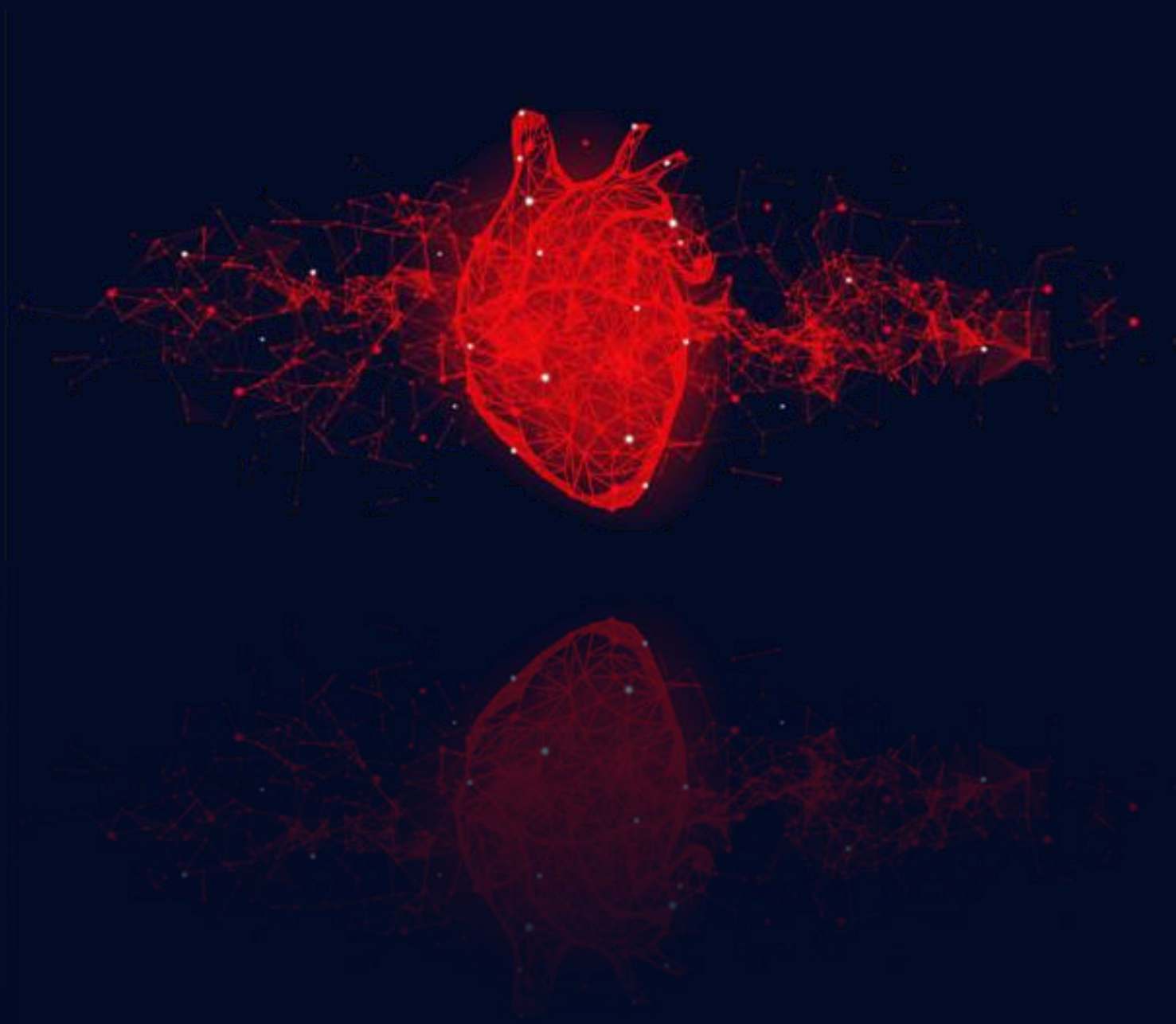
General Introduction



Lifestyle Disorders

A subset of non-communicable diseases, presently turning into a big economic burden worldwide, is interestingly a consequence of lately modified socio-economic stature globally. Lifestyle disorders (LSDs) have become major contributor to morbidity and mortality and at the same time the single biggest obstacle to the development across the world (Ponthière, 2011). LSDs are essentially the reflection of an individual's altered living pattern. Lack of sleep, adequate physical activity, nutrition, chronic stress, smoking, alcoholic abuse and disturbed circadian rhythms forms the core reasons of LSDs (Tabish, 2017). The occurrence of these problems is not a one-day process; long term ignorance to health care and disturbed way of living gradually starts reflecting into one's body in form of one or the other LSD. Broadly the risk factors have been classified into 3 categories.

(1) *Modifiable behavioural risk factors*: smoking, alcohol consumption, lack of physical activity, untimed and unhealthy diet consumption, and disturbed circadian rhythms.
(2) *Non-modifiable risk factors*: Gender, age, and genetic makeup.
(3) *Metabolic risk factors*: hypertension, hyperglycaemia, obesity etc.

Some of the highly prevalent LSDs are obesity, diabetes, cardiovascular diseases (CVD), cancers, strokes, Chronic Obstructive Pulmonary Disease (COPD), arthritis, and mental health issues. These LSDs that share same causative agents are at times interlinked and appears as a comorbidity in an individual, also referred to as metabolic syndrome. About 25% of the world population suffers from metabolic syndromes. World Economic Forum report predicts for about $200 billion loss to Indian economy,

due to unhealthy diet and lifestyle (Lozano *et al.*, 2012). LSDs are reversible and can be combated with awareness of a healthy diet, activity, and correct circadian rhythms.

Circadian Rhythms

Organisms have evolved to adapt to the cyclic environmental pattern on earth that substantially bifurcates in day and night based on earth's axial rotation of ~24 hours. The word circadian is derived from a Latin phrase 'Circa diem' meaning 'about the day' and is essentially referred to as circadian rhythms owing to its perpetual cycling pattern. Circadian timing aligns oscillation in the biological processes such as food intake, energy expenditure, alertness, activity to the earth's solar day, manifesting a rhythm in physiology and behaviour of organisms ranging from bacteria to mammals. Environmental cues such as light/dark cycle, temperature changes, availability of food, etc. acts as zeitgebers (ZT) that synchronizes the endogenous timings of every cell in an organism and aligns it with the environment (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Wehrens *et al.*, 2017). The word 'Zeitgebers' is the combination of 2 German terms; 'zeit' meaning time and 'geber' meaning giver. Biologically zeitgebers is used to refer to the day length or the time of biological rhythm (Daan and Merrow, 2002).

Across the life forms, internal timing systems can be witnessed at a transcriptional level that gives rise to divergent gene networks in different organism, oscillating on a 24h bases. Organismal circadian system consists of three components: the input, oscillator, and an output. Principally it operates at two levels, cellular and systemic. Light is a major input signalling for circadian rhythms at the systemic scale and resets the suprachiasmatic nuclei (SCN), also annotated as master pacemaker (Jin *et al.*, 1999). A defining feature of the SCN is the robust networking among its oscillator cells

compared with that in peripheral tissues. The main function of SCN is to rhythmically connect other region of brain and other peripheral tissue via neuronal or hormonal signalling. The SCN is a paired structure of ventral hypothalamus and contains about 50,000 neuronal connections in human. Moreover, SCN also controls endocrine system and their circadian secretion signals such as melatonin from the pineal gland and glucocorticoids and catecholamines from the adrenal cortex via the hypothalamic–pituitary–adrenal axis (Engeland and Arnhold, 2005; Bornstein *et al.*, 2008). Together it maintains vital physiological activities such as endocrine rhythms, feeding/fasting cycles, sleep/wake cycles and metabolic rhythms (Dibner *et al.*, 2010). However, other time cues such as work schedule, exercise schedule, meal timings, resting period also impact and alter the circadian pattern of an individual. All the circadian synchrony is the result of molecular framework; these genes controlling the circadian biology of the body is annotated as clock genes (Fig. 1).

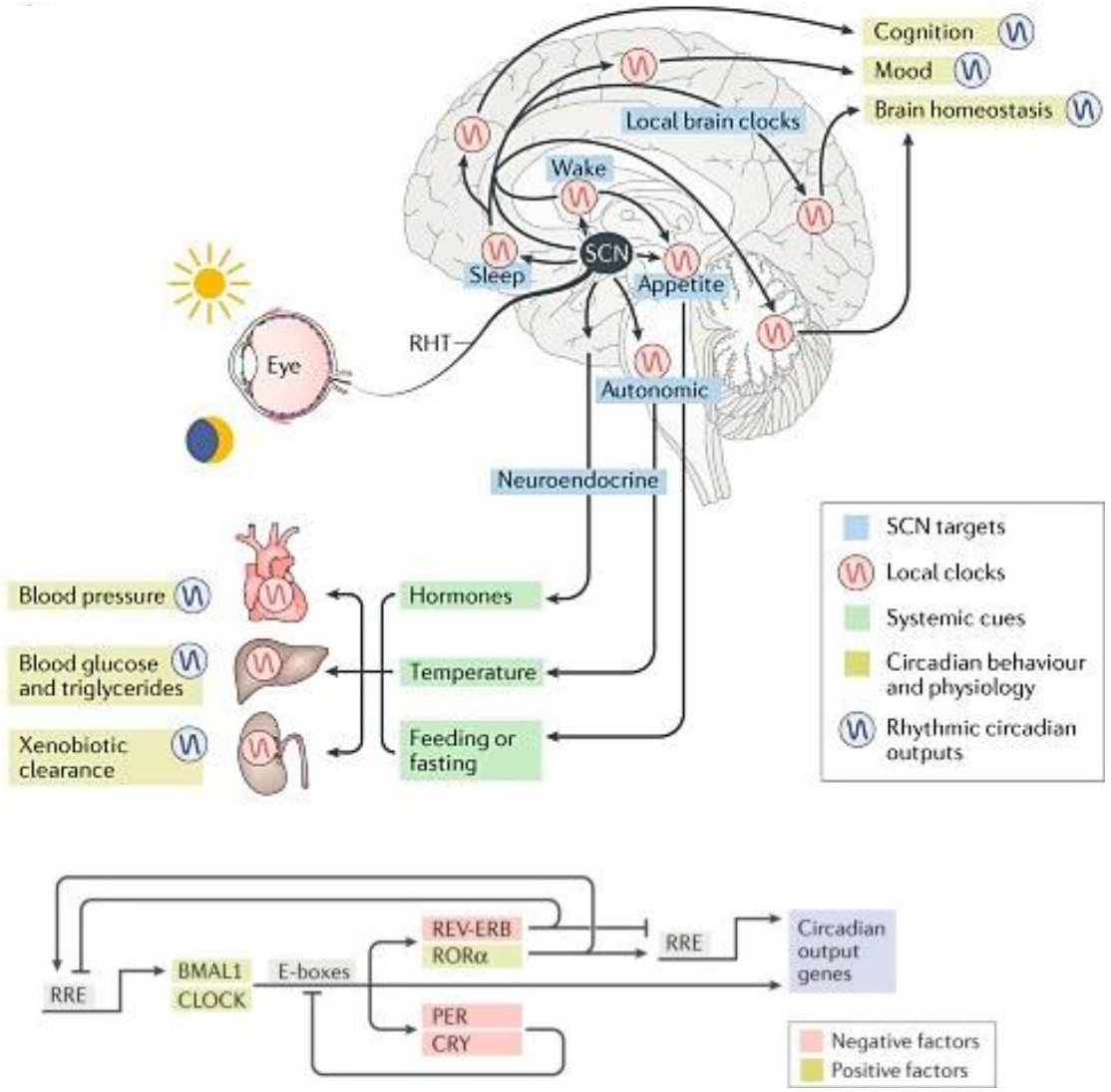


Fig. 1: Schematic representation of circadian rhythm operating in the biological system (Hastings *et al.*, 2018).

