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An Update on Myocardial Infarction

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Abstract

Myocardial infarction is described as a type of acute coronary syndrome caused by pathological event in the setting of myocardial ischemia with the marked cardiac insult and injury. The diagnosis is secured when there is a rise and/or fall of cardiac troponin levels along with supportive evidence in the form of typical symptoms, suggestive electrocardiographic changes, or imaging evidence of new loss of viable myocardium or new regional wall motion abnormality. The early rehabilitation for acute Myocardial infarction is directed towards reinstatement of perfusion as soon as possible to retrieve as much of the jeopardized myocardium as possible. This may be accomplished through medical or mechanical means, such as percutaneous coronary intervention, or coronary artery bypass graft surgery. The objectives of medical therapy for myocardial infarction are to decrease morbidity and to avert complications. The main targets of emergency department pharmacotherapy are rapid intravenous thrombolysis and/or rapid referral for percutaneous coronary intervention (PCI), optimization of oxygenation, reduction of cardiac workload, and pain control.

Keywords: Myocardial infarction; prognosis; cardiac biomarkers

Introduction

Myocardial infarction is particularly the utmost severe manifestation of coronary heart disease and it is a final outcome of either acute or chronic myocardial ischemia that results due to a mismatch in oxygen demand and oxygen supply. Myocardial infarction leads to myocardial injury or necrosis which is characterized by a rise in cardiac biomarkers, along with supporting clinical evidence conforming electrocardiogram changes, or imaging validation of new injury of viable myocardium or acute abnormality in regional wall motion [1]. The clinical manifestations of myocardial infarction include severe and sustained chest pain, often together with breathlessness, nausea, and sweating. Myocardial infarction causes ischemic attacks, angina,

severe to severe life-threatening arrhythmias and congestive heart failure as complications. Thus, clinical doubt of myocardial infarction should lead to aggressive treatment depending on the severity and type of infarction as well as supportive care and employing secondary prevention strategies. Prognosis mainly depends on the severity of clinical signs observed, the presence of concurrent disease if any, and response to initial therapy by the patient [2].

Definition

Myocardial infarction (i.e., heart attack) is defined "pathologically as myocardial cell death due to prolonged ischemia. Diminished cellular glycogen, and relaxed myofibrils and sarcolemmal disruption which are the first ultrastructural changes followed by mitochondrial abnormalities". The clinical definition of Myocardial infarction denotes the presence of acute myocardial injury detected by abnormal cardiac biomarkers in the setting of evidence of acute myocardial ischemia [3].

Epidemiology

Globally, cardiovascular diseases are the foremost cause of mortality and myocardial infarction being the key component of CVD. Worldwide, CVD led to 17.9 million deaths in 2016 signifying 31% of total global deaths. It is expected that by 2030 due to CVD 2,36,00,000 individuals will expire. More than 75% of CVD deaths occurred in developing countries. Of this 82 % of CVD deaths occurred in low- and middle-income countries [4]. The South Asian countries of India, Pakistan, Nepal, Bangladesh, and Srilanka accord to the utmost incidence of CVDs. In developed countries, the prevalence of age-specific myocardial infarction is seen more in older above 75 years compared to younger below 45 years whereas the contrast of this i.e., the highest prevalence of myocardial infarction seen in younger than 45 years of age compared to those elder than 60 years in South Asian counties [5]. Sex-wise prevalence of myocardial infarction in men was about three times greater than for women. The Global Burden of Disease survey estimated that the age-standardized CVD death rate of 272 per 100000 people in India, which is higher than the global average of 235 per 100000 people [6].

The prevalence of cardiovascular disease in India has increased over the past 2 decades owing to population growth, aging, and a stable age-adjusted cardiovascular disease mortality rate. The Age-Standardized Prevalence of cardiovascular disease per 100,000 was 5450 during 1990 in India where as in 2016 Age-Standardized Prevalence of cardiovascular disease per 100,000 was found to be 5681. The estimated number of prevalent cases of cardiovascular disease in 1990 was 25.6 million whereas there was two-fold increase in

estimated number of prevalent cases of cardiovascular disease in 2016 to 54.6 million. Death rates due to cardiovascular diseases in India are projected to have increased from 155.7 to 209.1 per 100,000 amid 1990 and 2016, though this number appears to be almost entirely owing to population aging. There is considerable state-level variability in the burden of cardiovascular disease in India, including a 9-fold dissimilarity in the burden of ischemic heart disease disability-adjusted life years per 100,000 persons between the states with the highest in Punjab and lowest in Mizoram burdens. Reasons for state-level alterations in mortality and morbidity are innumerable, and are likely driven by modifications in risk factor burden, treatment, control, management of acute manifestations of cardiovascular disease, and, conceivably, baseline event rates [7].

Pathological Characteristics

The pathological hallmark feature of myocardial infarction is small intramural foci of coagulative necrosis and perinuclear edema in the cardiac myocytes as well as swollen mitochondria and dilated sarcoplasmic reticulum. Severe mitochondrial changes, dehiscence of the intercalated disc, lamellar degeneration and granular degeneration were also observed [8]. Apoptosis (cellular and organellar shrinkage) and oncosis (cellular and organellar swelling) injury are seen in infracted myocytes. Apoptosis mainly occurs due to activation of caspase enzyme cascade and oncosis occurs due to progressive myocardial membrane dysfunction and damage from infraction, which leads to decrease in adenosine triphosphate (ATP) levels thereby causing energy depletion [9].

The histologic findings of Myocardial Infarction are observed Within 60 seconds of the onset of myocardial ischemia there will be loss of myocardial contractility and in 20 to 40 minutes loss of viability (irreversible injury) occurs due to coronary occlusion of blood flow. Within 12 to 24 hours Hyper-eosinophilia of myocytes cytoplasm and some chromatin condensation in the nucleus is observed followed by neutrophil infiltration at the border areas within 24 hours. In between 24 to 48 hours of infracted period coagulation necrosis is documented with some nuclear pyknosis, early karyorrhexis, and karyolysis. In this period elongation of sarcomeres are noted. In 3 to 4 days central portion of the infarct shows loss of myocytes nuclei and striations in smaller infracts. Infiltration and fragmentation of neutrophils are seen within infract, followed by an influx of inflammatory cells including mast cells, macrophages, and fibroblasts in the border areas of the myocardium. Myocardial recovery occurs within 4 to 6 hours if reperfusion takes place after the onset of angina or electrocardiographic changes in the myocardium, and

subendocardial without transmural extension is seen in the myocardial infarct [10, 11].

Presentation

The classic symptom of myocardial infarction is typically intense and unremitting precordial chest pressure/pain. The intense instinctual chest pain is retrosternal and frequently transmits up-to shoulder, spine, jaws, then towards left-arm and right-arm. The chest pain is usually designated as a crushing substernal pain/pressure sensation that is moreover observed as squeezing, sharp, aching or burning. In most of the cases Levine's sign, in which the patients clench their fists held over the sternum to describe ischemic chest pain may be seen [12].

The other symptoms of myocardial infarction include anxiety, nausea, vomiting, lightheadedness (with or without syncope), diaphoresis, and absence of chest-wall tenderness on palpation. Myocardial infarction is more often accompanied by wheezing, dyspnea mainly due to a decrease in circulation to the left ventricle, which causes left ventricular failure and consequently leads to pulmonary edema. In the patients with a confirmed myocardial infarction, women had greater rates of atypical clinical manifestations such as paroxysmal dyspnea, epigastric pain through sensation of acid reflux or congestive heart failure [13].

Classification

Myocardial infarction characterization is done to accommodate the increased use of high-sensitivity cardiac troponin above the 99th percentile upper reference limit includes the following criteria's: [14]

1. Type 1 myocardial-infraction is spontaneous and is instigated through atheroma-thrombotic CAD besides is related to atherosclerosis plaques wearing down and-or split, cleft, or partition in artery of myocardium.
2. Type 2 myocardial-infarction happens because of optional to ischemia which may be because of augmented exigency of oxygen or declined oxygen distribution, e.g., fixed myocardial atherosclerosis, myocardial embolism, coronary artery dissection, respiratory failure, severe anemia, hypotension, etc.,
3. Type 3 myocardial-infarction is associated with abrupt cardiac death, as well as heart failure, is frequently accompanied by signs of coronary ischemia escorted through predictable fresh ECG ischemic alterations either ventricular fibrillation either sign of fresh thrombus

in myocardial artery through angiography either or both at postmortem examination. In this type, demise occurs earlier then serum sample is collected or formerly the releases of myocardial injury markers inside systemic circulation.

4. Type 4 myocardial-infarction is related to myocardial technical cardiac damage correlated with cardiac revascularization procedure of PCI. In this Type 4a myocardial-infarction related by percutaneous coronary intervention. In type 4b be related by scaffold thrombosis or stent related through percutaneous coronary intervention recognized through angiography or by postmortem examination whereas type 4c myocardial-infarction occurs due to restenosis associated with PCI
5. Type 5 myocardial infarction is accompanied by CABG

Based on the ECG supervised classification technique, myocardial infarction is categorized into two types:

1. NSTEMI: Non-ST segment elevation myocardial-infarction, revealing incomplete or sporadic obstruction in blood vessel.
2. STEMI: ST-segment elevation myocardial-infarction or heart attack, usually produced through comprehensive in addition to continuing obstruction in blood vessel

Causes of Myocardial Infarction

Myocardial infarction may occur due to cardiac and non-cardiac ailment which may lead to a mismatch of oxygen demand and supply [15]. Causes of myocardial infarction or ischemia stratified by etiology include the following:

1. Primary myocardial ischemia

- Myocardial artery dissection
- Distal microembolization
- Intraluminal myocardial thrombus
- Atherosclerotic plaque estrangement

2. Imbalance between oxygen reserve or exigency producing cardiac ischemia

- Septic Shock
- Hypovolemic Shock
- Cardiogenic Shock

- Respiratory catastrophe
- Myocardial embolism
- Tachy-arrhythmias
- Aortic valve syndrome
- Hypertrophic cardiomyopathy
- Endothelial dysfunction of myocardium
- Brady-arrhythmia
- Hypertrophy of Left ventricle
- Increased blood pressure
- Myocardial artery vasospasm
- Aortic dissection

3. Damage not associated with cardiac ischemia

- Myocardial contusion
- Myocardial operation
- Ablation
- Cardiotoxic drugs
- Cardioversion
- Cytokine-mediated injury
- Myocarditis
- Pacing
- Rhabdomyolysis

4. Multifactorial or indeterminate etiology

- Acute heart failure and Chronic heart failure
- Burns
- Critical illness
- Infiltrative diseases (Amyloidosis and Sarcoidosis)
- Pulmonary embolism and Pulmonary hypertension
- Acute kidney injury and Chronic kidney disease
- Strenuous exercise
- Takotsubo cardiomyopathy
- Stroke
- Subarachnoid hemorrhage

The major etiological factor responsible for the development of myocardial infarction is atherosclerosis. In 90% of cases of myocardial infarction obstruction of a coronary artery due to rupture and erosion of atherosclerotic plaque leads to development of coronary thrombosis. This coronary thrombosis by the different cascade of mechanisms causes the activation of platelets and its aggregation which in turn activates the coagulation cascade thereby leading to vasoconstriction of endothelium leading to coronary occlusion which decreases the supply of oxygen to myocardial tissue there by leading to myocardial infarction.

