000). A strong positive correlation has also been reported between high levels of soluble HSP60 and coronary heart disease (Zhang et al., 2008). However, endothelial cells are not the only contributors to the presence of soluble HSP60 in serum. In a recent study, Martinus and Goldsbury (2018) had reported the secretion of HSP60 from monocytes exposed to hyperglycemic condition wherein, the secreted HSP60 imposed a paracrine inflammatory action on endothelial cells. HSP60 secretion has also been documented from injured cardiomyocytes contributing towards its presence in circulation (Gupta & Knowlton, 2007). HSP60 in circulation can act as an endothelial stressor in susceptible regions leading to commencement of atherogenic events.

Table 2: Role of classical atherogenic risk factors as endothelial stressors

| ***Risk Factor*** | ***Endothelial stress*** | ***References*** |
|---|---|---|
| *Chlamydia pneumoniae* infection | Surface expression of HSP60 in HUVEC | Kreutmayer et al., 2013 |
| | Co-localization of hHsp60 (eukaryotic), Chlamydial Hsp60 and TLR4 on the surface of ECs isolated from porcine coronary artery | Deniset et al., 2012 |
| Smoking | Cigarette smoke extract induced surface expression and secretion of HSP60 in HUVECs. | Kreutmayer et al. 2011 |
| Hypertension | Increasing concentrations of sodium chloride correlated to increased intracellular and surface expression of HSP60 in HUVEC that further correlated with increased apoptosis | Jakic et al. 2017 |
| Bacterial LPS | Surface expression of HSP60 on endothelial cells of rabbit aorta. | Wick et al., 2008; Seitz et al., 1996 |
| | Upregulated HSP60 in human arterial endothelial cells. | Amberger et al 1997 |
| Shear stress | Upregulation of HSP60 in HUVEC and in ligated right carotid artery in Lewis rats. | Hochleitner et al., 2000 |
| Pro-inflammatory mediators | Platelet-derived growth factor BB and IL-8 added to HUVECs induced Hsp60 secretion. | Zhao et al., 2015 |
| | Treatment of HUVECs with TNF-α entailed increased expression of Hsp60 and ICAM-1 | Wu et al., 2012 |
| | IL-1β, TNF-α, IFN-γ upregulated HSP60 in human arterial endothelial cells. | Amberger et al 1997 |
| Oxidized LDL | Upregulation of ICAM-1 and VCAM-1 in human arterial endothelial cells. | Amberger et al 1997 |
| | Surface expression of HSP60 in HUVEC. | Grundtman et al 2011 |

*HSP60-specific immune reactions in atherosclerosis*

The identification of HSP60 as an atherogenic autoantigen involved a series of experiments conducted in normocholesterolemic rabbits. In the earliest experiment, the rabbits showed differential development of atherosclerotic lesions on immunization with complete and incomplete Freund's adjuvant, where the only missing component was inactivated mycobacteria (Xu et al., 1992). Based on the experimental observations of mycobacterial HSP65 induced rheumatoid arthritis (Anderton et al., 1994), the same was considered as a potential candidate for the observed mycobacteria mediated development of atherosclerosis. Subsequently, rabbits were immunized with mycobacterial HSP65 and development of characteristic atherosclerotic lesions was recorded, even though the serum cholesterol levels were found to be normal (Xu et al., 1992). Further, the presence of HSP60-reactive T-cells was recorded in the early lesions of these rabbits (Xu et al., 1993). Consequently, it was concluded that increased levels of HSP60 and presence of HSP60-specific T-cells in the intima triggers the development of atherosclerosis. Further, time course study of the atherogenic development in rabbits either immunized with mycobacterial HSP65 or fed with cholesterol-rich diet revealed that the early inflammatory stage of atherosclerosis is reversible, if the initial stimulus is removed, which in this case was mycobacterial HSP65 or cholesterol rich-diet (Xu et al., 1996).

At the same time, a randomized prospective community-based study of atherosclerosis, the Bruneck study was initiated wherein, 1000 clinically healthy volunteers of both the sexes, aged 40-89 years had participated. An important observation of this study was significantly high titres of antibodies to mycobacterial HSP65 in individuals with atherosclerotic lesions detected by ultrasound imaging. Also, these autoantibodies strongly cross-reacted with HSP60 from other bacterial species as well as with human HSP60 (Willeit & Kiechl, 1993).