Molecular Clock work

Circadian rhythms are endogenously operated at molecular levels through clock genes that interact to bring about cyclic changes in the system. The molecular clock is self-sustained, autonomous and present in each cell type wherein it functions via interconnected negative feed-back loops (Abe *et al.*, 2002; Takahashi, 2017). Mammalian circadian clock is arranged with a central clock in the brain, gaining environmental ques and its output to the periphery is utilized in maintaining synchronized biological clocks of the peripheral organs. Master transcription factors include CLOCK (Circadian locomotor output cycles kaput) and Bmal1/2 (brain and muscle Arnt-like protein) that dimerize with each other and interacts with E-box (5′-CACGTG-3′) or E' box (5′- CACGTT-3′) promoter elements to drive transcription of period (Per1-3) and cryptochrome (Cry1/2) genes that form the negative limb of feedback loop (Kume *et al.*, 1999). CLOCK and BMAL1 also encodes bHLH-PAS proteins that forms the positive limb of feedback circuit (Jin *et al.*, 1999; Preitner *et al.*, 2002; Canaple *et al.*, 2006). PER and CRY proteins forms complexes in the cytoplasm orchestrating their physiological functions. At a certain threshold, these complexes migrate into the nucleus, inhibiting the action of CLOCK and BMAL1 and thus their transcription. Inhibitory PER/CRY complexes are phosphorylated by casein kinase Iε (CKIε) and undergoes proteasomal degradation and then ubiquitination. This lifts the inhibition on CLOCK and BMAL1, allowing the feedback loop to restart in 24h. This primary feedback loop system is intertwined with numerous other feedback loops that cumulatively functions in the system. Critically, REV-ERB α/β (retinoic acid receptor-related orphan receptors) and RORα bind to enhancer elements on the Bmal1 promoter to inhibit or promote transcription, respectively. Oscillations of REV-ERBα and RORα drive the rhythmic expression of BMAL1. BMAL1/CLOCK complex acts directly on

the REV-ERBα gene, driving an 'accessory' loop (Fig. 2) (Preitner *et al.*, 2002; Sato *et al.*, 2004; Canaple *et al.*, 2006).

SIRT1 (Sirtuin1), an NAD+ dependent deacetylase, known to be at a cellular metabolic juncture also plays an important role in circadian clock circuit. SIRT1 deacetylates Bmal1 and inhibits Clock: Bmal1 transcriptional activity. SIRT1 also deacetylates PER1 resulting in its decreased stability and degradation. Further, Clock binds to nicotinamide phosphoribosyltransferase (NAMPT) and elevate its expression that culminates into an increased NAD+ and SIRT1 activity (Tong *et al.*, 2015). Overall circadian components are closely functioning with the metabolic genes bringing circadian co-ordination with physiological cues.

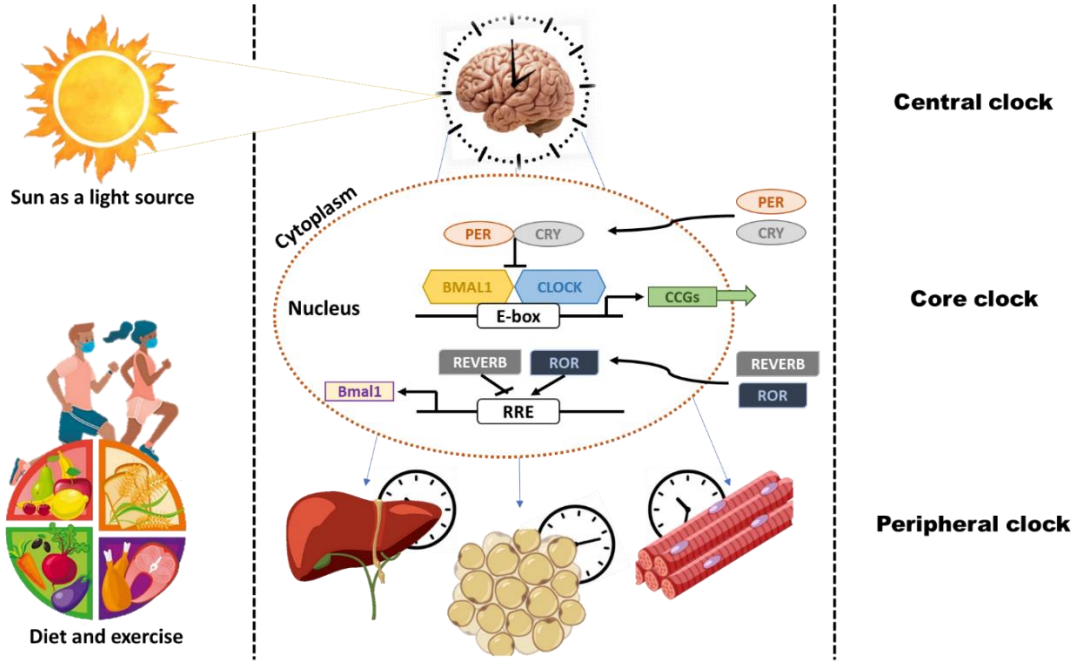


Fig. 2: Diagrammatic representation of core and peripheral circadian clocks in the body and factors manifesting alterations.

Circadian rhythms and metabolism

Circadian rhythms and energy metabolism are reported to be closely associated with each other physiologically. About 40% of the genes exhibit 24 h oscillations in transcript abundance throughout the body (Zhang *et al.*, 2014). However, the identity of those cyclic genes differs significantly between organs, suggesting organ specific integration with circadian cues. A study, meticulously demonstrates that clock proteins are directly regulated by energy sensors that also has an ability to modify their expression or stability (Lamia *et al.*, 2009). AMP kinase (AMPK) senses AMP/ATP ratio and is activated at higher ratio or by low energy state. Moreover, AMPK nuclear localization and activity are shown to be having circadian pattern in mouse liver, exhibiting a molecular clock control over its function. Further clock is also reported to be sensitive to NAD+/NADH ratio that directly alters on dietary pattern. NAD+ is co-factor for multiple metabolic enzymes including SIRTs that has deacetylation activity; thereby transduce changes in metabolic status by transcriptional/chromatin modifications (Ramsey *et al.*, 2009; Amjad *et al.*, 2021). Further, BMAL1: CLOCK heterodimer directly activates the transcription of genes coding for the NAMPT which is the rate-limiting enzyme of the NAD^+ salvage pathway, resulting in circadian oscillations of the intracellular NAD+ levels that further reflects on regulation of energy metabolism, DNA damage repair and stress response. A study showcased that homozygous CLOCK mutant mice exhibited hyperphagic symptoms and altered feeding patterns, leading to obesity (Rudic *et al.*, 2004; Turek *et al.*, 2005). A whole-body deletion of BMAL1 or CLOCK mutant also displayed reduced gluconeogenesis, altered glucose tolerance and increased body weight (Rudic *et al.*, 2004; Lamia *et al.*, 2008).

Interesting is the reciprocal control between the circadian clock and metabolism. Its sensitivity to variety of genetic and behavioural factors leads to disruption of the rhythms and consequently aggravated metabolic dysfunction (Table 1). Shift-work jobs altered feeding patterns, constant exposure to ALAN (artificial light at night), untimed physical workout etc. bring about changes in energetics and individual's metabolism that further manifest alterations in circadian rhythm, also referred to as chronodisruption (CD). Present lifestyle broadly includes untimed feeding, high calorie diet, altered sleep regimen, shift work, transcontinental travel and lack of adequate physical activity which cumulatively brings about disturbed circadian patterns and consequential metabolic dysfunction. Pathological conditions like diabetes, obesity, hypertension, CVDs happen to be the consequence of the mentioned alterations.

Table 1: Metabolic phenotypes observed after genetic clock disruption.

Gene	Organ/Cell Type	Metabolic phenotype	Reference
Clock mutant	Global	Decreased gluconeogenesis, blunted circadian oscillations of liver PEPCK, increased BW (on chow or HFD diet)	(Rudic *et al.*, 2004)
Clock mutant (D19) (C57 background)	Global	Hyperphagic and altered feeding pattern; when fed a HFD, become obese, insulin resistant, hypoinsulinemic, hyperlipidemic, hyperleptinemia	(Turek *et al.*, 2005)
Clock mutant (D19) (ICR background)	Global	Protected from HFD-induced obesity due to reduced intestinal fat absorption	(DeBruyne *et al.*, 2015)
Bmal1 -/-	Global	Exacerbated insulin-induced hypoglycemia due to decreased gluconeogenesis, elevated plasma TG levels	(Rudic *et al.*, 2004)
Bmal1 -/-	Global	Higher BW, glucose intolerance, increased insulin sensitivity	(Lamia *et al.*, 2008)
Bmal1-/- (Esr-Cre) (tamoxifen induced)	Global, in the adult	Unchanged BW and blood Glc, normal glucose tolerance and insulin sensitivity despite lack of circadian wheel-running activity, and dampened liver clock gene expression.	(Yang *et al.*, 2016)
Bmal1 -/- (Pdx Cre)	β-cell	Elevated blood glucose and impaired glucose tolerance,	(Marcheva *et al.*, 2010;

		hypoinsulinemia due to impaired insulin secretion	Perelis *et al.*, 2015)
Bmal1 -/- (aP2-cre, adiponectin cre)	White and brown adipocytes	Normal glucose tolerance and insulin sensitivity, increased BW gain upon HFD likely due to reduced lipolysis, changes in PUFA levels, reduced energy expenditure and attenuated food intake rhythms	(Paschos *et al.*, 2012)
Bmal1 -/- Alb-Cre	Liver	Hypoglycemia during the fasting period due to increased glucose clearance	(Lamia *et al.*, 2008)
Bmal1 -/- (Mlc1fCre or iaHSA)	Skeletal muscle	Normal fasted glucose, reduced insulin-stimulated glucose uptake by skeletal muscle due to decreased Glut4, altered glycolytic genes, altered AA homeostasis, shift toward an oxidative fiber type	(Hodge *et al.*, 2015; Harfmann *et al.*, 2016)
Cry1 overexpression	Liver	Decreased gluconeogenesis, Protected from HFD-induced insulin resistance	(Zhang *et al.*, 2010)
Cry1/Cry2 DKO	Global	Glucose intolerance due to high corticosterone levels and increased GR signalling in liver, lower BW	(Lamia *et al.*, 2011; Kettner *et al.*, 2015)
Per1/Per2 DKO	Global	Disrupted bile acid homeostasis	(Ma *et al.*, 2009)
Rev-erbα	Global	Increased plasma lipid (TG) levels, disrupted bile acid	(Duez *et al.*, 2008;

		homeostasis, altered body temperature control, reduced exercise capacity due to altered muscle mitochondria biogenesis and function	Gerhart-Hines *et al.*, 2013)
Rev-erbβ in the Rev-erbα global KO or Rev-erbα mut/Rev-erbβ KO	Liver	Altered glucose and lipid plasma levels, derepressed hepatic lipogenesis	(Bugge *et al.*, 2012; Cho *et al.*, 2012)

Chronodisruption and Cardiovascular disorders

Circadian rhythms are closely associated with several aspects of cardiovascular physiology. Each organ has its own peripheral clock that has a beneficial role in maintaining organ homeostasis. Stringently controlled circadian protocols like forced desynchrony (FD) or constant routine (CR) protocols are employed to determine the effect of circadian system on cardiovascular physiology (Anea *et al.*, 2009; Scheer and Shea, 2014). The FD protocol uncouples circadian cycles from daily behavioural/environmental rhythms. Using these carefully controlled experimental paradigms circadian rhythms have been observed in heart rate, blood pressure (BP), circulating epinephrine and norepinephrine, heartbeat dynamics, plasminogen activator inhibitor 1 (PAI-1), cardiac vagal modulation, platelet aggregability and immune responses (Bridges *et al.*, 1993). Collectively, the circadian system influences cardiovascular risk factors with possible contributions to the adverse event timed with peak in the morning (Behrens *et al.*, 1995).

The rise in heart rate, BP, vascular tone, coagulation activities in the early morning underlies the frequent onset of acute myocardial infraction. Further, ventricular

arrhythmias viz. ventricular tachycardia or ventricular fibrillation also tend to develop in the morning. Studies have showed that onset of stent thrombosis as well circadian oscillation with peak incidences around 0700 hours. Acute aortic dissection also exhibited time-of-day variation with one primary morning peak (0800 – 1100 h) and a secondary evening peak (1700-1900 h) (Kozák *et al.*, 2003).

Vascular functions like endothelium dependent vasodilatory activity also show circadian oscillations; not only in the in-vitro vascular endothelial cultures but also in human subjects. Critically, vasodilation decreases in the morning hours. Rho and Rho-associated protein kinase (ROCK), its effector, phosphorylates myosin light chain in vascular smooth muscle cells (VSMCs) and acts as a major regulator of vascular contraction (Saito *et al.*, 2013). RORα enhances expression of ROCK2 in circadian manner, thus governing vascular constriction (Mohri *et al.*, 2003). Endothelial Nitric Oxide (NO) has vital role in regulating vascular integrity and vasomotor tone. Nitric oxide synthase (eNOS), an enzyme responsible for NO production also exhibit time-of-day variation (Denniff *et al.*, 2014). Likewise, internal clock of smooth muscle cells contributes to circadian exhibition of blood pressure. Overall, a close-knit association of circadian rhythm and cardiovascular disorders is well established and chronodisruption is acclaimed as one of the major root causes for manifestation and aggravation of several CVDs (Fig. 3).