Non-modifiable and modifiable risk factors for atherosclerosis as well as Non-atherosclerotic causes of myocardial infarction are enumerated in figure 1. Apart from these causes myocardial infarction can occur due to hypoxia from pulmonary disorders or carbon monoxide poisoning. In addition, childhood myocardial infarction occurs due to uncharacteristic source by left myocardial artery or inflammation of coronary vasculature. In relationship with coronary artery stenosis intrauterine myocardial infarction can occur.

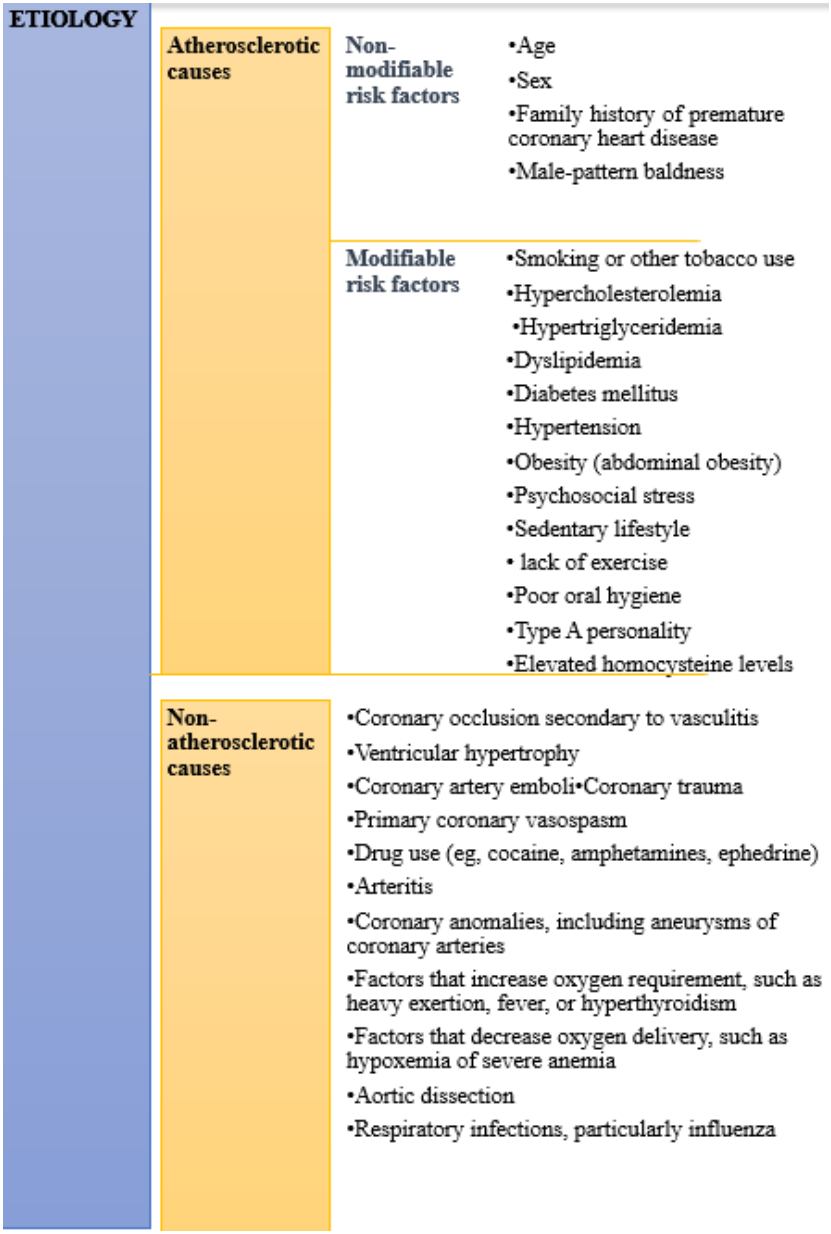

Fig 1: Etiology of myocardial infarction

Diagnosis

A diagnosis of myocardial infarction is done by assimilating the history of the presenting illness through a physical examination with

electrocardiogram (ECG) tracings, cardiac biomarkers and through angiography. According to the fourth universal definition of myocardial infarction, the elevation of cardiac troponin (cTnT) values by a minimum incentive over the ninety-ninth percent higher locus limit is the main diagnostic criteria. Myocardial infarction in a scientific scenery is utmost frequently be recognized through individual's past besides electrocardiogram. The main motives for estimation of biomarkers and ECG tracing or imaging is towards:

- Resolving existence and nonexistence in myocardial-infarction aimed at analysis as well as differential analysis.
- To distinguish the nature and type of myocardial infarction.
- To know the extent of infarct size.
- To characterize the locus of myocardial infarction.
- To distinguish myocardial infarction or recurrent ischemia.
- To identify the early as well as late complications of myocardial infarction
- To determine the prognosis of the patient

Physical examination: Physical examination outcomes for myocardial infarction can vary according to the experienced symptoms; some patients may be calm and comfortable while others may be in distressed and severe pain [16]. But most of the patients with myocardial infarction generally lie quietly with pale and diaphoretic appearance.

- Due to tachycardia secondary to sympathoadrenal release heart rate is usually increased and due to supraventricular arrhythmias, atrial flutter or fibrillation, an augmented idioventricular rhythm, ventricular tachycardia, or ventricular ectopy irregularity in pulse rate can be noted.
- In response to vasoconstriction of peripheral artery consequential from sympathetic retort for anxiety, cramp, spasms, and hypertension due to improper functioning of ventricles can be seen in most of the subjects
- Due to anxiety or pulmonary congestion respiratory rate may be increased
- Within 24-48 hours of ischemic attack increase in body temperature above 102°F may be seen.
- Enlargement of neck veins can be observed when there is

involvement of right ventricle with acute inferior wall myocardial infarction.

- Diminished contractility of compromised left ventricle causes dyskinesis, lateral dislodgment of the apical impulse; a palpable S_4 gallop, as well as a soft sound of S_1, were noted. Due to sliding contact of inflammatory roughened myocardial surfaces, a pericardial friction rub could be audible.
- In Acute left ventricular myocardial infarction, which is secondary to pulmonary venous hypertension wheezing or rales sound can be heard. At lung base, Egophony can be auscultated due to Unilateral or bilateral pleural effusions from extensive acute left ventricular myocardial infarction.
- Often tricuspid incompetence is seen in patients; hepatojugular reflux can be grasped even when hepatomegaly is not manifested.

Electrocardiogram tracing:

The ECG is the first tool for the preliminary assessment and triage of patients in whom myocardial infarction is suspected. In STEMI, a distinctive elevation surpassing one millimeter in two functionally adjoining leads in ST segment of EEG which can be noted within minutes to hours of inception of pain and in first few days' inversion of T waves is observed and followed by the evolution of Q waves in ECG tracing [17]. Whereas in NSTEMI depression of ST-segment pursued by T-wave reversal deprived of the progression of Q waves remain detected. Apart from this different ECG abnormalities can be seen depending on the localization of infarct or ischemia on walls or surfaces of myocardium such as "Posterior wall (R/S ratio above 1 in V_2 besides V_1, then T-wave fluctuations with V_9, V_8, and V_1), Right ventricular (RV_5, RV_4), Anterolateral (V_6 through V_1), Anteroseptal (V_3 through V_1), Lateral wall (aVL, V_4 through V_6, I) and Inferior wall (aVF, II, III,)" [18].

Cardiac Biomarkers:

Different cardiac biomarkers or cardiac enzymes have conventionally continued to be utilized for assessment and diagnose of acute myocardial-infarction patient. Cardiac biomarkers are the proteins that are released from the damaged myocardial cells through their injured cell membranes into the circulation. Until the 1980s, the elevation of enzymes aspartate aminotransferase and lactate dehydrogenase were used to evaluate the myocardial injury [16] (Rashid et al., 2019). Raise of muscle/brain isoenzyme of creatine kinase, creatine kinase, myocardial-specific troponins I and T, and myoglobin occurs in all patients with myocardial infarction and necrosis. B-

type natriuretic peptide which is a biomarker for heart failure is produced mainly by the ventricular myocardium in retort to ventricular wall stress is now used in hazard identification besides prediction of individuals by severe myocardial infarction early next to an acute coronary event. Glycogen phosphorylase isoenzyme BB which is elevated within 1 to 3 hours after myocardial ischemic attack is a new cardiac biomarker which is considered to improve timely diagnosis of an acute coronary event [19]. Another biomarker for detecting ischemia is Ischemia modified albumin which is produced when circulating serum albumin interacts with the ischemic cardiac muscle. A leukocyte enzyme myeloperoxidase is a novel cardiac marker produced from the free radicals generated by oxygen and it has been associated with the reduction of nitrous oxide thereby causing vasoconstriction, formation of soft plaque creations embedded with lipid and instability of atherosclerotic plaques. Myeloperoxidase levels have been associated with the antagonistic events related to coronary artery disease and cardiovascular outcomes. A nonspecific marker of inflammation secreted by macrophages and T cells; C-reactive protein acts as prognostic indicator associated with myocardial atherosclerotic plaques formation. Increased of CRP activity can forecast the adverse cardiovascular outcomes that may occur during primary or secondary management stages. It is a nonspecific indicator but when used in combination with B-type natriuretic peptide, cardiac troponin it may have diagnostic value as cardiac biomarker for acute coronary events in the emergency department.

Other Laboratory studies:

Apart from biomarkers complete blood cell count (Before thrombolytic agents are administered anemia which causes reduced oxygen supply and the levels of prothrombin time, partial thromboplastin time, and international normalized ratio should be checked), comprehensive metabolic panel (Before initializing ACE Inhibitors, levels of potassium and magnesium should be assessed) and lipid and lipid profile may be helpful for an accurate diagnosis of myocardial infarction. Blood oxygenation ought to be tested as well as constantly adjusted in the event that somewhat medical verdicts recommend hypoxemia. This hypoxemia may result from atelectasis, aspiratory blockage or ventilatory damage auxiliary to hitches of myocardial-infarction else intemperate sedation else analgesic drugs.

Imaging Techniques used:

The use of different types of imaging techniques for diagnosis of myocardial-infarction remains comprehensive nevertheless, these were predominantly adopted to approve or avert any coronary artery disease.