Thus, the levels of these autoantibodies served as an atherosclerotic marker that also proved to have prognostic value. Later two similar clinical studies were conducted using the approach of Bruneck study, wherein young males aged 17-18 years (Atherosclerosis Risk factors in Young Males-ARMY) and young females (Atherosclerosis Risk factors in Young Males-ARMY) aged 19-21 years had participated (Knoflach et al., 2003; Knoflach et al., 2009). A significant correlation was observed between intima-media thickness and T-cell reactivity against HSP60 in otherwise clinically healthy individuals, which was not observed in elderly participants of Bruneck study. Similar trend towards correlation was observed between anti-HSP60 autoantibodies and early atherogenic changes in ARMY participants. Also, active and passive smoking was observed to be the most important atherogenic risk factor in ARMY and ARFY cohorts, respectively.

Investigations of cellular and humoral immune responses to HSP60 were carried out in $LDLR^{-/-}$ mice immunized with mycobacterial HSP65. The transfer of autoantibodies and lymphocytes from these mice to syngeneic mice led to formation of fatty streaks (George et al., 2001). Also, passive transfer of monoclonal antibodies against HSP60 or polyclonal antibodies to HSP60 purified from humans with atherosclerosis by affinity chromatography resulted in atherosclerosis in $LDLR^{-/-}$ mice (Mandal et al., 2005). These observations implied towards the role of anti-HSP60 antibodies as endothelial stressors that entail subintimal T-cell infiltration. However, it should be noted that stressed endothelial cells expressing HSP60 on their surface are lysed by anti-HSP60 monoclonal or affinity purified polyclonal human anti-HSP60 antibodies via complement-mediated or antibody-dependent cellular cytotoxicity mechanisms (Mayr et al., 1999; Schett et al., 1995). Also, the functional effects of anti-HSP60 autoantibodies have been proposed to differ based on their HSP60 epitope specificity (Metzler et al., 1997). Thus, both cellular

and humoral immune responses against HSP60 participate in atherosclerosis but, T-cells seems to play a bigger role with respect to the primary atherogenic initiation.

*Novel intracellular functions of HSP60*

Apart from surface expression and secretion, the cytosolic expression of HSP60 preceded by its upregulation in stressed vascular cells is also an event of atherogenic relevance. Cytosolic presence of HSP60 has been reportedly associated with various intracellular signaling pathways in various cell types (Table 3). With respect to atherosclerosis, intracellular overexpression of HSP60 has been associated with increased proliferation of primary rabbit VSMCs (Deniset et al., 2018). Further, HSP60 upregulation has been observed in SMCs present in atherosclerotic lesions specimens of human carotid and aorta, emphasizing the atherogenic relevance of HSP60 overexpression induced VSMC proliferation. Thus, it is evident that endogenous HSP60 has pleiotropic effects in various cell types but, not much is known about its intracellular novel functions in context of atherosclerosis.

Table 3: Non-canonical functions of endogenous HSP60

| ***Function*** | ***References*** |
|---|---|
| Endogenous HSP60 stimulates maturation of Caspase 3 | Xanthoudakis et al., 1999 |
| Endogenous HSP60 associates with α3β1-integrin allowing for integrin signaling | Barazi et al., 2002 |
| Cytosolic accumulation of HSP60 may be pro-apoptotic or anti-apoptotic | Chandra et al., 2007 |
| Endogenous HSP60 inhibits digoxin induced vascular endothelial cell apoptosis by inhibiting Caspase 3 | Qiu et al., 2008 |
| Cytosolic HSP60 interacts with surviving and p53 to inhibit tumor cell apoptosis | Ghosh et al., 2008 |
| Cytosolic HSP60 stimulates NF-κB activation | Chun et al., 2010 |
| Intracellular HSP60 interacts with β-catenin to enhance metastasis | Tsai et al., 2009 |
| HSP60 binds to clusterin to form a complex that suppresses tumor | Chaiwatanasirikul & Sala, 2011 |
| Endogenous HSP60 is involved in intracellular cholesterol transport | Olvera-Sanchez et al., 2011 |
| Intracellular HSP60 upregulation stimulates cell proliferation in VSMCs | Deniset et al., 2018 |

Overall, it can be said that HSP60 activates immune system is a multiple ways and its surface expression and secretion from stressed or damaged endothelial cells remains to be the most important atherogenic event that holds a central position in atherogenic initiation.