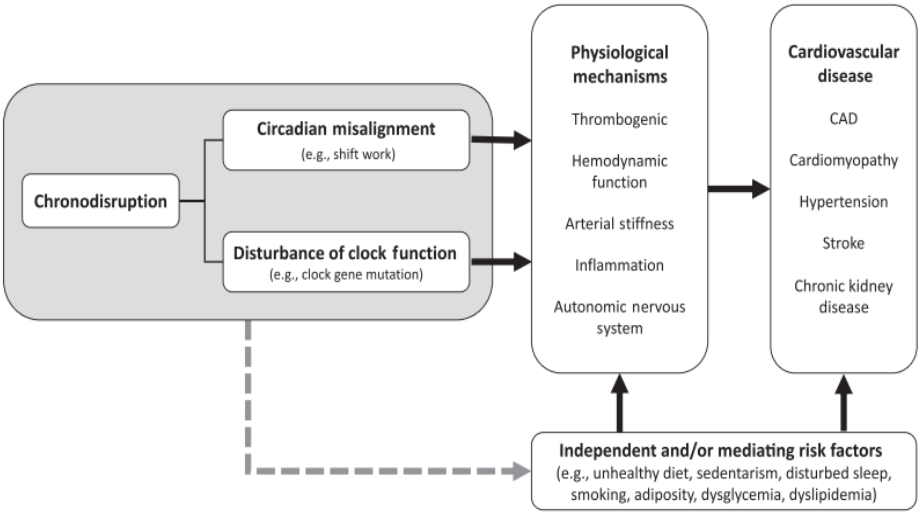


Fig. 3: Represented are the pathological implications of chronodisruption (Chellappa *et al.*, 2019).

Cardiovascular Disorders

Cardiovascular disorders are wide spectrum of disorders related to heart and the vascular system physiologically responsible for blood circulation and supply in the body (Fig. 4). CVDs can broadly be divided into 4 main types as follows

1. *Coronary Artery disease*

   Blocked or reduced flow of oxygen rich blood to the heart muscle leads to development of coronary artery disease. This condition puts an additional strain on heart that can further culminate into angina (chest pain caused by restricted blood flow to the heart muscle), heart attack (suddenly blocked blood flow to the heart muscle) and heart failure (inability of heart to pump blood)

2. *Strokes and Transient ischaemic attacks*

   Stroke is where the blood supply to the brain in cut off causing brain damage and in some cases death.

Transient ischaemic attack (TIAs) or mini stroke is same but here the blood flow is temporarily disrupted. This leads to inability of facial movements, weakness or numbness in arms and slurred speech.

3. *Peripheral arterial disease*

   Blockage in the arteries caused peripheral arterial disease. This is majorly observed in legs and can cause cramping leg pain, hair loss on legs and feet, numbness in leg and persistent ulceration in the legs.

4. *Aortic disease*

   A group of conditions affecting aorta manifest aortic disease. Aorta is the largest blood vessel of the body that carries blood from the heart to rest of the body. One of the most common aortic diseases is aortic aneurysm wherein the aorta becomes weakened and bulges outwards.

There are several causative agents that have been studies to have critical role in developing CVDs. Some of these causative factors include high blood pressure, smoking, high cholesterol, diabetes, inactivity, obesity, genetic factors, ethnic background, age, gender, alcohol abuse, diet etc.

CVDs are the leading cause of death globally, taking about 17.9 million lives each year. This is about 32% of deaths worldwide (Fig. 5) (Mensah *et al.*, 2019). CVDs claim about 4.77 million lives in India and is prevalent in about 7% rural and 13.2% urban Indian population (Fig. 6) (Prabhakaran *et al.*, 2018). Atherosclerotic CVDs tops the chart for mortality by CVDs globally.

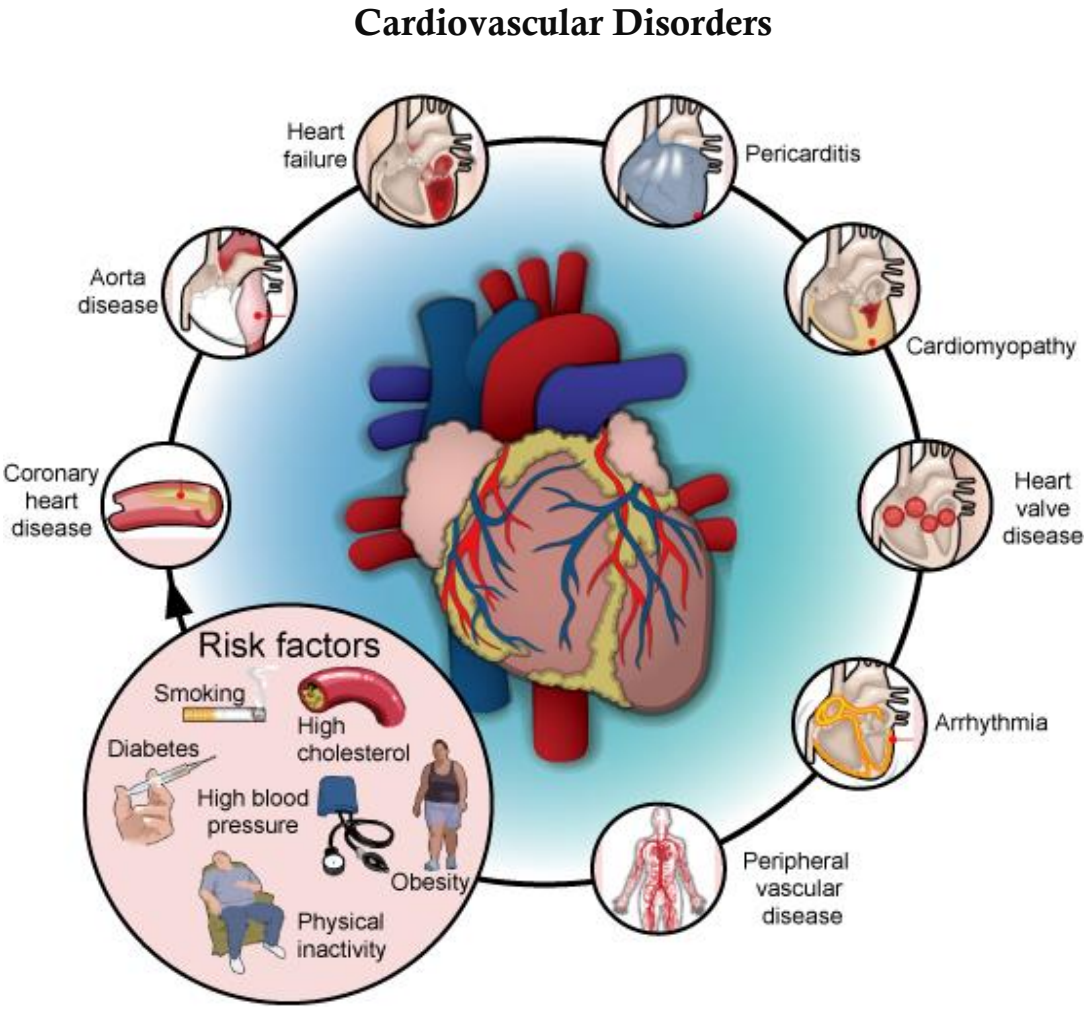


Fig. 4: The diagram depicts cardiovascular disorders and the potential risk factors.

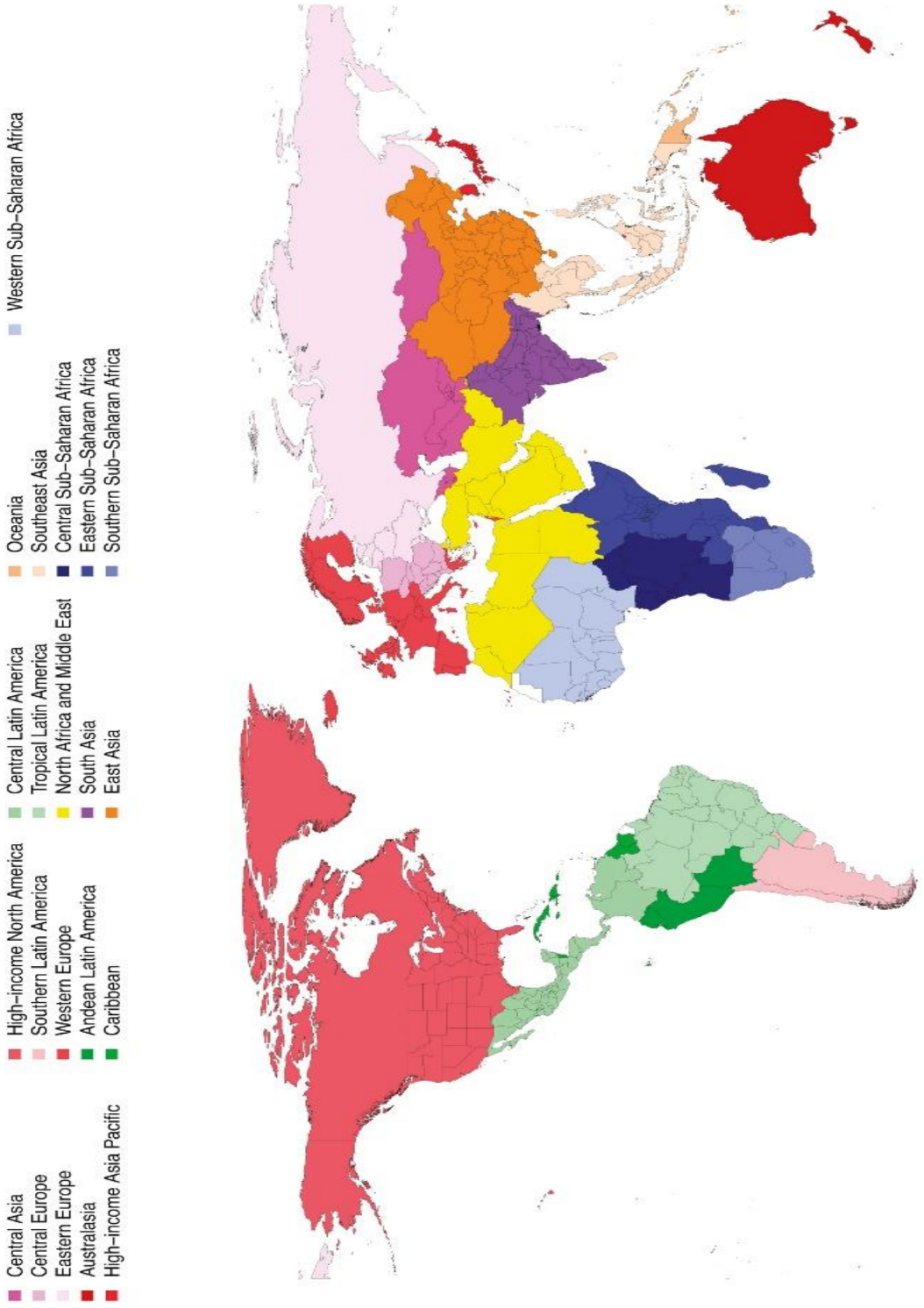


Fig. 5: The global burden of cardiovascular diseases marked in national and sub-national locations. Data from Institute of health metrics and evaluation. (Mensah *et al.*, 2019)