Additionally, imaging might aid to express the framework and degree of myocardial perfusion abnormalities. The techniques that are employed are Chest radiography (to know the extent of clinical noteworthy temporal inconsistencies from diagnostic and post-therapeutic gaps), Positron-emission tomography (used for quantitative valuation of the circulation and amount of damage of regional myocardial perfusion and myocardial oxidative metabolism), Computed Tomography (for the evaluation of chambers dimensions and wall thickness, and presence or absence of intracardiac thrombi), Magnetic Resonance Imaging (for straight imagining of the cardiac tissue with exceptional description of the endocardial, epicardial and pericardial boundaries), Ultrasonography (helped to predict cardiac events when patients undertook reperfusion), Nuclear Imaging(useful for measuring the collateral flow, infarct size, and endangered myocardium, and for validating the prognosis of subjects with infarction) and Angiography (used for the assessment of bypass-graft patency, Kawasaki disease and anomalous coronary arteries) [20. 21].

Pathophysiology:

Whatever may be the etiology, the final common pathway of myocardial infarction includes occlusion of the coronary artery, myocardial ischemia (resulting in hypoxia), which leads to release of inflammatory pro-apoptotic cytokines, and ultimately necrosis of myocardial tissue [22]. The main pathophysiology of myocardial infarction includes damage and dysfunction of the endothelium due to plaque formation and myocardial injury which causes ischemic damage to the cardiac tissue with the depicted in figure 2 and figure 3 respectively. A dynamic interaction among damaged intima, platelet aggregates, and spasm is hypothesized to occur as a prelude to thrombosis in acute transmural myocardial infarction.

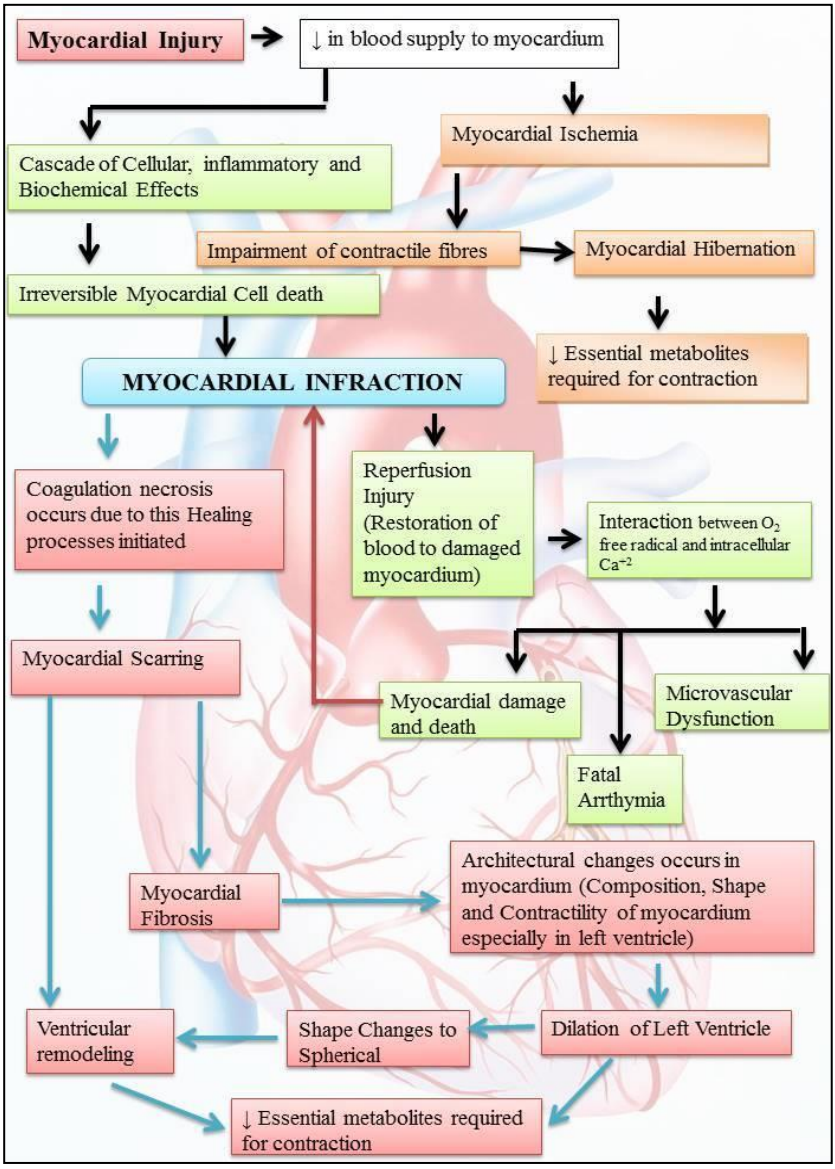

Fig 2: Pathophysiological insight on the myocardial injury which causes ischemic damage to the cardiac tissue

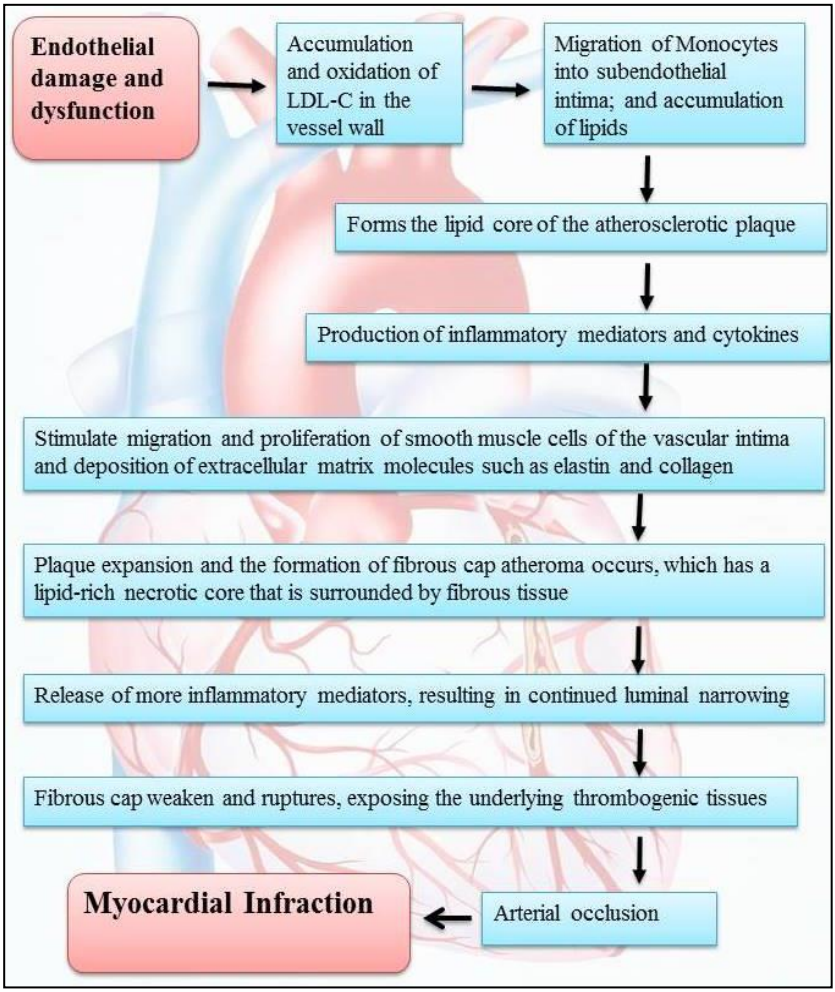

Fig 3: Pathophysiological insight on endothelium damage and dysfunction due to plaque formation

Metabolic consequences of myocardial infarction:

Acute myocardial infarction causes acute metabolic stress which causes the release of Adrenocorticotropic hormone, leading to adrenal stimulation and increased secretion of cortisol. Apart from this stress causes increased sympathetic nervous system stimulation thereby releasing catecholamine, which in turn causes mobilization of lipids there by increasing plasma free fatty acid levels. Release of catecholamine also suppresses the secretion of insulin, thereby increasing blood glucose levels and may also probably contribute to glucose intolerance.

The localized metabolic response occurs from coronary occlusion includes a rapid breakdown of glycogen in the ischemic area with the release of lactate and hydrogen ion. Due to intracellular accumulation of hydrogen ion thereby causing calcium displacement from the attaching site on actin and myosin occurs and from accumulation of lactic acid decrease in pH is noted which leads to a decrease in myocardial contractility. Total arterial occlusion causes impairment or stoppage of oxygen supply and lack of oxygen breaks down the tissue adenosine triphosphate, thereby causing the accumulation of its byproduct adenosine which acts as local vasodilators. So, the breakdown of ATP now initiates compensatory vasodilator response and develops the collateral circulation. Collateral blood supply may provide sufficient oxygen to the ischemic area to preserve the sustainability of myocardium provided that contraction has concluded. Because of inadequate myocardial blood supply by collateral arteries else due to decreased rate of collateral flow or due to continued contraction of parts of the ischemic area or due to early decrease in adenosine triphosphate levels and release of catecholamine from storage sites in myocardium ischemic area undergoes infarction.

Due to lack of oxygen during infarction increase utilization of glucose occurs than free fatty acids for ATP production. Even though free fatty acid is normally a significant source of energy for cardiac tissue, the consumption of fatty acid as supplier of energy is lessened due to oxygen dearth. So, in the infarcted area, the energy production mainly occurs from carbohydrate metabolism by accelerating movement of glucose into myocardial tissue, glycogenolysis and augmented glycolysis in the myocardial tissue. The myocardial processes accountable to this change include a decrease in ATP levels and increased in its byproduct such as adenosine monophosphate and inorganic phosphate as well as the stimulating effects of endogenous catecholamines, which causes ATP breakdown and increases the production of cyclic AMP. In spite of this compensatory mechanism, cell death may proceed in most of myocytes. This cell death doesn't mean the termination of cellular activity, since there's a seriously amalgamation of protein followed by mending of wound, which comes about in scar arrangement [23].

Oxidative stress and myocardial infarction:

Oxidative stress that occurs due to an imbalance between reactive oxygen species generation and antioxidants in the cellular and subcellular levels plays a significant role in a myocardial infarction as they cause cardiac ischemia and reperfusion injuries. The sources of generation reactive oxygen species in cardiac myocytes could be mitochondrial electron transport chain, NADPH oxidase, nitric oxide synthase, xanthine oxidase and

lipoxygenase/cyclooxygenase and the auto-oxidation of various substances, mainly catecholamines (Misra et al., 2009). [19]. Under oxidative stress, reactive oxygen species attacks biomolecules which leads to damage of nuclear and mitochondrial DNA; cross-linking of protein and lipid peroxidation ensues, resulting in protein denaturation and loss of enzyme and membrane pump function. Damage to intracellular and sarcolemma membranes due to reactive oxygen species impairs ATP-dependent Na^+ and calcium reuptake mechanisms. Decline in the activity of the sarcoplasmic reticulum membrane calcium pump, which plays a vital part in cardiac calcium handling occurs due to reactive oxygen species. Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 production activated by reactive oxygen species contribute to intracellular calcium $^+$ dysregulation and an increase in its concentration. Myocardial cytosolic calcium overload leads to myofibrillar hyper contracture, cytoskeletal damage and myocardial cell disruption via activation of calcium dependent proteases and phospholipases. Mitochondrial calcium overload causes disorganized ATP synthesis and utilization, causing necrosis [24].

Disproportionate levels of reactive oxygen species activate a number of regulatory chain reactions such as stimulation of endothelial cell migration and angiogenesis; triggering of the cascade of transcriptional factors, adhesion molecule expression and proinflammatory cytokines (TNF-α, IL-1β, IL-6) which cause massive recruitment of inflammatory cells, especially neutrophils, occurs. Neutrophils then penetrate the endothelium of the blood vessels of infarcted area and highly express NADPH-oxidase, which causes more free radical generation and gives rise to a vicious cycle of damage. Apart from neutrophils, fibroblasts, smooth muscle cells, and T lymphocytes generate reactive oxygen species thereby causing the formation of white thrombi in micro vessels due to attraction and activation of leucocytes leading to increased platelet aggregation, microvascular cell edema and dysfunction, resulting in augmentation of ischemia and tissue stunning.

Risk factors associated with Myocardial Infarction:

A substantial proportion of patients with coronary artery disease do not have traditional risk factors of the disease. The common risk factors of atherosclerosis explain disease occurrence in only half of the diagnosed cases. The INTERHEART study recognized abnormal lipids (ApoB/ApoA ratio), smoking (Current smokers and former smokers), hypertension, diabetes, abdominal obesity, psychosocial influences (financial pressure, global tension, locus of control, depression, as well as life occasions counting conjugal division, work misfortune, family struggle), dietary risk (ingesting of

salty snacks, fried foods, meats), and alcohol, and regular physical activity (moderate or strenuous exercise) account for most of the risk of myocardial infarction globally [25].

According to India CLEN supported study predictors for myocardial infarction includes waist-hip ratio (≥0.9 (Men), ≥0.8 (Women)), body mass index (≥25 kg/m^2), stress at work in last 1 year, stress at home in last 1 year, financial stress, hypertension, family history of CHD, past history of gingival sepsis (painful teeth, painful gums, lost teeth), tobacco smoking, raised total serum cholesterol, *Chlamydia pneumoniae* (IgG antibodies positivity), *Helicobacter pylori* (IgG Antibodies positivity) and raised C-reactive protein [26].

Treatment

Rapid diagnosis and management are the key components to decrease mortality in patients with myocardial infarction along with understanding the pathology and disease progression. The different treatment strategies used for the management of myocardial infarction is illustrated in figure 4

The main treatment goals for patients with STEMI consist of immediate reperfusion therapy, followed by stabilization of the acute coronary lesion, treatment of residual ischemia, potential revascularization and continuing secondary prevention. Reperfusion therapy in patients with STEMI is accomplished either with percutaneous coronary intervention or by intravenous thrombolytic therapy (Alteplase, Reteplase, Streptokinase, and Tenecteplase). Adjunctive antithrombotic therapy consists of antiplatelet drugs and anticoagulants such as aspirin, $P2Y_{12}$ receptor inhibitors (Clopidogrel, Ticagrelor, Prasugrel), unfractionated heparin or low molecular weight heparin, direct thrombin inhibitors (Lepirudin, Desirudin, Bivalirudin, and Argatroban) and Glycoprotein IIb/IIIa inhibitors (Abciximab, Eptifibatide and Tirofiban) are used to avert further thrombosis and allow the endogenous fibrinolytic mechanisms to dissolve the thrombus formed. Antiplatelet treatment should be continued for extended period to decrease the risk of developing recurrent thrombosis and to inhibit progression to complete blocking of the coronary artery.

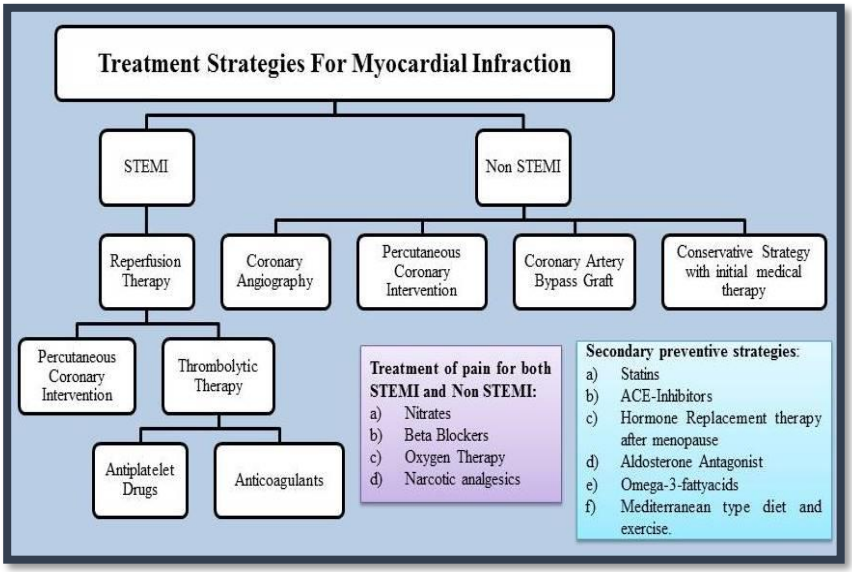

Fig 4: Different treatment strategies employed for myocardial infarction

For NSTEMI, the treatment strategies mainly include initial aggressive approach with myocardial angiography, by resolved for revascularization through PCI intervention else CABG else through conventional approach through early therapeutic approach as well as imaging strategies through noninvasive myocardial route. Irrespective of stratagem, patients need intensive usage of drugs to treat the imbalance of oxygen and decrease the disease progression [27].

To reduce myocardial oxygen demand both in case of STEMI and NSTEMI anti-ischemic therapy primarily beta blockers and nitrates are employed. For the management of recurrent or persistent symptoms even after treatment with a full dose of nitrates and beta blockers, calcium channel blockers are prescribed. After the patient has been stabilized by the immediate initial treatment, now the goals of management include prevention of long-term complications and to decrease the risk factors associated with myocardial infarction, as well as aggressive lifestyle modifications to restore the normal functioning of the heart. This multidimensional goal is accomplished with the employment of significant components in management, which includes usage of cardio-protective drugs, myocardial restoration, and changes in eating habits and physical activity apart from patient education. Cardioprotective drugs in secondary management mainly include reductions in elevated blood pressure by inhibitors of the renin-angiotensin-aldosterone system such as

angiotensin-converting enzyme inhibitor, angiotensin-receptor blockers or aldosterone blockers; Beta blockers such as metoprolol, carvedilol, or bisoprolol and most potent statins like rosuvastatin and atorvastatin. Nonpharmacological secondary prevention includes lifestyle modifications and cardiac rehabilitation which mainly comprises dietary changes such as decrease in saturated fat and salt intake, usage of omega-3 polyunsaturated fatty acids, mainly derived from fish oil and Mediterranean regimen accompanied by nuts and/or extra-virgin olive oil as well as limiting alcohol consumption; smoking termination; vaccination, apart from enhancement in exercise and physical activity [28]. Post-menopausal women who are at higher risk of cardiovascular disease, hormone replacement therapy, either with estrogens alone or in different combinations with progesterone and estrogens should be considered [29].

Medication Summary

The key objective of myocardial infarction pharmacotherapy is to avert further complications and decrease the morbidity. The foremost aim of emergency department pharmacotherapy is to provide quick intravenous thrombolysis and/or prompt recommendation for percutaneous coronary intervention, optimization of oxygenation, lessening of myocardial workload and control of pain. The different classes of drugs used for the management of myocardial infarction are beta-adrenergic blockers, PCSK9 inhibitors, angiotensin-receptor blockers, angiotensin-converting enzyme inhibitors, anticoagulants medication, thrombolytics, glycoprotein IIb/IIIa inhibitors, vasodilators, antiplatelet medication and analgesic medication.

Antiplatelet Therapy

These agents decrease blood coagulation capacity by antagonizing processes that impedes with the process of platelet activation. In the controlling of myocardial infarction, they have a significant advantage in terms of mortality. In emergency coronary artery bypass graft (CABG), there is an enhanced danger of bleeding which can be reduced by these agents [30].

- Aspirin an irreversible cyclooxygenase inhibitor, early aspirin administration in acute myocardial infarction patients showed a 23 percent reduction in the first month's cardiac mortality rate.
- Clopidogrel an Adenosine diphosphate receptor (ADP-receptor) inhibitor exerts beneficial effect on numerous hemorrhagic factors and can protect against atherosclerosis, not only by platelet function inhibition, but as well as modifications in the hemorrhagic profile. Clopidogrel use in acute coronary syndrome patients showed to

reduce cardiovascular mortality in myocardial infarction and stroke.

- Ticagrelor and its major metabolite by prevention of platelet activation and signal transduction by interacting with platelet P2Y12 Adenosine diphosphate receptor, decreases the frequency of cardiovascular thrombotic events in acute coronary syndrome patients for instance unstable angina, NSTEMI and STEMI apart from reducing the occurrence of stent thrombosis in subjects with stent positioning for acute coronary syndrome therapy and is stated more than a year earlier in patients with MI history. Patients can move from clopidogrel to ticagrelor without interrupting the impact of antiplatelet.
- Prasugrel, a thienopyridine prodrug that prevents platelet aggregation and activation by irreversibly binding of active metabolite to adenosine phosphate platelet P2Y12 receptor, thereby suggested for decrease of thrombotic cardiovascular development in subjects suffering from acute coronary syndrome accomplished through PCI who have either unstable angina or NSTEMI or STEMI while being treated from the immediate or delayed PCI. Patients with past episodes of stroke or temporary ischemic attacks are not advised to use prasugrel.
- Vorapaxar, a Protease-activated receptor-1 antagonist irreversibly reduces cardiovascular thrombotic episodes in patients with peripheral artery disease otherwise MI history. It is not used as monotherapy, but added to aspirin and/or clopidogrel.

Anticoagulants Agents

This agent protects or decreases blood coagulation by hindering the cascade of coagulation by coagulation factors that occur after the original aggregation of the platelet. Agents associated with this class prevent the development of thrombus accompanied with MI and thereby reducing the death proportion through decreasing likelihood of deadly MI, cerebrovascular accidents, as well as cardiovascular deaths. The two choices for primary anticoagulation treatment are fractionated low-molecular-weight subcutaneous heparins and unfractionated intravenous heparin [31].

- Bivalirudin, a synthetic congener of recombinant hirudin which is more potent and highly specific inhibitor of thrombin is recommended as anticoagulant in unstable angina patients undergoing PCI. It has Impending benefits over conventional heparin therapy such as rapid onset of action, predictable antithrombotic

response, inhibitor of both clot-bound and circulating thrombin, lack of natural inhibitors (example platelet factor 4, heparinase), and sustained effectiveness even after plasma clearance. The use of bivalirudin is shown to be effective in management of both stable and unstable angina, NSTEMI, STEMI patients undergoing percutaneous coronary intervention (PCI).