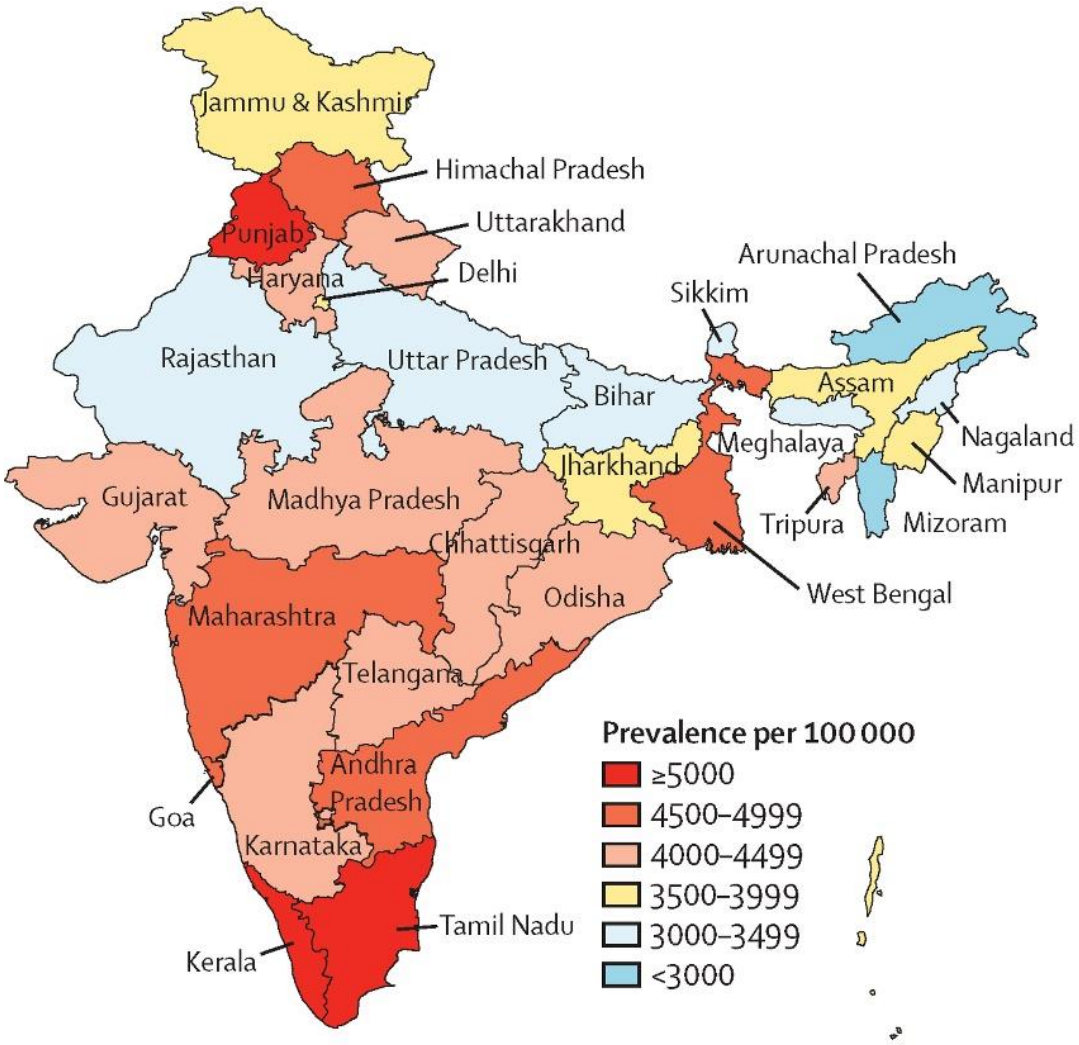


Fig. 6: Prevalence of cardiovascular diseases in India. (Prabhakaran *et al.*, 2018)

Atherosclerosis, an overview

Atherosclerosis is accumulation of fatty/fibrous material in the inner most layer of the arteries, referred to as intima. The term atherosclerosis is derived from the Greek word 'athero' meaning 'gruel' or 'porridge', reflecting the appearance of the lipid material found in the core of atheromatous plaque. Excessive lipid deposition in the inner layer over a period of time progresses into an atheromatous plaque or atheroma. Progressive atherosclerosis witnesses higher fibrous plaque and calcium accumulation (Shioi and Ikari, 2017). Advanced atheromatous plaque can encroach upon the arterial lumen, obstructing the blood flow and eventually causing tissue ischemia (Fig. 7). Other instance to flow limiting obstruction is plaque rupture, that provokes the formation of a thrombus that can occlude the lumen probably causing a severe ischemia (Bentzon *et al.*, 2014). Atherosclerosis majorly happens in larger blood vessels of the body and can lead into progressive/chronic damage to the concerned organ. Atherosclerosis in the coronary artery can cause acute coronary syndrome including myocardial infarction or chronic conditions such as stable angina pectoris (chest pain or discomfort caused by insufficient perfusion of the heart muscle) (Lindenholz *et al.*, 2020). Atherosclerosis causes many ischaemic strokes and transient cerebral ischaemic attacks (Valanti *et al.*, 2021). It can lead to the formation of aneurysms including those that form in the abdominal aorta. Atherosclerosis in the peripheral arteries can cause intermittent claudication ulceration and gangrene that can jeopardize limb viability (Fig. 8) (Levin *et al.*, 2020).

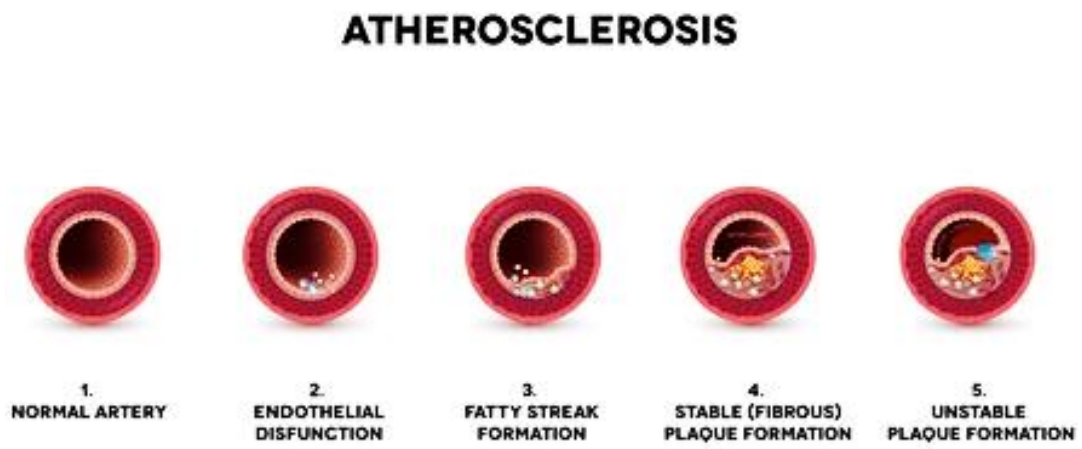


Fig. 7: Schematic representation of arterial status at different stages of atherogenic progression.

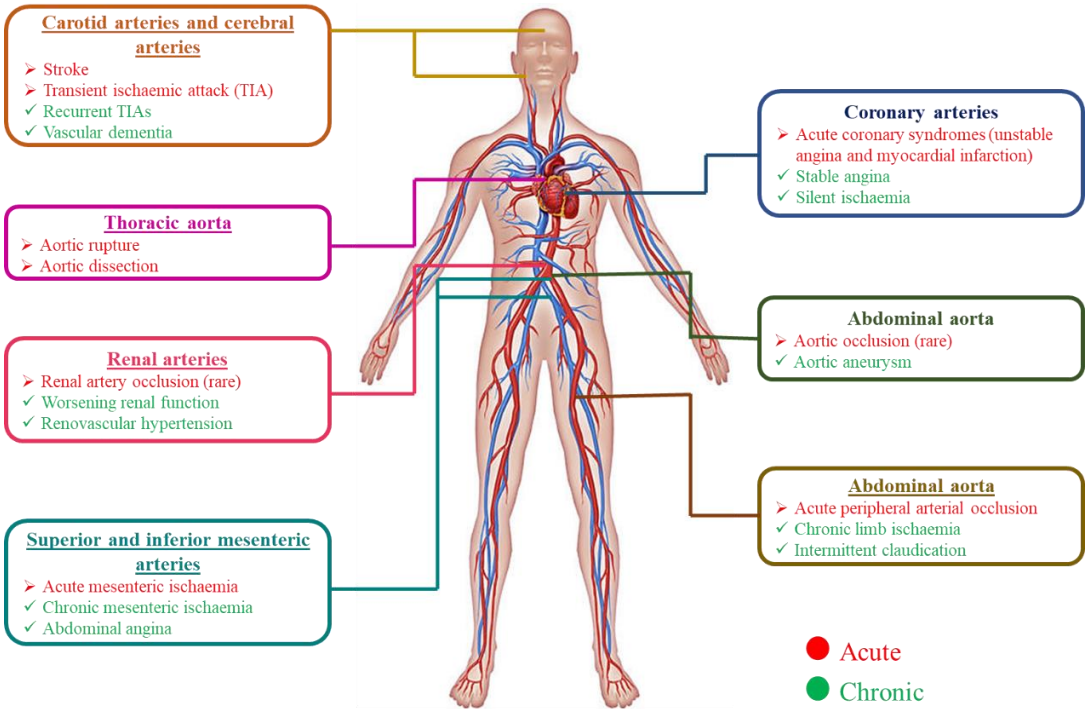


Fig. 8: Diagrammatic representation of arterial positioning and related acute or chronic pathological conditions.

Atherogenic pathophysiology

Broadly atherosclerosis can be classified into three main stages of (i) Initiation (ii) Progression and (iii) Complications.

Atherogenic initiation

Basic arterial physiology exhibits trilaminar structure. The outermost layer termed as adventitia constitutes of nerve endings, mast cells and vasa vasorum, the micro-vessels nourishing the outer layer of media. Tunica media consists of quiescent smooth muscle cells and a well-organized extracellular matrix comprising elastin, collagen, and other macromolecules. The inner most layer, intima is made with endothelial cells supported by internal elastic lamina, these are in direct contact with the blood flow. Preliminary phase of lesion initiation has low-density lipoproteins (LDL) particles accumulating in the intima, where that undergo oxidative and other modifications rendering them pro-inflammatory and immunogenic changes (Valanti *et al.*, 2021).

Monocytes exhibiting pro-inflammatory palette of function enters the intima by binding to adhesion molecules expression by activated endothelial cells. Chemoattractant cytokines or chemokines promotes migration of monocytes into the arterial wall, wherein they mature into macrophages. These Macrophages express scavenger receptors (SR) that facilitates binding of LDL and furthers foam cell formation (Moore *et al.*, 2018).

In response to the mediators elaborated by accumulating leukocytes, the smooth muscle cells (SMCs) in the tunica media can migrate into the intima. The smooth muscle cell chemoattractant platelet-derived growth factor arising from macrophages and deposited by activated platelets at sites of endothelial breaches or intraplaque haemorrhage

probably participates in this directed migration of medial smooth muscle cells into the intima.

- *LDL cholesterol and modifications*

LDL cholesterol (LDL) is physiologically composed of phospholipid monolayer, esterified and unesterified cholesterol particles, Apolipoprotein B100 (ApoB100) and triglycerides (TGs) (Fig. 9). Conventionally LDL is connotated as 'bad cholesterol' pertaining to its capability of arterial accumulation and retention, culminating in development of fatty streaks, and thus acting as a potent atherogen (Chakrabarti, 2021). LDL accumulation in sub-intimal layer is accomplished by either infiltration or binding with proteoglycans. The cumulative exposure of an artery to LDL over years remains a principal determinant of disease initiation and progression. However, years of research suggest that it's not the LDL but the oxidized form of LDL that's a troublemaker (Goldstein *et al.*, 1979; Steinbrecher *et al.*, 1984). Amongst several other sources, the accumulated LDL undergoes oxidative modification by reactive oxygen species (ROS) sourced through metal ion catalysis (Fenton reaction), altered cellular respiration, 12/15-lipoxygenase and myeloperoxidase (Alouffi *et al.*, 2018). The pathophysiological conditions fail to illicit fall in expression of scavenger receptors and high affinity LDL receptors even on higher cellular cholesterol content. Macrophage overloading with cholesterol ester drives foam cell formation, a hall mark of early atherogenic lesion. Constituted oxidized LDL (ox-LDL) particles further induces inflammatory reaction and stimulates humoral and adaptive immunity (Fig. 10).

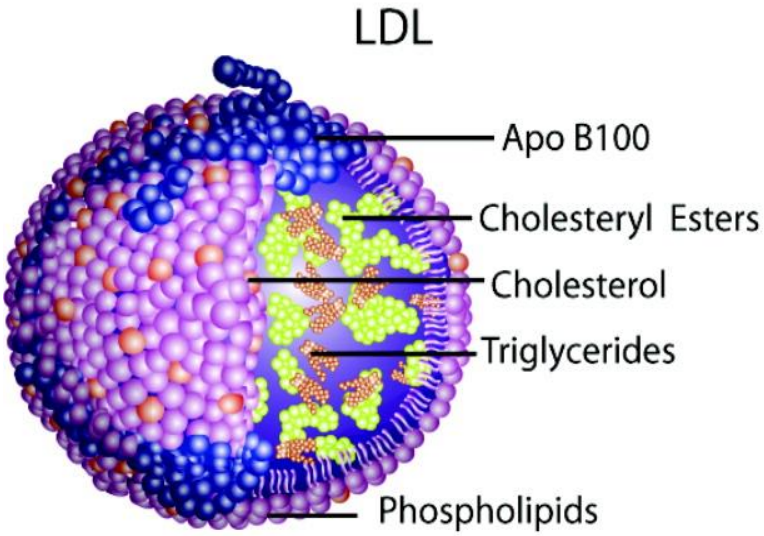


Fig. 9: physiological composition of Low-Density Lipoprotein (LDL) particle.

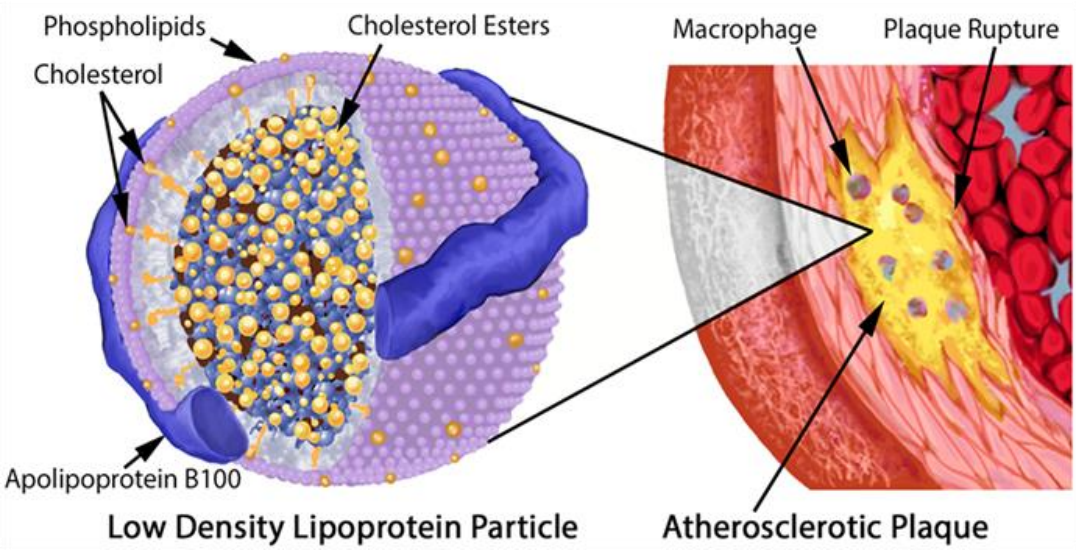


Fig. 10: physiological status of oxidized LDL (ox-LDL) and its positioning in atheromatous plaque.

- *HDL cholesterol and Triglycerides*

HDL cholesterol (HDL) is commonly annotated as a 'good cholesterol'. Physiologically it comprises of TGs, cholesterol esters, apo -proteins, phospholipids and unesterified cholesterol. Broadly, HDL metabolism consists of five main processes: (1) Apo A-I synthesis and secretion into plasma as nascent HDL; (2) free periphery cholesterol uptake; (3) maturation with cholesterol esterification; (4) delivery of cholesteryl ester to the liver, steroidogenic organs, and apo B-containing lipoproteins; and (5) Apo A-I catabolism (Fig. 11) (Ben-Aicha *et al.*, 2020). HDL concentrations are constantly associated inversely with the risk of atherosclerosis. HDL is proposed to have an anti-atherogenic properties that includes reverse cholesterol transport, antioxidant & anti-inflammatory properties, endothelial maintenance function, deterrence of cell apoptosis and maintaining low blood viscosity via permissive action on red cell deformity. However current human genetic evidence does not support a protective role of HDL against atherosclerosis. Adding on to that several HDL raising therapies have not obtained major successful outcome against cardiovascular disorders. Some studies suggest that probable disparity in the observational data is because HDL tracks inversely with TG concentrations. And substantial human genetic evidence now supports a casual role of TGs in atherosclerosis. On contrary to HDL, convincing human genetic evidence supports the strong observational relationship between lipoprotein(a) and atherosclerotic risk.

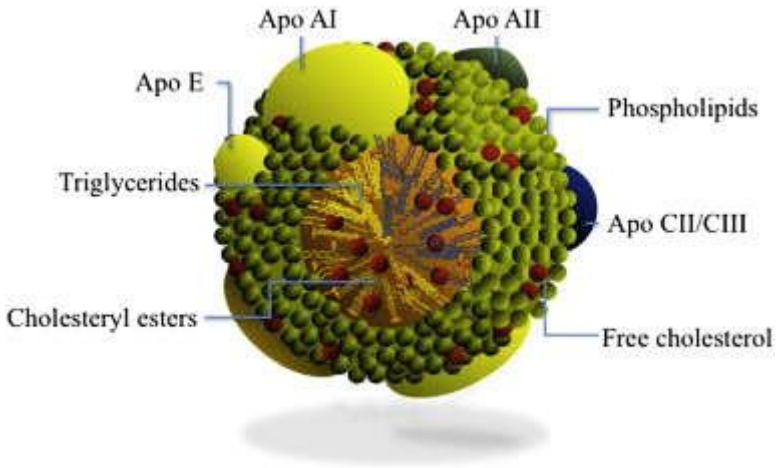


Fig. 11: physiological composition of High-Density Lipoprotein (LDL) particle.

- *Inflammation*

Atherogenic risk factors include hypertension, tobacco chewing, smoking, metabolic syndrome cluster, blood pressure, visceral obesity etc. are studied as potential triggers to elicit inflammatory response. Studies have shown that inflammation can alter the function of the cells of the arterial wall in a manner that initiates atherosclerosis. Explanatorily, angiotensin II, participating in the pathogenesis of hypertension, can unleash inflammatory pathways governed by the master transcriptional regulator nuclear factor-κB (NF-κB) pathway (Chen *et al.*, 2019a). Visceral adipose tissue containing inflammatory cells can elaborate multiple mediators of inflammation. Tobacco use can also elicit an inflammatory response in the airways and alveoli that can affect distant artery walls, as they release soluble inflammatory mediators such as cytokines that can activate cells in the intima (Ghio *et al.*, 2022). C-reactive protein (CRP) is an inflammatory biomarker that also predicts cardiovascular risk.

Several experimental bases have established role of adaptive immunity in atherosclerosis. Human atherogenic lesions contain T lymphocytes and display markers

of adaptive immune activation. Some T cell subtypes (type 1 T helper (TH1) cells) promote experimental atherosclerosis, whereas others (regulatory T (Treg) cells) seem to mitigate the same (Ou *et al.*, 2018). Numerous experimental works majorly conducted on mice, has rigorously demonstrated causal role of adaptive immunity in modulating experimental atherosclerosis (Roy *et al.*, 2022). Further studies of human atherosclerotic plaques and biomarker investigations provide support for the contribution of inflammation and immunity in atherosclerosis.

- *Endothelial cell activation*

Endothelial monolayer basically provides an interface between the blood flow and arterial intima. Alterations in this monolayer is the site of atheroma initiation, occurring in early phase of atherosclerosis. Exposure to atherogenic risk factors as mentioned earlier can compromise the capability of production of endogenous vasodilators like nitric oxide by endothelial cells (ECs) (Radomski *et al.*, 1987). Consumption of cholesterol rich diet can activate expression of adhesion molecules like vascular cell adhesion molecule 1 that facilitate binding of blood leukocytes to the endothelial surface and chemo-attractants that promote entry of the bound leukocytes into the intima (Luchetti *et al.*, 2021). The local haemodynamic environment also impacts EC functions. Blood flow changes are sensed by flow-dependent ion channels or surface structures like members of the integrin family of transmembrane proteins. The downstream transcriptional mechanisms that transduce the effects of flow into altered gene expression include Krüppel-like factor 2/4 (KLF 2/4). Such abnormal flow patterns disturb the physiological homeostasis, athero-protective functions of the endothelium, reversing tonic vasodilatation, anti-thrombotic and anti-inflammatory

properties and mechanisms resisting thrombus formation (Parmar, 2008). Atherogenic plaque usually forms at the site of flow disturbances like the aortic arch, where the laminar shear stress predominates. Cumulatively, exposure to the atherogenic risk factors, their downline mediators, blood flow perturbations etc promotes atherogenic initiation by alterations the endothelial homeostasis also referred to as EC activation. Activated ECs are exhaustively shown to recruit monocytes to the lesion prone area by capturing (adhesion), rolling and transmigration.

Progression of Atherosclerosis

On establishment the atherogenic plaque progress by continual accumulation of lipid and/or lipid-engorged cells. These lipid engorged cells widely consist of monocyte derived macrophages that are believed to progress in formation of lipid-laden foam cells fostering atheroma (Chistiakov *et al.*, 2017). However, recent experimental data suggest that metaplasia of smooth muscle cells may also give rise to foam cells resembling macrophages. Arterial arrangement physiologically has SMCs placed in the media layer, however intimal site of atheroma development exhibits SMC presence. SMC migration from media to intima contributes to its accumulation in the atheromatous plaque. These cells proliferate over the years and elaborate extracellular matrix macromolecules that encompass much of the bulk of an established plaque (Basatemur *et al.*, 2019).

Extracellular matrix of atheroma consists of collagen, elastin, proteoglycans and glycosaminoglycans. These macromolecules can entrap lipoproteins and promote lipid accumulation within the intima. Inflammatory leukocytes not only arrive in the intima by infiltration but can also proliferate within the lesion. Atheroma not only consist of

macrophages but also T cells that probably orchestrate negative or positive impact based on the T cell type present. These cells, at times drain from the plaque and reach to near lymph nodes acting as either T cells or B cells. Along with these, the resultant cytokines also play a vital role in modulating immune response.

In advanced stages the macrophages and SMCs undergo programmed cell death forming a nidus of a lipid rich or necrotic core of advanced atheroma. System is unable to clear the dead cells leading to a compromised efferocytosis that contributes to necrotic core formation. Later stages witness calcification in the plaque. Microscopic spotty calcification of plaque is associated to mechanical instability and indicates higher probability of rupture and resultant thrombosis. Large range calcification promotes plaque stability and lowers the probability of triggering thrombotic event.

Atherogenic Complications

In much of the disease course the atherogenic plaque expands outward radially in an abluminal direction preserving the calibre of arterial lumen. But eventually this plaque begins to encroach upon the arterial lumen and can lead to obstruction in blood flow and formation of flow limiting lesions. Atheroma of advanced stages that have ruptured often is noted to have large lipid core covered by thin fibrous cap (<60 μm). Lesions with this characteristic are termed as 'vulnerable plaques.' On the other hand, plaques with limited lipid accumulation and thicker fibrous cap are referred to as 'stable plaque' (Truong *et al.*, 2021). Rupture of a plaque exposes internal content out to the blood compartment. Tissue factor produced from macrophages and SMCs, considered as a thrombogenic material of necrotic core can cause an episode of thrombosis and

eventually initiate the most dreaded complications of atherosclerosis (Asada *et al.*, 2020).

Diagnostics

There have been several technological advancements for detection of atherosclerosis, including invasive and non-invasive methods. However, the policy regarding appropriate screening strategy for atherosclerotic detection and prevention of its complications remains variable across different guidelines. Diagnostic strategies are employed highly on presented individual and clinically evident risk factors. Diagnostics are broadly divided as direct or indirect atherogenic plaque visualization. The direct strategies include Ultrasonography, Doppler Ultrasonography, CT angiography, MRI, Intravascular Ultrasonography and Optical coherence tomography. The indirect methodologies include PET and Invasive angiography. Utilization of these diagnostics is purposed upon the clinical condition of the subjected patient (Table 2) (Doukky *et al.*, 2017).

Table 2: Diagnostic tests for atherosclerosis.

Test	Clinical Applications	Imaging characteristics	Advantages	Disadvantages
Direct Atherogenic Plaque Visualization				
Ultrasonography or Doppler ultrasonography	Carotid arteries, intracerebral arteries (with transcranial Doppler ultrasonography), abdominal aorta, lower extremity vessels	Enables differentiation of some plaque components	Non-invasive and no radiation exposure required	Can only be used in large-calibre and superficial vessels
CT angiography	Differentiation between calcified and noncalcified plaque	Most vasculatures	Non-invasive	Radiation exposure
MRI	No evaluation of plaque components	Aorta and carotid artery	Non-invasive and no radiation exposure required	Limited to large-calibre vessel; and smaller ones like carotid artery
Intravascular Ultrasonography	Plaque tissue characterization by analysis of radiofrequency backscatter from the ultrasound signal: 'virtual histology'	Coronary artery evaluation	Excellent for evaluation of plaque burden and composition	Invasive; radiation exposure and iodinated contrast agent (for catheter positioning) required; limited availability

Optical coherence tomography	High-resolution imaging for plaque characteristics	Routine clinical application restricted to very selected cases of coronary artery evaluation	Excellent near-field resolution permitting visualization of the intimal morphology in detail	Invasive; radiation exposure and iodinated contrast agent (for catheter positioning) required; limited availability and limited penetration, limiting the evaluation of deeper areas of the plaque
Indirect atherogenic plaque analysis				
PET	Identifies glucose uptake by inflammatory and other cells in plaques	Applications restricted to research	Evaluates plaque metabolism	Radiation exposure required; can only be used in large-calibre vessels
Invasive angiography	Classic reference standard for the evaluation of luminal stenosis	Most vascular territories	Widely available; good visualization of the lumen	Invasive; radiation exposure and iodinated contrast agent required; does not visualize the plaque directly

Managements and Treatments

Atherosclerosis is a multifactorial disease and hence its management should incorporate all the known treatable risk factors. It being a lifestyle disorder, the primordial prevention initiates with adapting healthy lifestyle that is beneficial in reducing or reversing the pathogenicity at any stage of atherogenic progression, often referred to as a secondary treatment (Moran *et al.*, 2014). Presently, couple of primary treatment option that still remain debatable encompass lipid lowering drug, anti-platelet therapy, aspirin therapy and revascularization (Mora and Manson, 2016; Yuksel *et al.*, 2018; Kouhpeikar *et al.*, 2020; Reale *et al.*, 2022).