- Heparin increases the activity of enzyme inhibitor antithrombin III thereby preventing the formation of new clots (thrombus) and extension of existing clots within the vessels apart from preventing the re-accumulation of a clot next to extemporaneous fibrinolysis.
- Enoxaparin irreversibly inactivates clotting factor Xa and potentiates the activity of antithrombin III. It is used for the management of acute STEMI with or without PCI. Prophylactically used for the treatment of non-Q-wave myocardial infarction and unstable angina along with aspirin.
- Dalteparin low molecular weight heparin that is used for the management of acute coronary syndrome succeeded by percutaneous intervention

Glycoprotein IIb/IIIa Inhibitors

These drugs interfere with cross-linking platelets and thrombus formation derived from platelets, thus acting as powerful inhibitors of platelets aggregation. These drugs are used in controlling of ACS patients undergoing PCI, unstable angina and NSTEMI patients undergoing PCI as well as to avert acute myocardial ischemic difficulties in unstable angina that is unsympathetic to conservative treatment. The only glycoprotein IIb/IIIa antagonists permitted for medical use are abciximab, tirofiban and eptifibatide [32].

Nitro-Vasodilators

The drugs belonging to this class for example nitroglycerine causes the vasodilatation thus decreasing the LVEDP and ventricular filling pressures thereby redistributing blood so that more is pooled in peripheral veins and improving the oxygen supply to heart which in turn dilates epi-cardial and collateral blood vessels consequently improving blood supply to the ischemic myocardium. Nitoglycerine by activating guanylate cyclase (atrial natriuretic peptide receptor A) it stimulates the formation of intracellular cyclic guanosine 3',5'-monophosphate thereby subsequently causing relaxation of cells of smooth muscles and vasodilation which increases the blood flow to the myocardium and due to venodilatation it decreases myocardial preload and additionally reduces cardiac wall stress. Nitrates are used only for

symptomatic relief and have nil influence on mortality rate in myocardial infarction [33].

Beta-adrenergic blockers

Beta adrenergic receptor antagonists are competitive blockers that antagonizes the adrenergic beta receptors sites for the endogenous catecholamines i.e., adrenaline and noradrenaline, of the sympathetic nervous system. In the management of myocardial infarction these drugs reduce myocardial ischemic potential via reducing the myocardial oxygen demand by decreasing the heart rate, myocardial contractility, blood pressure thereby subsequently relief from ischemic chest pain. Apart from this these drugs have the potential antiarrhythmic properties and they decrease the risk of ventricular fibrillation thereby reducing the risk of sudden cardiac death that may occur due to excessive release of catecholamines or ischemia. Beta blockers apart from management of myocardial infarction these agents are utilized as antihypertensives to reduce blood pressure due to their negative inotropic effect that reduces cardiac output, negative chronotropic property that decreases heart rate at rest as well as at exercise, decreases in cranial sympathetic outflow and decreasing the release of renin from kidneys thereby improving and preserving the hemodynamic status by reducing myocardial congestion, acting on cardiac contractility and reducing cardiac energy expenditure. The drugs in this class that are for the management of myocardial infarction are metoprolol, esmolol and atenolol [34].

Angiotensin-Converting Enzyme Inhibitors

These agents inhibit the angiotensin-converting enzyme, thereby reducing the activity of the renin–angiotensin–aldosterone system by preventing the conversion of angiotensin-I to angiotensin-II, thereby reducing the secretions of adrenal cortex and subsequent release of aldosterone is inhibited. These drugs reduce the arteriolar resistance and raise the venous capacity, thereby decreasing the cardiac index, stroke volume and cardiac output. Angiotensin-Converting Enzyme inhibitors by preventing release of bradykinin and formation of angiotensin II causes vasodilatation of arteries and veins thereby decreasing the cardiac preload, cardiac afterload and arterial blood pressure. It inhibits the cardiac and vascular remodeling associated with myocardial infarction thereby preventing ventricular remodeling and ventricular hypertrophy thus reducing the mortality rates after myocardial infarction. Angiotensin-Converting Enzyme inhibitors such as captopril, enalapril, quinapril and lisinopril have the utmost advantage in ventricular dysfunction patients [35].

Angiotensin-Receptor Blockers

These agents modulate the renin angiotensin aldosterone pathway by antagonizing the activation of angiotensin-II AT_1 receptors thereby causing vasodilatation, reducing secretion of vasopressin, and decreasing the formation and release of aldosterone from adrenal cortex. These drugs elicit anti-hypertensive effects by antagonizing angiotensin receptor induced vasoconstriction, adrenaline discharge, water reabsorption and hypertrophic responses. These are used mainly as an alternative in patients who can't tolerate adverse effects such as persistent dry cough, angioedema and hyperkalemia associated with ACE inhibitors. Receptor blockers of angiotensin such as irbesartan, candesartan, valsartan, azilsartan, eprosartan mesylate and losartan should be used in individuals with STEMI who cannot tolerate angiotensin-converting enzyme inhibitors [36].

Thrombolytics

This is the mainstay pharmacological reperfusion therapy for treatment of acute myocardial infarction. Fibrinolytic therapy is mainly used to repair the coronary circulation through an earlier obstructed vessel flow through prompt and widespread elimination of a pathologic intraluminal embolus or thrombus that has not been dissolved by the natural fibrinolytic agent plasmin which breaks down the clots by digesting the fibrinogen and fibrin present in a clot. The first generation of fibrinolytic drugs (such as urokinase, streptokinase, reteplase, acetylated plasminogen streptokinase activator complexes, and novel plasminogen activator) extensively induced stimulation of clot-associated plasminogen and circulating plasminogen thereby consistently producing a systemic lytic state characterized by reduction of plasminogen, circulating fibrinogen, and hemostatic proteins and by noticeable rise of concentrations of fibrinogen degradation products in plasma. The second generation of fibrinolytic drugs (such as eg, alteplase tenecteplase) specially activates plasminogen in the fibrin area, slightly than in the circulation, as with free plasminogen thereby showing the selectivity towards all the clots. Alteplase is effective in prompting recanalization of epicardial coronary vessel triggered by an acute thrombotic obstruction in 80-90% of infarct-related arteries within 90 minutes [37].

Analgesics

One of the obvious clinical markers of myocardial infarction is the angina chest pain. Pain relief is essential for quality patient well-being, but also as the pain produces systemic circulatory properties such as enhanced heart rate, stroke volume and blood pressure which can undesirably affect the

equilibrium between cardiac metabolic necessity and supply and results in extension of infarct. Analgesic drugs mainly safeguard the comfort of patient, relax the pulmonary edematous state, reduce sympathetic-adrenal response and possess sedating as well as tranquillizing effect that shows beneficial effect for patients who encounter chest pain. Narcotic analgesic mainly morphine is the drug of choice for treatment of severe pain in acute myocardial-infarction because of their enhanced haemodynamic effect [38].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) Inhibitor

These drugs show plausible decreases the residual cardiovascular disease risk by prominently causing decrease in the circulating low-density lipoprotein particle concentrations. They offer a valid therapeutic target for avoidance of cardiac arrest, strokes and PCI. PCSK9 inhibitors used for the prophylaxis of myocardial infarction include evolocumab and alirocumab that also reduces the risk associated with stroke, myocardial infarction and unstable angina [39].

Complications

Myocardial Infarction has dramatic and potentially lethal Complications which include ischemic, mechanical, arrhythmic, thrombotic or embolic, inflammatory and psychosocial complications [40].

- Ischemic complications occur due to failure of primary percutaneous coronary intervention which includes re-occlusion of an infarct-related artery, infarction in a separate territory (recurrent infarction) and post-infarction angina.
- The foremost mechanical complications of myocardial infarction consist of ischemic mitral regurgitation, free wall split, ventricular septal fissure, and in the chronic phase of mechanical complications negative remodeling and aneurysm formation could arise.
- Arrhythmic complications such as life-threatening arrhythmias (total atrioventricular block, ventricular tachycardia, fibrillation and flutter), reperfusion arrhythmia (idioventricular rhythm, ventricular ectopic or tachycardia), atrial fibrillation, persistent tachycardia, sinus tachycardia, sinus bradycardia, besides additional supraventricular tachycardias might as well happen.
- Thrombosis and embolic complications such as Deep vein thrombosis and pulmonary embolism can be noted. Mural thrombosis and systemic embolism may be associated with myocardial infarction.
- Inflammatory complications which include early pericarditis,

Dressler's syndrome and post-myocardial infarction syndrome.

- Psychosocial complications which include anxiety and depression following myocardial infarction may be noted.

Prognosis

Myocardial-infarction is related by thirty percent death rate; nearby fifty percent of the patients expire before entering into the hospital and further five percent to ten percent of patients expire in a period of one year after their episode of myocardial infarction. Patients admitted to coronary care units with acute myocardial infarction have an in-hospital mortality of 15 % to 20 %. Within one year almost half of all patients with myocardial infarction are re-hospitalized for different myocardial index events such as recurrent MI, heart failure, arrhythmias, angina, and stroke.

Some risk factors for death in myocardial infarction includes age, diabetic status of individual, earlier cardiovascular and/or peripheral vascular disorders, hemodynamic parameters (such as heart failure, cardiac arrest on admission, decreased systolic blood pressure, or Killip class of two or greater patients), ST-segment deviation, increased serum creatinine, peripheral vascular disease, and raise of cardiac markers (B-type natriuretic peptides as well as high sensitive CRP). Assessment of left ventricular ejection fraction may upsurge the predictive power of mortality. Prognosis of myocardial infarction will become worse if a mechanical complication such as a myocardial free wall or papillary muscle rupture occurs [41].

In myocardial infarction, morbidity and mortality are associated with the functional severity of the disease and risk assessment. The Killip classification will be extensively used in individuals staging by acute myocardial infarction in an effort to myocardial menace prediction, which comprises of [42]:

- Killip class I comprise of subjects with nil medical manifestation of cardiac arrest
- Killip class II comprises of subjects with crackles or decrease in functioning of lungs, S_3 gallop, as well as elevated blood pressure in jugular vein
- Killip class III comprises of subjects with forthright severe water retention in lungs
- Killip class IV comprises of subjects with hypotension or cardiogenic shock, as well as an indication of decreased stroke volume which includes decrease in urine output, cyanosis, or compromised phycological condition.

The risk of death after the episode of myocardial infarction is assessed by considering the predictors of mortality. Risk scores measure the outcome after an episode of acute myocardial infarction. The most regularly used scoring system is the TIMI (Thrombolysis in Myocardial Infarction) risk scores for STEMI and NSTEMI. The higher the score, the more the patient is at risk of death from cardiac events [43].