Lifestyle Interventions

Lifestyle interventions basically furnish fundamental form of therapy. Prominent advantage is targeting multiple risk factors effective at any stage of the pathogenicity. Abstinence from tobacco, alcohol and smoking remains imperative accompanying indulgence in intake of healthy diet as a routine. Along with correcting lipid ratios and lowering obesity, a healthy diet also alters gut microbiota and prevents them from producing metabolites harmful to the vasculature. According to a study, Smoking cessation remains the most clinically effective and cost-effective strategy for the prevention of the disease (Adamson *et al.*, 2022). Effective management of diabetes mellitus, blood pressure reduces the risk of microvascular complications and macrovascular disease.

Lipid lowering pharmacological therapy

Lipid lowering treatment remains gold standard for atherosclerotic management. Epidemiological, genetic and mendelian randomized studies along with randomized clinical trials have shown LDL to be a causal risk factor and hence it being a therapeutic target holds vital value. Several patient studies have shown improvement in CVDs, post lipid lowering treatment. Substantial records of safety have been accumulated for the use of statins with rare serious complications. Cumulatively supporting the current concept of lipid lowering by targeting LDL cholesterol (Navarese *et al.*, 2018). The intensity of lipid lowering treatment depend highly on individual's patient history and response to the prescribed therapy. Broadly, pharmacological treatment should start with a statin. Non-HDL represents the cholesterol in all atherogenic particles. However, in some cases discordance between LDL and non-HDL concentration might exist in insulin resistant states in which non-HDL might predict the disease better than LDL cholesterol. Thus, having reached the desired LDL goal, non-HDL cholesterol should serve as a secondary target for treatment, especially in individuals with diabetes mellitus.

Statins

Statin acts by inhibiting the functioning of 3-hydroxy-3-methylglutaryl- CoA reductase (HMG-CoAR), the rate limiting enzyme in cholesterol synthesis. Several randomized clinical trials have confirmed the decreased CVD events on statin mediated lowering of LDL. A meta-analysis from statin-based trials shows that the treatment with a statin is related to a log-linear reduction of 22%, in the risk of major CVD events per millimole per litre reduction in LDL. Statins at highest dose shall attain the treatment goal, if not

so a combinatorial therapy with statin and a non-statin drug is better employed (Gencer *et al.*, 2020).

Non-statin lipid lowering drug

Statins unarguably remain the first preferential theraputant against atherosclerosis, however in an un-met need situation other options ought to be explored. An early clinical trial has shown bile-acid sequestrants as an LDL lowering agent, however owing to gastrointestinal adverse effects, drug interactions and elevated TGs limit their widespread use (Wanis *et al.*, 2020). Ezetimibe inhibits cholesterol absorption by enterocytes and augment expression of liver LDL receptors. In combination with statins, Ezetimibe reduces LDL by 15-20% (Boutari *et al.*, 2021). PCSK9 binds to LDL-R and promote their intracellular degradation. Their inhibition can be used in combination with statins, recommended for high-risk patients. Studies with anti-PCSK9 monoclonal antibodies have shown reduction up to 60% in LDL (Ginsberg *et al.*, 2018). Further, the use of omega-3 fatty acid supplements in combating CVD had shown controversial results in past. However, recently a clinical trial has shown that among statin treated patients with elevated TGs the risk of ischaemic events and cardiovascular death was significantly reduced on receipts of 2g icosapent ethyl twice daily (Bhatt *et al.*, 2019).

Antiplatelet and Anti-inflammatory drugs

Platelets play a vital role in atherosclerosis associated thrombosis. Anti-platelet therapy does not fall under routine treatment of atherosclerosis pertaining to elevated chances

of bleeding. However, there are secondary preventions that exceed the bleeding hazard. Inflammation is preliminary response that pertains throughout since pathogenesis of the disease. Anti-inflammatory drugs have been tested in different clinical trials of their therapeutic potency. Anti-IL-1β antibody canakinumab is shown to reduce major adverse cardiovascular events by almost 15% in patients after myocardial infraction (Thompson and Nidorf, 2018). However, in an investigative analysis, incidences of fatal lung cancer also fell by about 77% in those treated with canakinumab (Schieker *et al.*, 2020). Further, studies also used colchicine as an alternative anti-inflammatory drug. Likewise, methotrexate was also utilized as a theraputants against CVDs. However, these treatments did not lower any biomarkers of inflammation (CRP, IL-1β or IL-6).

Gasotransmitters as therapeutants

Vasculature is highly responsive to endogenous gasotransmitters and some of the vital functions including vasodilation and vasoconstriction are under regulation of these gasotransmitters. Systemically there are three known gasotransmitters, Hydrogen Sulphide (H_2S), Carbon Monoxide (CO) and Nitrogen oxide (NO). Recent studies have shown therapeutic potential of these gases in atherogenic pathology. H_2S facilitates vascular dilation by forming polysufides (H_2S_n) and activating TRPA1 channel (Miyamoto *et al.*, 2017). H_2S is also studied to upregulate SIRT1 to exert some of its protective effects. Viz. H_2S upregulated SIRT1 to inhibit endoplasmic reticulum stress in PC12 cells and to prevent H_2O_2 -induced or nicotinamide-induced senescence in HUVECs (Suo *et al.*, 2013; Li *et al.*, 2014b; Zheng *et al.*, 2014). Moreover, H_2S is also studied for improving mitochondrial functions. Apart from that NO has also been

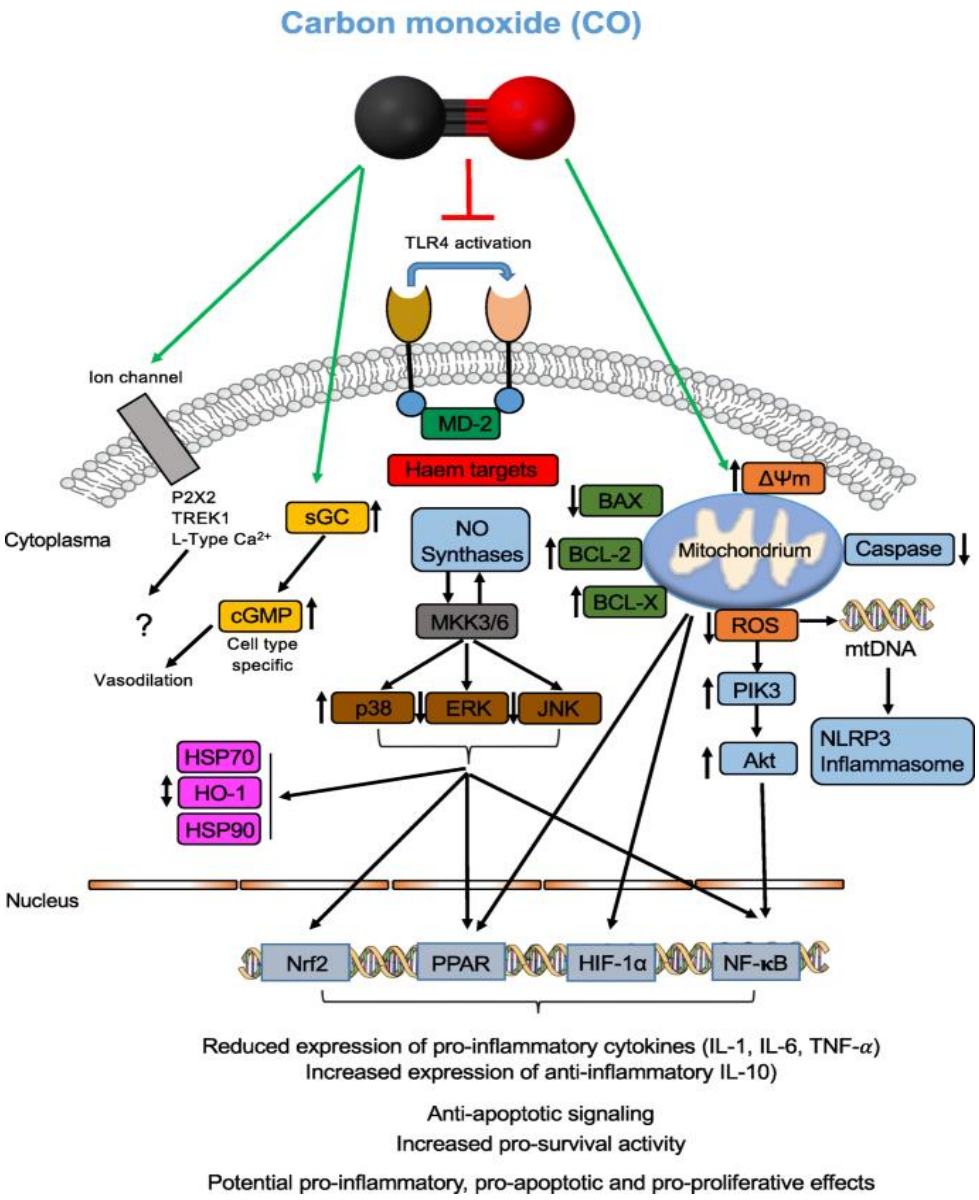
studied broadly for its vasorelaxing properties, vital in scenario of plaque obstructing arterial lumen. NO releasing drugs have been used to for improving various aspects of atherosclerosis viz. Nitroglycerin and sodium nitroprusside have been found to specifically inhibit platelet interaction with damaged coronary arteries (Folts *et al.*, 1991). S-nitroso-N-acetylpenicillamine, (SNAP), widely studied nitrosothiol, has been found to inhibit macrophage dependent oxidation of LDL (Hogg *et al.*, 1995) and to completely reverse endothelin-1 mediated constriction in human arteries (Wiley and Davenport, 2000). In recent years CO has also come to light as a therapeutic agent for atherosclerosis. Delimited concentrations of CO have been proven to have therapeutic potential. CO is studied to modulates the TLR signaling pathway by inhibiting the translocation of TLR to lipid rafts through the suppression of NADPH oxidase-dependent ROS generation (Nakahira *et al.*, 2006). CO mediates the anti-apoptotic action in the intrinsic pathway is the prevention of the association of Bid and Bax, which are pro-apoptotic members of the Bcl-2 family at the outer mitochondrial membrane. CO inhibits the expression and mitochondrial translocation of Bax (Wang *et al.*, 2007). CO has also been studied to be beneficial for hypertensive patients, it can be an adjunctive therapy in acute myocardial infarction, as an antiapoptotic agent in reperfusion injury etc. CO has been shown to have wide implications in cardiovascular disorders (Fig 12). Moreover, a sophisticated class of molecules have been identified for slow and sustained release of CO, Carbon Monoxide Releasing Molecules (CORMs). CORMs are extensively being studied for their therapeutic potential in wide range of pathological conditions and are claimed to have promising therapeutic value.

Carbon Monoxide and physiological entrainments

Carbon monoxide is a stable oxide of carbon that is produced when there is partial oxidation of carbon-containing compounds. It was discovered in the 1960s that CO can be endogenously produced in the body by heme oxygenase (HO) metabolism of heme to produce CO, iron, and biliverdin. Heme Oxygenase-1 (HO-1) catalyses the reaction and is also the rate limiting enzyme. CO has colloquially gained a bad connotation as a poisonous gas attributed to its irreversible heme-binding property. Excessive amount of CO competitively binds to heme forming CO-Hb, impending HbO_2 formation that turn out to be fatal in extreme cases. It was later studied and understood that CO not only had vital physiological properties but also therapeutic potential that was solely attributed to the 'concentration of CO'.

Physiologically CO is instrumental in several processes such as anti-inflammatory (Goebel *et al.*, 2008), anti-thrombotic (Kramkowski *et al.*, 2012), anti-oxidative (Brugger *et al.*, 2010), anti-proliferative (Song *et al.*, 2004), anti-apoptotic (Schallner *et al.*, 2012), anti-atherosclerotic (Ishikawa, 2003), neuroprotective (Stifter *et al.*, 2017), and vasodilative (McRae *et al.*, 2019). Of note, CO may dose-dependently exert certain "pro"-effects: pro-inflammatory, pro-apoptotic, and pro-proliferative, depending on the individual setting (Tamion *et al.*, 1999; Almeida *et al.*, 2016; Wu *et al.*, 2019). Fredenburgh and colleagues extended their previous research in baboons and published the first-in-man study regarding the effects of inhaled CO in sepsis induced ARDS patients (NCT 02425579) (Fredenburgh *et al.*, 2018). In this placebo-controlled clinical trial the patients received treatment with 100 or 200 ppm inhaled CO respectively that aimed to achieve an algorithm-specified CO-Hb of 6–8%. The authors successfully demonstrated safe CO-inhalation in their setting and that CO-Hb may not only be measured but can be predicted accurately using the Coburn-Forster-Kane

equation. Moreover, the author found a significant reduction in mtDNA, while there was no reduction in any other biomarker (IL-18 or RIPK3). In spite, of the small cohort in the phase-1 trial, there were no statistical differences in PaO2/FiO2 ratio, the oxygenation index, lung injury score, lactate, or the SOFA score. These results are most promising and show not only the feasibility and safety of inhaled CO, but the translation of pre-clinical data into a relevant clinical setting. For sure, further studies are needed to confirm these results in a larger population.



Goebel et al, 2020

Fig 12a: Schematic representation of biological pathways altered by carbon monoxide.

Carbon monoxide and Cardiovascular disorders

CO is instrumental in critical physiological function of vasorelaxation by mainly activating large-conductance calcium-activated potassium channel (BKCa channel) and partly by activating soluble guanylyl cyclase in smooth muscle cells. CO dilates the arteries and arterioles by binding to tetrameric voltage-gated big potassium channels that conduct large amounts of potassium ions (K+) across the cell membrane in smooth muscle cells of the blood vessel (Yuan *et al.*, 2010). The vasorelaxation activity of CO is mediated by binding with the α-subunit of BKCa channels and activation of the channels. CO may be one of the solutions in ischemic injury resulting from the circulatory insufficiency caused by unwanted vascular constriction. For example, vasodilatory action could be helpful in acute coronary syndrome, especially in variant angina, by dilating coronary arteries. CO may also increase the renal blood flow, glomerular filtration rate and decrease the renal vascular resistance in acute kidney injury by dilating the renal artery and arteriole. Moreover, in pulmonary hypertension with or without heart failure, CO could be a potential solution to enable the release of the pressure in pulmonary and systemic vasculature.

Carbon monoxide and mitochondrial dysfunction

CO is also cytoprotective in nature pertaining to its function against both intrinsic and extrinsic apoptotic pathways (Elmore, 2007). Apoptosis initiated by the intrinsic pathway is related to mitochondrial membrane permeability (Queiroga *et al.*, 2012). Mitochondrial membrane permeabilization leads to the process of irreversible programmed cell death via the following mechanisms: (1) the extinction of mitochondrial transmembrane potential; (2) the uncoupling of oxidative phosphorylation; (3) excessive production of reactive oxygen species; (4) cessation of

ATP synthesis; and (5) the release of pro-apoptotic proteins (Kroemer *et al.*, 2007). The main mechanism by which CO mediates the anti-apoptotic action in the intrinsic pathway is the prevention of the association of Bid and Bax, which are pro-apoptotic members of the Bcl-2 family at the outer mitochondrial membrane. CO inhibits the expression and mitochondrial translocation of Bax (Wang *et al.*, 2007). CO also prevents the activation of Bid by inhibiting the activity of caspase-8, which activates Bid by cleavage to truncated Bid (tBid) (Kantari and Walczak, 2011). The activated tBid is a direct activator and inducer of Bax, causing a conformational change that enables Bax oligomerization and insertion into the outer mitochondrial membrane (Desagher *et al.*, 1999; Eskes *et al.*, 2000). CO is also shown to operate by other mechanisms in the extrinsic pathway, such as the activation of the p38 mitogen-activated protein kinase signaling pathway and NK-kB upregulation; the resultant FADD-like ICE inhibitory protein activation inhibits the TNF-α/Act-D induced caspases-8 cleavage (Brouard *et al.*, 2000; Kim *et al.*, 2006).

Furthermore, CO could promote the uncoupling of mitochondrial respiration and modulate the production of reactive oxygen species (ROS). In mitochondrial oxidative phosphorylation, 1–3% of the consumed oxygen is incompletely reduced to anion superoxide, which is the primary ROS produced by the electron transport chain, and this generation of a moderate free radical can lead to a more reactive or secondary ROS derivative, even under physiological conditions (Queiroga *et al.*, 2012). Under pathological conditions, the reversion of electron flow might result in persistent and augmented generation of ROS; thus, mild mitochondrial uncoupling is an inherent cellular mechanism to limit excessive oxidative stress. The uncoupling of mitochondrial respiration could be activated by CO through the stimulation of the activity of uncoupling proteins and/or the ATP/ADP translocator, which play an important role in

uncoupling reactions at a low level of CO (Iacono *et al.*, 2011). This activation of uncoupling in mitochondrial respiration may prevent the excessive generation of ROS and consequently indicate the anti-oxidant properties of CO.

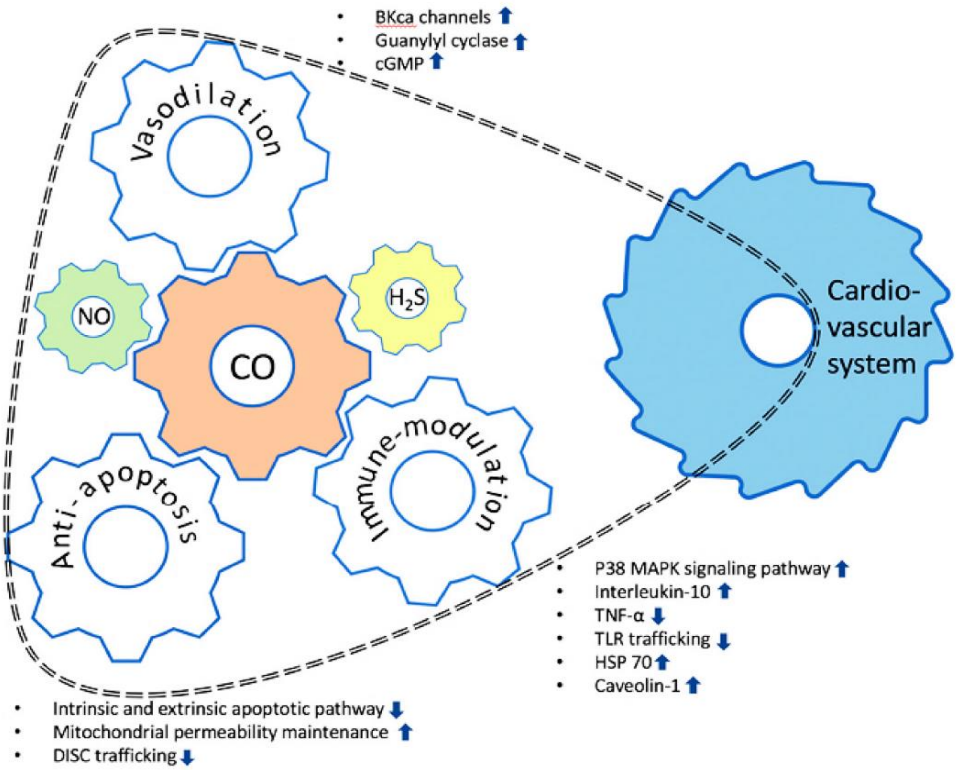


Fig. 12b: Schematic diagram of the effects of carbon monoxide on the cardiovascular system. Carbon monoxide (CO) can influence the cardiovascular system through (1) the vasodilatory effect, (2) anti-apoptotic activity, and (3) immune-modulation mechanisms. The roles of CO in the cardiovascular system may be executed in conjunction with other gasotransmitters, such as nitric oxide (NO) and hydrogen sulfide (H_2S). The possibility of action on the cardiovascular system is represented by the dotted line. The upward pointing arrow and the downward pointing arrow mean activation and inhibition for each pathway, respectively (Kim and Choi, 2018).

Novel therapeutic strategies

Classical cornerstone therapies of lipid lowering, targeting inflammation and hypertriglyceridemia are being constantly evolved. However, novel therapeutants have come to light with their unique mode of action and physiological targets. In recent decades there has been a dramatic increase in studying messenger molecules that are functionally active in CVDs to hypoxia and ischemia/reperfusion. Along with gasotransmitters other type of molecules that have come to light in past decade was non-coding RNAs. Non-coding RNAs are specific class of RNAs that are not functional in protein translation, on contrary these are functionally active based on their physiological type. These RNAs include miRNAs, lncRNAs, siRNAs, snoRNA, snRNA, exRNA etc. Each of these non-coding RNA has its unique mode of action and physiological importance. Epigenetic modifications in atherogenic pathology are gaining attention with emerging genomic approaches that profile DNA methylation, chromatin accessibility, post-translational modifications, post-transcriptional modifications low or single cell populations are poised to enhance our spatiotemporal understanding of atherogenesis. miRNAs are being widely studied for their utilization not only as theraputants but also as biomarkers in regard to various pathologies.

Micro RNAs

micro RNAs (miRNAs) were first discovered in 1993 by Ambros and Ruvkun group in C. elegans. miRNAs are small non-coding RNAs of about 20-22 nucleotides. miRNAs mostly are transcribed from DNA into primary miRNA (Pri-RNA) and processed further into precursor miRNA (Pre-miRNAs) and mature miRNAs. Majorly, miRNAs interact with 3'UTR of target mRNA to suppress its translation (Treiber *et al.*, 2012).

However, miRNAs are also devised to interact with other regions including the 5′ UTR, coding sequence, and gene promoters. miRNAs have also been shown to activate gene expression in some conditions. Recent studies have shown that miRNAs can also shuttled between different subcellular compartments to control the rate of translation and even transcription in some cases (O'Brien *et al.*, 2018). miRNAs are involved in several biological processes and their aberrant expression has been associated with manifestation of several pathological conditions. miRNAs have also been documented for their signalling role and are shown to be secreted in extracellular matrix and in circulation. Recent studies vouch for their potential role as biomarkers and/or therapeutic target in several sever pathologies.

miRNA Biogenesis

miRNA biogenesis initiates with the processing of RNA polymerase II/III, either post-transcriptionally or co-transcriptionally. Broadly miRNAs are classified into two categories; (i) intragenic miRNAs, that are processed mostly from the introns and relatively few exons of the protein coding gene. This class comprises of about half of all the known miRNAs. (ii) the other is intergenic miRNAs that are transcribed independent of the host gene and are regulated by their own promoters. Couple of miRNAs are transcribed as one long transcript and are called clusters that might have similar seed region and are called *family*. miRNA biogenesis is classified into (i) Canonical and (ii) Noncanonical pathways (Fig. 13) (O'Brien *et al.*, 2018).

Canonical pathway

The canonical biogenesis pathway is dominant pathway by which miRNAs are majorly processed. Herein, pri-miRNAs are transcribed from their genes and processed into pre-miRNAs by microprocessor complex, DiGeorge Syndrome Critical Region 8 (DGCR8), an RNA binding protein and ribonuclease III enzyme and Drosha. DGCR8 identifies N6-methyladenylated CGAC and other motifs in pri-miRNA and Drosha cleaves the pri-miRNA duplex at the base of hairpin structure resulting into two nucleoide 3'overhang on pre-miRNA. On generation, these pre-miRNAs are exported to cytoplasm by exportin5 (XPO5)/RanGTP complex and further processed by Dicer (RNase III endonuclease) to generate mature miRNA. The directionality of the miRNA strand determines the name of the mature miRNA form; 5p strand arises from the 5′ end of the pre-miRNA hairpin and the 3p strand originates from the 3′ end. Both these strands can further be loaded into Argonaute (AGO) protein in ATP dependent manner. AGO loaded 3p or 5p strand highly depends on the type of miRNA and cellular environment. In case of predominant loading, that usually takes place based on thermodynamic stability at 5' end of the miRNA, the strand forming AGO complex is deemed as guide strand of miRNA. The other one is referred to as passenger strand that is unwound from the guide strand through various mechanisms based on the degree of complementarity. Passenger strands with no mismatch are cleaved by AGO2 and degraded by cellular machinery producing a strong strand bias.

Non-canonical Pathway

Multiple non-canonical miRNA biogenesis pathways have been elucidated till date. These pathways are different in combinatorial usage of proteins involved in canonical

pathways including Drosha, Dicer, Exportin5 and AGO2. Broadly, non-canonical pathways can be groups as Drosha/DGCR8 independent or Dicer independent pathways. Mirtrons is type of miRNA processed by Drosha/DGCR8 independent pathway, produced from the introns of mRNA during splicing. Secondly, there is 7-methylguanosine (m7G)-capped pre-miRNA, these nascent RNAs are directly exported to the cytoplasm through exportin 1 without the need for Drosha cleavage. On the other hand, Dicer-independent miRNAs are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts. AGO2 complex is required to complete their maturation within the cytoplasm owing to their insufficient length as Dicer-substrates. This in turn promotes loading of the entire pre-miRNA into AGO2 and AGO2-dependent slicing of the 3p strand and the 3′ -5′ trimming of the 5p strand completes their maturation (Havens *et al.*, 2012).