TIMI Score for STEMI includes

- Patient with history of diabetes mellitus, hypertension or the history of sternal pain (1 point)
- Systolic blood pressure more than 100 mmHg (3 points)
- Heart rate greater than 100 beats per minute (2 points)
- Patients fitting in Killip class II-IV (2 points)
- Patients with age of 75 years or more (3 points)
- Patients with age in between 65-74 years old (2 points)
- Patients with age of fewer than 65 years (0 points)

TIMI risk score: 0 points (0.8% mortality); 1 point (1.6% mortality); 2 points (2.2% mortality); 3 points (4.4% mortality); 4 points (7.3% mortality); 5 points (12% mortality); 6 points (16 %); 7 points (23% mortality); 8 points (27% mortality); 9-14 points (36.0% mortality).

TIMI Score for NSTEMI includes

- Patients with age of fewer than 65 years (1 point)
- Patients with three or more *Coronary artery disease* risk factors (1 point)
- Patients with known *Coronary artery disease* with more than 50% stenosis (1 point)
- Patients using Aspirin in the past 7 days (1 point)
- Patients having severe angina in the earlier 24 hours (1 point)
- Patients diagnosed with the raised cardiac biomarkers (1 point)
- Patients having ST deviation greater than 0.5mm (1 point)

TIMI risk scores: 0-1 points (3% to 5% mortality); 2 points (3% to 8% mortality); 3 points (5% to 13% mortality); 4 points (7% to 20% mortality); 5 points (12 % to 26% mortality); 6-7 points (19% to 41% mortality).

Alternative Medicines for management of Cardiovascular Disease-Myocardial Infarction:

Vitamin and Nutraceutical Supplements:

Table 1: Vitamin and Nutraceutical Supplements in management of Myocardial infarction

S. No	Nutraceutical	Estimated Average Requirement (Adults)
1.	Calcium	525 mg/day
2.	Carotenoids	β-Carotene 20-50 mg/day
3.	Chromium	25 µg/day
4.	Coenzyme Q10	300 mg/day
5.	Flavonoids	200-1000 mg/day
6.	Folic acid	150 µg/day
7.	L-carnitine	2 g/day
8.	Magnesium	Males: 250 mg daily Females: 200 mg daily
9.	Manganese	1.4 mg daily
10.	Molybdenum	50-40 µg/day
11.	Niacin	Males: 15 mg daily Females: 11 mg daily
12.	Pyridoxine (vitamin B6)	1.4-1.6 mg/day
13.	Thiamine	0.8-1.0 mg/day
14.	Selenium	Males: 75 µg daily Females: 60 µg daily
15.	Cynacobalamin	1.25 µg daily
16.	Ascorbic acid	25 mg daily
17.	Cholecalciferol	15 µg daily above 55 years
18.	Alpha-tocopherol	Males: >4 mg daily Females >3 mg daily
19.	Phylloquinone	1 µg/kg/day
20.	Zinc	Males: 7.3 mg daily Females: 5.5 mg daily
21.	Omega-3 fatty acids	EPA 1500 mg/day

Potential Herbal Medicines in Cardiovascular Diseases [44]:

Table 1: Potential Herbal Medicines in Cardiovascular Diseases

S. No	Herb	Possible Cardiovascular Indications
1.	Astragalus (*Astragalus membranaceus*)	Heart failure and coronary heart disease
2.	Asian ginseng (*Panax ginseng*)	Anti-anginal, anti-hypertensive, anti-diabetic and anti-hyperlipidaemic

3.	Flaxseed oil (*Linum usitatissimum*)	Anti-atherosclerotic, anti-hyperlipidaemic, anti-hypertensive and anti-arrhythmic
4.	Garlic (*Allium sativum*)	Anti-hypertensive, anti-hyperlipidaemic, antithrombotic and inhibitor of platelet aggregation
5.	Ginkgo (*Ginkgo biloba*)	Management of Coronary heart disease, Cerebral insufficiency, peripheral vascular disease and antithrombotic
6.	Grape (*Vitis vinifera*) seeds	Anti-atherosclerotic, anti-hypercholesterolemic, anti-hypertensive and management of chronic venous insufficiency
7.	Green tea (*Camellia sinensis*)	Anti- hypercholesterolemic, anti-diabetic and prevention of cardiovascular events.
8.	Hawthorn *(Crataegus)*	Anti-hypertensive, anti-hyperlipidaemic, anti-hypertensive and anti-arrhythmic and management of congestive heart failure
9.	Milk thistle (*Silybum marianum*)	Cardioprotective effect
10.	Soy (*Glycine max*)	Anti- hypercholesterolemic and anti-hypertensive
11.	Hawthorn (*Crataegus* species)	Heart failure, angina, hyperlipidaemia
12.	Danshen (*Salvia miltiorrhiza*)	Anti-anginal, anti-hypertensive, anti-ischemic stroke, antithrombotic and anti-hyperlipidaemic
13.	Lingzhi (*Ganoderma lucidum*)	Anti-hyperlipidaemic, anti-hypertensive and anti0diabetic
14.	Foxglove (*Digitalis* species)	Management of Heart failure and atrial fibrillation

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Chapter - 2

Secretory phospholipase A2 Group IIA: A Potential Therapeutic Target in Inflammation

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Abstract

Inflammation is a biological response of vascularized tissues to infections and damaged tissues caused by injury, infection, malignancy, and cellular changes. The function of inflammation is lifesaving. However, if prolonged, it results in undesirable consequences, such as systemic shock, circulatory collapse, arthritis, and local tissue injury in many organs. Inflammation is mainly mediated by PLA2 enzyme that catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid (AA) and lysophospholipids, which are the rate-limiting precursors for the production of pro-inflammatory lipid mediators. The enzymes cyclooxygenase 1/2 (COX 1/2) and lipoxygenase (LOX) catalyze the arachidonic acid into pro-inflammatory mediators such as prostaglandins, leukotrienes, and thromboxanes, respectively. The lysophospholipid is converted into platelet activation factors (PAF) by acetyltransferase. An elevated level of sPLA2 Group IIA (sPLA2GIIA), which is the type of PLA2 enzyme is found to be present in various chronic inflammatory diseases including arthritis, inflammatory bowel disease, psoriasis, and septic shock. The recent reports revealed the role of sPLA2GIIA in different types of cancers, including breast, colon, pancreas, and prostate cancer. Some of the studies have revealed that the eicosanoid pathway is activated in prostate cancer and the cyclooxygenase (COX) and lipoxygenase (LOX) products contribute to the progression of the disease *via* promoting cell proliferation, motility, invasion, and angiogenesis. Thus, inhibition of sPLA2GIIA by molecules from plant or natural source can lead the market as potent anti-inflammatory drug. In this context, many plant extracts and its constituents are reported for their anti-inflammatory activity through the inhibition of $sPLA_2$s. However, effective and specific inhibitors of sPLA2GIIA are still not available to date.

Keywords: secretory phospholipase A2, cyclooxygenase, lipoxygenase, arachidonic acid, Non-Steroidal anti-inflammatory drugs (NSAIDs).

Inflammation

Inflammation is the complex stereotypical reaction of the body expressing the response of the host against cellular injury which brings about restoration of tissue structure and function. It is the body's first reaction to invasion by an infectious agent, chemical and even just physical or traumatic damage. The aim of inflammation is to promote healing of the damaged tissue. Inflammation promotes healing by isolating the damaged area and by mobilizing effectors cells and molecules to the site of inflammation. Inflammatory reactions were phenomenologically described in ancient times. In the IV century, Celsius articulated the signs of inflammation that in a familiar classic view: rubor, dolor, calor, and tumor (Table 1). The famous Roman physician Galen, who lived and worked in the II century A.D. proposed a fifth sign *functiolaesa*. These cardinal signals are the main basis for describing any type of inflammation even today. The inflammatory symptoms are the results of plasma extravasations and increased leukocyte infiltration to the site of inflammation [1-2].

Table 1: The cardinal signs of inflammation and their causes

English	Latin	Cause
Redness	*Rubor*	Vasodilation
Swelling	*Tumor/Turgor*	Vasodilation
Heat	*Calor*	Increased vascular permeability, increased granulation of tissue
Pain	*Dolor*	Physical and chemical stimulation of nociceptors
Loss of function	*Functiolaesa**	Disruption of tissue structure and function

**Functiolaesa* is not really unique to inflammation and is a characteristic of many disease states.

However, the protective inflammatory reactions become detrimental when the inflammatory response is excessive in magnitude and/or duration resulting in severe tissue damage. Such inflammatory reactions might result in several types of autoimmune diseases and allergic reactions.

Despite the complexities associated with the inflammatory responses. They can be divided into four general components, namely,

i) Inflammatory inducers,

ii) Inflammatory sensors,

iii) Inflammatory mediators, and

iv) Target tissues.

The inflammatory inducers may be exogenous or endogenous, which results in tissue stress, injury and malfunction. The signals by inflammatory inducers are detected by the specific receptors of sensor cells (e.g. tissue resident macrophages and mast cells) and in response they produce inflammatory mediators. Different combinations and amount of mediators are produced from the sensory cells depending on the nature of inducer. The action of inflammatory mediators ultimately results in the elimination of inducers and restoration of tissue homeostasis [3-4].

Causes of inflammation

Inflammation can be initiated by various factors, including physical and chemical agents, injuries, tissue necrosis, wounds, trauma, inappropriate immune reactions, genetic defects, and infection from pathogens such as viruses, bacteria, fungi, parasites and protozoans which cause inflammation by invading the host cell and destroying it. Physical agents (radiation and burns) and chemicals (acids, alkalis and oxidizing agents) can initiate inflammatory responses by damaging the tissue. Environmental agents such as pollen can trigger allergic and hypersensitivity reactions which can lead to chronic inflammatory conditions. Loss of blood flow to the tissue leads to lack of oxygen and nutrients to the cells which can also lead to inflammation. Bacterial entry to the cell results in the release of endotoxins which can cause severe inflammatory disorders such as sepsis [4-6].