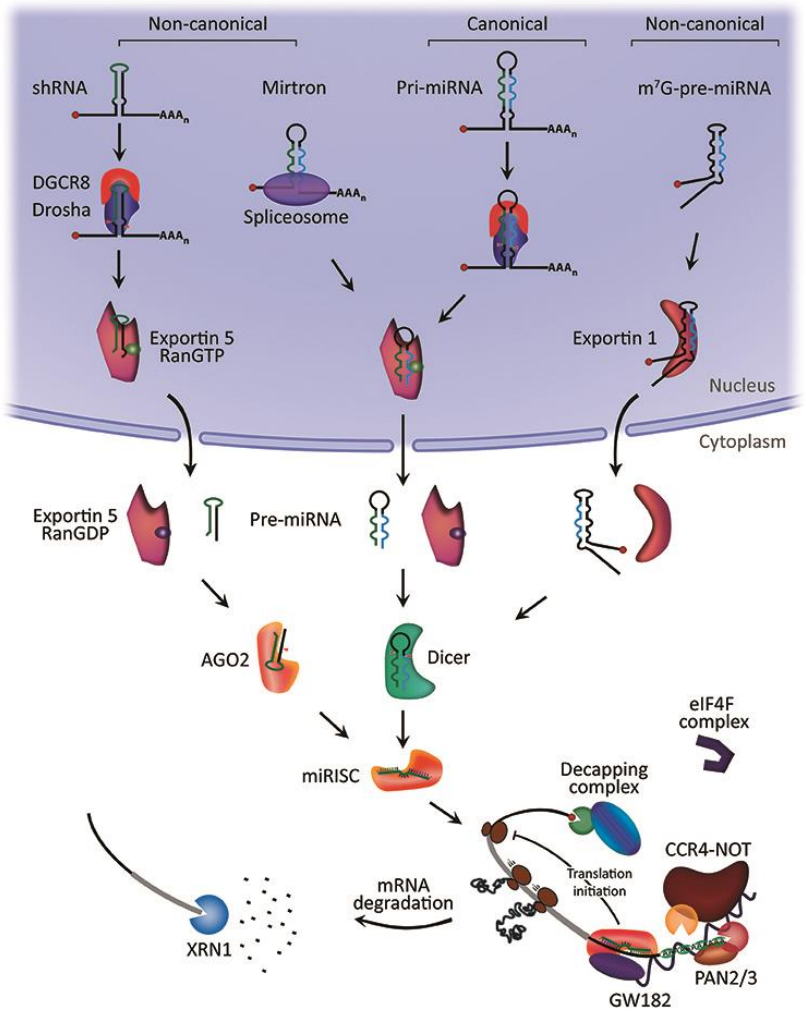


Fig. 13: Pictorial representation of canonical and non-canonical miRNA biogenesis pathways operating in biological systems (O'Brien *et al.*, 2018).

Regulation by miRNAs

Overwhelming number of studies have shown that miRNAs bind to complementary seed sequence in the 3'UTR of their target mRNA induce translational repression and mRNA deadenylation and decapping. However, miRNA binding sites have also been identified in other region of mRNA, including 5'UTR and coding sequence as well as in the promoter region. Site specific binding of miRNA orchestrates different effects

viz. miRNA binding to 5'UTR and coding region have silencing effects on gene expression whereas miRNA interactions with promoter region induces transcription. However, these interactions and consequential activities still warrants further investigation. Systemic miRNA functions are extremely robust. The contributing factors to this robustness of miRNA mediated gene regulation includes functionalized compartmentalization and shuttling of miRISC within the cells. Herein, the availability and abundancy of miRNAs and their target mRNAs are also contributing factors in determining which genes are regulated. Studies have also shown that miRNA suppression of mRNA targets is not ubiquitous between cell types. Alternative splicing and alternative polyadenylation effect 3'UTR and cell type specific RNA binding proteins that further affects target mRNA secondary structures to change the available pool of MREs. This renders subsets of mRNAs sensitive or insensitive to miRNA-mediated gene regulation in a cell type/state-specific manner (Huang *et al.*, 2020).

miRNAs in atherosclerosis

miRNAs have been implicated in several key physiological processes including cell survival, cell apoptosis, differentiation, and various other metabolic processes. Atherogenic pathophysiology is multifactorial and involves operation of several signalling pathways right from initiation up to progression of the pathogenicity. Studies have shown implications of miRNAs in these metabolic pathways altering the pathogenicity. Present day miRNAs are being considered as important regulators in atherosclerosis and their aberrant expression is associated with initiation and progression of the disease. miRNAs are reported to regulate cell migration, differentiation, lipid flux regulation, cytokine production etc in atherosclerosis.

Pertaining to wide spectrum of implications, miRNA have also been proposed as biomarker in diagnosis, prognosis and even therapy (Schober *et al.*, 2015; Torres *et al.*, 2019; Huang *et al.*, 2020; Sun *et al.*, 2020).

miRNAs act as both inter- and intra- cellular molecules that potentially alters endothelial cell profile. Initiation of atherosclerosis is marked by endothelial cell dysfunction. Preliminary response to EC dysfunction in atherogenic pathogenicity is expression of adhesion molecules intracellular adhesion molecule (ICAM)-1, VCAM-1, and E-selectin facilitates the recruitment and migration of leukocyte to the marginal area of vessel. miR-17-3p, miR-31, and miR-126 have been reported to modulate inflammation by regulating the adhesion molecules (Suárez *et al.*, 2010). miR-146a has been reported to destabilize the plaque and modulate inflammation by activating nuclear factor (NF)-κB signal-transduction pathway that is also studies to be modulated by miR-10 (Guo *et al.*, 2010). miR-126-5p has been shown to orchestrate protective impact on ECs by inhibiting Notch1 inhibitor delta like 1 homolog (Dlt1) (Du *et al.*, 2022). Further, miR-223 has been reported to inhibit cholesterol biosynthesis and control HDL uptake (Vickers *et al.*, 2014). Likewise, several miRNAs such as miR-758, miR-302a, miR-301b, miR148a, miR-130b, miR-128-1, miR-26, miR-106, miR-33 and miR-144 have been shown to regulate foam cell formation and lipid uptake in macrophages and thus regulate second major event of atherosclerosis (Rotllan *et al.*, 2016). miRNAs have also been reported in regulating vascular smooth muscle cells. Viz. miR-221 and miR-222 are shown to be involved in VSMC proliferation (Liu *et al.*, 2009). miR-21 elevates BCL2 by targeting PTEN and promotes proliferation of VSMCs (Jazbutyte and Thum, 2010). Likewise, several miRNAs have been listed below in the table with their specific target and potential function (Fig. 14; Table 3).

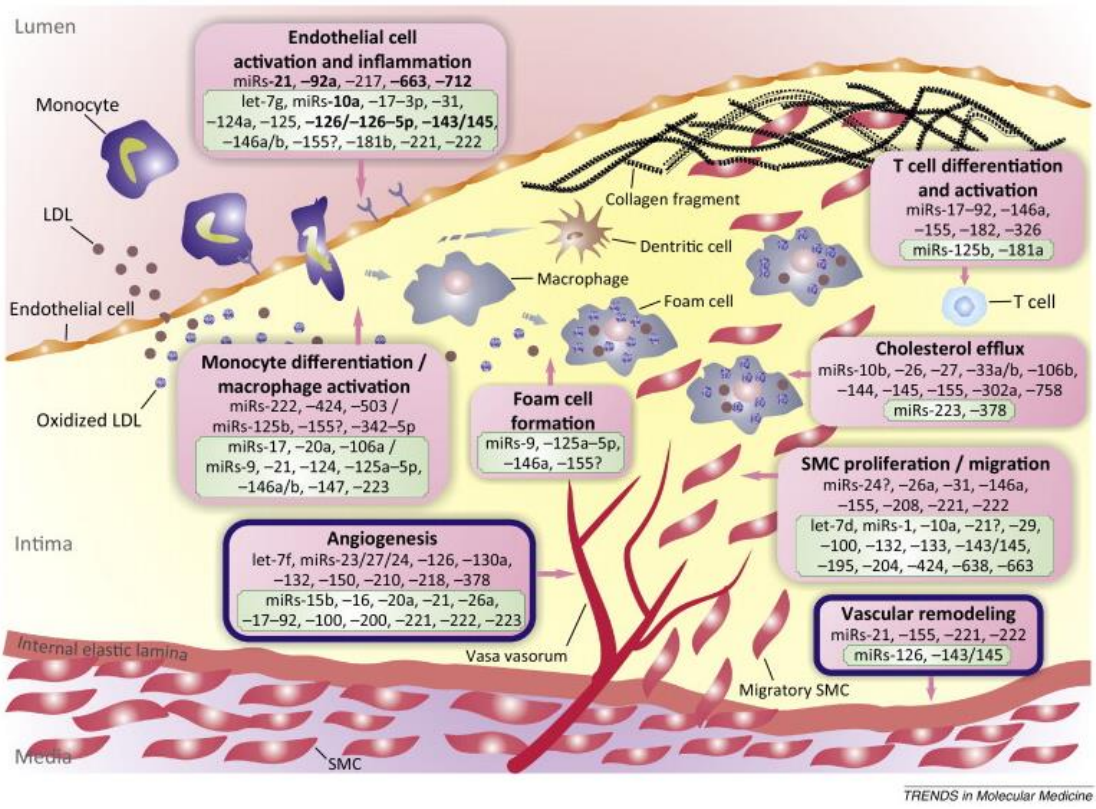


Fig. 14: Schematic representation of various miRNAs functional in different cells in atherogenic milieu (Andreou *et al.*, 2015).

Table 3: List of some of the miRNAs reported to be functional in atherogenic pathophysiology.

Cells	miRNAs	Target	Function
Endothelial cells	miR-17-3p	ICAM-1	Migration and inflammation
	miR-31	E-selectin	
	miR-126	VCAM-1	
	miR-146a	NF-κB signaling pathway, MAP kinase pathway, and HuR	Plaque destabilization
	miR-10	NF-κB signaling pathway	Inflammation
	miR-126	Notch1 inhibitor delta-like 1 homolog (Dlk1) PI3K pathway	Lesion formation Angiogenesis and proliferation
	miR-223	Associated genes with lipid and cholesterol metabolism	Cholesterol biosynthesis, and metabolism
	miR-26a	TRPC6	Cell death
Monocyte and macrophage	miR-124a	CCL2	Migration
	miR-106a miR-20a miR-17	Signal-regulatory protein α	Inflammatory responses
	miR-145	KLF4, angiotensin-converting enzyme, and actin polymerization	VSMC differentiation and plaque stability
	miR-223	CXCL2	Infiltration
	miR-342-5p	Akt pathway	Activation of inflammatory macrophages
	miR-146a	TLR4	Cytokine release

	miR-125a-5p	Oxysterol binding protein-like 9	Lipid uptake
	miR-155	HBP1	Formation of macrophage foam cell
	miR-758 miR-302a miR-301b miR-130b miR-128-1 miR-106 miR-33	ABCA1	Cholesterol efflux and formation of macrophage foam cells
	miR-148a	ABCA1 and LDL-R	Cholesterol efflux and formation of macrophage foam cells
	miR-144	Nuclear Receptor FXR	M1 & M2 polarization
	miR-223	Pknox1	
	miR-155	SOCS1	
	miR-19a	Fra-1	
	miR-33	Aldh1a2	
	miR-let7a	C/EBP-δ	
	miR-125a	KLF13	
	miR-21	STAT3	
	miR-214	β-catenin	
	miR-27a	sprouty2	
	miR-146a	Akt2	
	miR-124	CXCL1	

Vascular Smooth muscle cells	**miR-21**	**PTEN**	**High proliferation and low apoptosis**
	miR-221 miR-222	p57(Kip2) and p27(Kip1)	Proliferation
	miR-143	KLF5 and ELK-1	Differentiation

Functionally miRNAs are now ventured into as potential biomarkers or therapeutic targets. Various miRNA-based therapies are in preclinical stages and two of them are in clinical trial, but these are against non-atherogenic pathogenesis. One is miR-122 against hepatitis C virus, Miravirsen and the other is mimic of miR-34, MRX34, that stimulates antitumor responses in several oncogenic pathways. However, when it comes to miRNA in atherosclerosis, the research is still at its infancy but confers a promising future as potent biomarker in diagnosis, prognosis, and therapy.