Events and Phases of inflammation

Several cell types such as neutrophils or polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, mast cells, basophils and eosinophils are involved in inflammatory responses. They are widely distributed in the body; however, during infection, which concentrated at the site of damage and release chemical substances called as inflammatory mediators, i.e., histamine, leukotriene, bradykinin, platelet activating factor, complement components, cytokines, reactive oxygen species etc,. These inflammatory mediators bring about healing of the damaged tissue through various mechanisms (Figure 1). The overall events of inflammatory responses can be summarized as follows.

a) Local vasodilation increases regional blood flow to the inflamed area, and together with an increase in microvascular permeability due to alterations in the vascular endothelium. This leads to increased blood flow (hyperemia) that causes redness (erythroma) and the entry of fluid and plasma proteins to the tissue (edema).

b) Concomitantly, there is an up-regulation of expression of adhesion molecules on endothelial cells and the release of chemotactic factors

from the inflamed site. These factors facilitate the adherence of circulating cells to the vascular endothelium and their migration into the affected area.

c) Due to increased vascular permeability, inflammatory cells migrate to the site of injury. Neutrophils are the predominant cells to reach an inflamed region in the early phase. However, monocytes and lymphocytes are also recruited to the site of damage in the later phase.

d) These inflammatory cells also release hydrolytic and proteolytic enzymes, and generate reactive oxygen species that eliminate and digests invading organisms and clearing up of cellular and tissue debris

e) All these events ultimately results in the removal of injurious agents and restoration of normal tissue structure and function

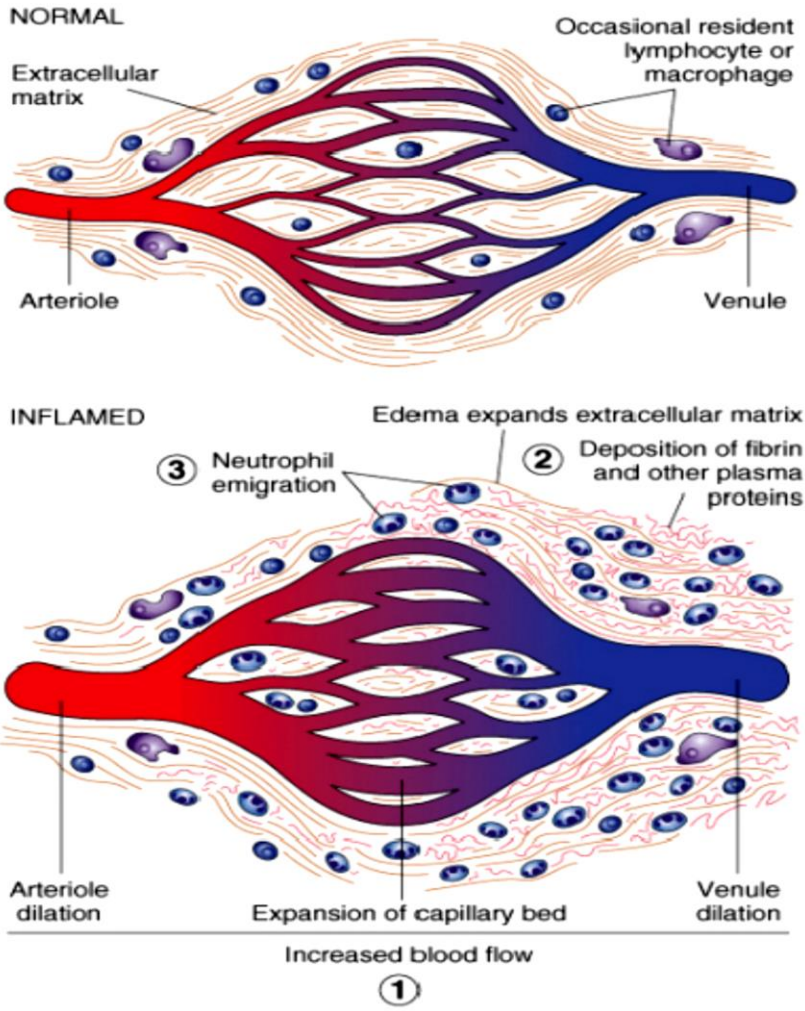

Fig 1: Events and phases of inflammation: During injury causes local vasodilation increases regional blood flow to the inflamed area 1) and up-regulate the expression of adhesion molecules 2). Further, the migrated inflammatory cells 3) release hydrolytic/ proteolytic enzymes 4) and generate reactive oxygen species that eliminate and digest invading organisms and clearing up the cellular and tissue debris.

Types of inflammation

Inflammation reactions are generally of two types, namely acute inflammatory and chronic inflammatory reactions, depending upon the severity ofthe inflammatory agents (Table 2).

a) Acute inflammatory reactions

Acute inflammatory reactions are short term response of the host against infection. These reactions are developed within a minute or hours and generally last for hours to a few days. Neutrophils, monocytes, eosinophils, basophils, macrophages and mast cells are the principal cell types involved during acute inflammatory responses. These reactions are generally mediated by histamine, bradykinin, lysosomal components and lipid mediators. Major outcomes of acute inflammation are healing and resolution of injury, abscess formation and progression to chronic inflammation depending on the type of inflammatory agent, severity of tissue damage and the ability of inflammatory cells to divide and replicate within the damaged tissue.

(b) Chronic inflammatory reactions

The purpose of inflammation is healing of the damaged tissue. However, persistence of the inflammatory agents for a longer period of time leads to chronic inflammatory condition or non-resolving inflammation, which ultimately results in loss of tissue or organ function. Chronic inflammation is characterized by the proliferation of blood vessels and connective tissue and is associated with thepresence of lymphocytes, macrophages and fibroblasts. These chronic reactions are generally mediated through lipid mediators, cytokines, proteases, complement system and reactive oxygen/nitrogen species. The chronic or non-resolving inflammation significantly contributes to the pathogenesis of arthritis, asthma, Alzheimer's disease, atherosclerosis, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, cystic fibrosis, dermatitis, gout, multiple sclerosis, psoriasis, systemic lupus erythematosis, tuberculosis.

Most of the aforesaid inflammatory reactions are mediated by chemical factors released mainly from vascular connective tissues such as plasma and circulating cells in response to inflammatory stimuli. Hence, it is prerequisite to understand the molecular basis of these chemical mediators to regulate inappropriate inflammatory responses.

Table 2: Comparison between acute and chronic inflammation

Comparison between acute and chronic inflammation		
Characteristics	**Acute**	**Chronic**
Causative agent	Pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions
Major cells involved	Neutrophils (primarily), eosinophils and basophils	Mononuclear cells (monocytes, macrophages, lymphocytes,

	(response to helminth worms and parasites), mononuclear cells(monocytes, macrophages)	plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months, or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis,

Various chronic inflammatory diseases

- **Rheumatoid arthritis (RA)** is an auto-immune disease, that is, a disease where a person‘s immune system attacks his or her own body tissues. This auto-immune reaction causes over-production of synovial (joint) fluid causes joints to become swollen and painful.
- **Alzheimer's disease** is the most common neurodegenerative disorder characterized by aggregates of fibrillar Aß derived from misprocessing of amyloid precursor protein (APP). Aß deposits are surrounded by activated microglia and astrocytes, which have been postulated to contribute to Alzheimer's pathophysiology by establishing a chronic inflammatory state.
- **Atherosclerosis** is the term for the process of fatty substances, cholesterol, cellular waste products, calcium and fibrin building up in the inner lining of an artery. It is a slow and progressive disease that may start in childhood. If left untreated, atherosclerosis can lead to heart attack or stroke.
- **Chronic Obstructive Pulmonary Disease, or COPD**, is a group of progressive lung diseases characterized by airflow obstruction or limitation that is not fully reversible. The restricted airflow is generally progressive and associated with an abnormal inflammatory response of the lungs to irritants. The family of diseases includes chronic bronchitis, emphysema and bronchiectasis.
- **Inflammatory Bowel Disease (IBD)** is the name of a group of disorders that cause the intestines to become inflamed (red and swollen). IBD can be painful and debilitating and causes chronic inflammation of the digestive tract. The two most common forms of IBD are ulcerative colitis and Crohn‘s Disease. Both conditions inflame the lining of your digestive tract and both can cause severe bouts of watery diarrhea and abdominal pain.

- **Psoriasis** is a common immune-mediated chronic skin disease that comes in different forms and differing levels of severity. It is a condition that is generally found on the knees, elbows, scalp, hands, feet, or lower back, and generally appears as patches of raised red skin covered by a flaky white buildup. It can cause intense itching and burning.
- **Multiple sclerosis (MS)** is an incurable inflammatory autoimmune disease that leads to irreversible damage to the brain and spinal cord. This disease is also associated with the reactivation of ancient viruses, which were inserted in our DNA during the evolution of humankind. It was therefore long thought that multiple sclerosis was due to a viral infection.
- **Pelvic inflammatory disease (PID)** is a general term that refers to infection and inflammation of the upper genital tract in women. It can affect the uterus (womb), Fallopian tubes (tubes that carry eggs from the ovaries to the uterus), ovaries, and other organs related to reproduction. This disease leads to infertility tubal (ectopic) pregnancy, chronic pelvic pain, abscess (sores containing pus) and etc.

Mediators of Inflammation

The inflammatory mediators can be derived either from inflammatory cells or from plasma. The cellular inflammatory mediators comprise histamine, serotonin prostaglandins, leukotrienes, cytokines, platelet activating factors (PAF), reactive oxygen and/or nitrogen species. While, the plasma derived mediators mainly include kinin system, coagulation system and complement cascade [4, 7-11]. The major source and biological actions of these inflammatory mediators are listed in table 3.

Table 3: Inflammatory mediators, their major source and mechanism of action

Mediators	Major Source	Action
Cellular Mediators		
Pre-formed mediators		
Histamine	Mast cells, basophils, eosinophils, leukocytes, platelets	Increased vascular permeability, vasodilation, smooth muscle contraction, endothelial activation
Serotonine	Mast cells, basophils, eosinophils, leukocytes, platelets	Increased vascular permeability, vasodilation
Lysosomal enzymes	Neutrophils	Increased vascular permeability, complement activation, hydrolysis of foreign bodies

Newly Synthesized mediators		
Prostaglandins	Leukocytes, platelets, mast cells, monocytes, macrophages	Increased vascular permeability, vasodilation, pain, fever
Leukotrienes	Leukocytes, mast cells, monocytes, macrophages	Increased vascular permeability, vasoconstriction, leukocyte adhesion and activation bronchospasm, chemotaxis,
Thromboxanes	Platelets	Platelet aggregation, blood vessel constriction, smooth muscle contraction
Cytokines	Lymphocytes, mast cells, macrophages, endothelial cells, leukocytes	Granuloma formation, pain, fever, anorexia, hypotension, expression of adhesion molecules, decreased vascular resistance
Chemokines	Leukocytes, activated macrophages	Chemotaxis, leukocyte activation
Platelet activating factor	Leukocytes, epithelial cells, mast cells, platelets,	Increased vascular permeability, vasoconstriction, degranulation, bronchoconstriction, leukocyte adhesion, chemotaxis, platelet aggregation and activation, oxidative burst
Nitric oxide	Macrophages, endothelial cells	Vascular smooth muscle relaxation, antimicrobial activity, tissue damage, vasodilation
Proteolytic enzymes (elastin, cathepsins, matrix metalloproteinases), ROS	Fibroblasts, leukocytes, monocytes, macrophages eosinophils	Degradation of extracellular matrix and basement membrane proteins, tissue damage, antimicrobial activity, increased vascular permeability, lipid peroxidation
Plasma derived mediators		
Kinin system (bradykinin, kallikrein)	Plasma (produced in liver)	Increased vascular permeability, vasodilation, pain, activate PLA_2,hypotension, release of prostanoids, cytokines and free radicals, mast cell degranulation
Coagulation/fibrinolysis system	Plasma (produced in liver)	Endothelial activation, leukocyte recruitment
Complement products(C3a, C3b, C4a, C4b, C5a)	Plasma (produced in liver)	Increased vascular permeability, smooth muscle contraction, leukocyte chemotaxis and activation, vasodilation, mast cell degranulation, stimulation of prostaglandin and leukotriene formation, phagocytosis

Inflammation and eicosanoids:

Among the vast variety of mediators, 'eicosanoids' which include prostaglandins, prostacyclin, thromboxanes and leukotrienes play vital role in the pathophysiology of several chronic inflammatory conditions. Hence, regulation of eicosanoids production is the major focus of research in the development of effective anti-inflammatory drugs.

Production and role of eicosanoids:

The eicosanoids include

i) Prostanoids such as prostaglandins (PGs), prostacyclins and thromboxanes (TXs) produced through the cyclooxygenase (COX) pathway.

ii) Leukotrienes (LTs), lipoxins (LXs), hepoxilins and monohydroxy fatty acids formed by the lipoxygenase (LOX) pathway.

iii) Epoxy and dihydroxy fatty acids derived via cytochrome P450 (CYP) enzymes.

iv) Isoprostanes, isoleukotrienes and other peroxidized fatty acid products are formed non-enzymatically [10].

Eicosanoids are involved in various physiological and pathophysiological inflammatory processes. They are synthesized by different cell types in the body and released in response to appropriate stimuli. The major precursor for the formation of these eicosanoids is the 20 carbon poly unsaturated fatty acid (PUFA), arachidonic acid (AA). Phospholipase A_2s (PLA_2s) are the principal enzymes responsible for the release of AA from membrane phospholipid. PLA_2 acts on the *sn*-2position of membrane phospholipid and liberates lysophospholipid and a free fatty acid which is generally arachidonic acid. The arachidonic acid is further metabolized by downstream enzymes (COX1/2LOXs,) to produce a group of inflammatory lipid mediators known as eicosanoids. COX catalyzes the formation of PGs, prostacyclins and TXs. LTs and LXs are the products of the LOX catalyzed reaction. The PGs and LTs are potent mediators of pain and inflammation. Among the PGs, PGE_2 plays an important role in inflammatory processes and is responsible for the classical inflammatory symptoms such as redness, edema and pain [11]. LTs are both autocrine and paracrine lipid mediators involved in the regulation of innate immune responses. Among the LTs, LTB_4 is involved in leukocyte chemotaxis and cysteinyl LTs (cysLTs) are mediators of allergic and hypersensitivity reactions.

Thus, LOX and COX pathway derived eicosanoids, play a critical role

during inflammatory reactions. Hence, regulation of these eicosanoids through the inhibition of the enzymes (PLA_2, LOX and COX) responsible for their production is the key strategy in controlling several chronic inflammatory diseases.

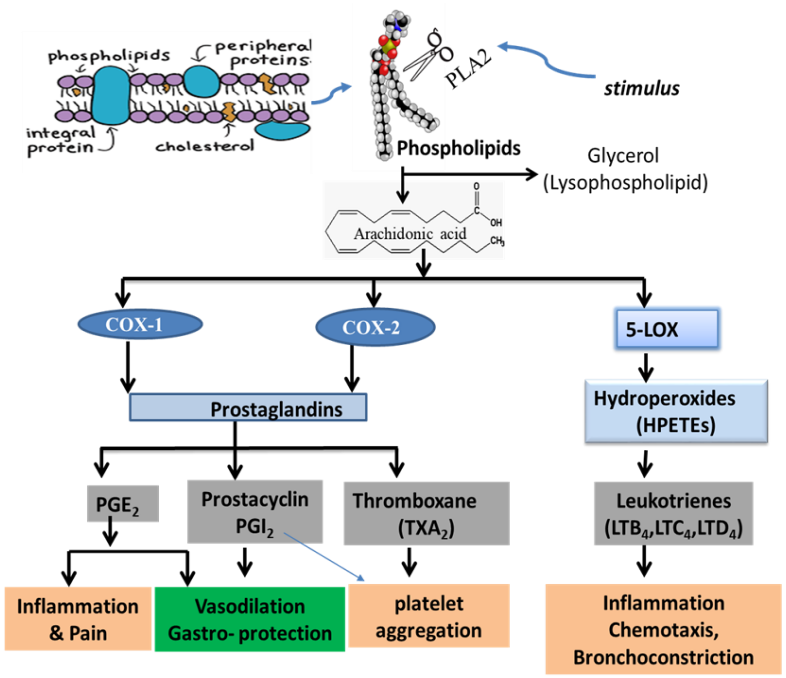

Fig 2: Synthesis of eicosanoids from arachidonic acid.

Lipoxygenases

The lipoxygenase (LOX) catalyze the conversion of arachidonic acid to epoxy intermediate LTA4, a common precursor for leukotrienes and lipoxins [18]. These LOXs are long single chain proteins of molecular mass of upto103 KDa, found in neutrophils, platelets and epithelial cells. Two isoforms of LOXs are encoded by distinct genes have been identified. 5-LOX is known to produce leukotrienes, which are critical mediators of inflammatory response seen in allergy and arthritis. Whereas the action of 15-LOXs has been implicated in both beneficial and deleterious events in human circulation and it is the major protein in biologically programmed degradation of mitochondria in reticulocytes. On the other hand 15-LOX has been implicated in the oxidation of low density lipoprotein (LDL), a critical step in the formation of atherosclerotic lesions, an inflammatory condition [19].

Cyclooxygenases

Cyclooxygenase (COX) enzymes also termed as prostaglandin H synthase or prostaglandin endoperoxide synthase, catalyze the conversion of arachidonic acid to PGH_2, the common precursor of the prostaglandin and thromboxane [8]. Two isoforms of COX encoded by distinct genes have been identified and called as COX-1 and COX-2. The COX-1 enzyme is expressed constitutively in most tissues and cells to control synthesis of prostaglandins, which are required in the maintenance of normal cells and organ function called as 'house keeping' enzyme. In contrast, COX-2 is an intermediate response gene that encodes a 71 KDa protein inducible in certain cells in response to inflammatory stimuli resulting in enhanced prostaglandin synthesis.COX-2 incorporates two oxygen molecules into arachidonic acid a cyclopentane ring to produce PGG_2. The acyclic peroxide group in PGG_2 is cleaved by a hydroperoxidase to yield PGH_2 that can be modified in several ways to produce PGF_2, PGE_2 or PGD_2.

The prostaglandin formed by COX-2 primarily mediates pain and inflammation [12]. COX-2 is induced by a variety of growth factors and is over expressed in rheumatoid arthritis and breast cancer [13-15]. It can be evident from the studies that, inhibition of COX-2 by non-steroidal anti-inflammatory drugs (NSAIDS) provides anti-inflammatory effect, whereas inhibition of COX-1 is responsible for gastric and renal side effects as well as anti-thrombic activity [16-17].

Phospholipases

Phospholipases constitute a class of hydrolases that catalyzes the hydrolysis of acyl esters (deacylase activity) and phosphate esters (phosphodiesterase or phosphomonoesterase, also known as phosphohydrolase or phosphatase activity or sometimes pyrophosphatase activity) on membrane phospholipids (diacylglycerophosphate esters and related compounds). They are named based on the site of the position they hydrolyze glycerophospholipid as phospholipase A_1, A_2, B, C and D. PLA_1 and PLA_2 hydrolyze the phospholipid at *sn*-1 and *sn*-2 position respectively, and release lysophospholipid and a free fatty acid. Phospholipase B (PLB) hydrolyses the phospholipid at both *sn*-1 and *sn*-2 positions. In addition, PLB possesses lysophospholipase activity. Phospholipase C(PLC) hydrolyzes the phosphate bond of phospholipids and releases diacylglycerol (DAG). Phospholipase D (PLD) also acts on the phosphate bond of phospholipid and releases phosphatidic acid. The site of action of different phopholipases is depicted in figure 3. Of these different classes of phospholipases, PLA_2 is very

well studied and has been largely implicated in the pathophysiology of several inflammatory disorders.

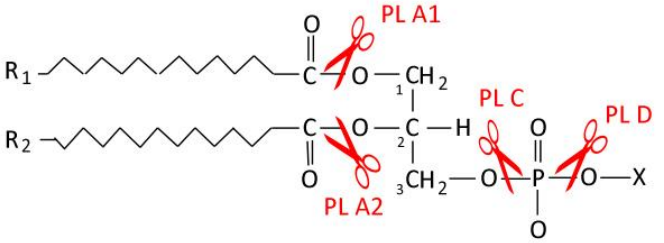

Fig 3: Site of action of different phospholipases on phospholipid.R_1 and R_2 represent fatty acid chain; X represents polar head group such as choline, serine, inositol and ethanolamine.

Phospholipase A_2

Phospholipase A_2s (PLA_2s) (EC 3.1.1.4) are a group of hydrolases that act on the *sn*-2 ester bond of various glycerophospholipids releasing lysophospholipids and free fatty acids (Figure 4), both of which have the potential to alter cell homeostasis. The released free fatty acid, mainly AA in vascularized tissues, is further metabolized by LOX and COX pathways to yield a series of inflammatory lipid mediators collectively known as eicosanoids. While, lysophospholipids serve as the precursors of another class of potent inflammatory mediator, i.e., PAF [20].

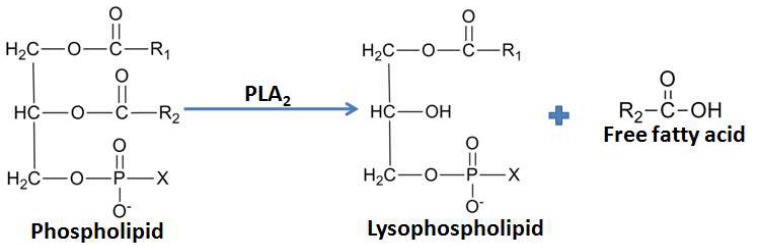

Fig 4: Reaction catalyzed by PLA_2 at the *sn*-2 position of phospholipid. R1 and R2 represent fatty acid chain; X represents polar head group such as choline, serine, inositol and ethanolamine.

Classification of phospholipase A_2

PLA_2s are classified into six families, namely, secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), intracellular calcium independent PLA_2 ($iPLA_2$), platelet activating factor acetyl hydrolase (PAF-AH) or lipoprotein-dependent PLA_2 ($LpPLA_2$), lysosomal PLA_2 ($LPLA_2$) and adipose specific PLA_2

(AdPLA_2). They are further divided into 16 groups and a number of subgroups, each differing in their structure, sequence and mechanisms of action [21]. Apart from these, few PLA_2 like proteins (otoconin 90/95, phospholipase B, patatin domain containing proteins andperoxiredoxin-6) have also been reported. The source, nature, substrate specificity and physiological and pathological functions of each PLA_2subgroup was summarized in table 4. Among this large collection of PLA_2s, secretory PLA_2s (sPLA_2s) are well characterized as the major contributors to the excessive production of pro-inflammatory lipid mediators in several chronic inflammatory conditions.

Table 4: Classification of phospholipase A2

Group	Subgroup	Sources	Size (kDa)	Head group/*sn*-2 specificity	Pathological Functions
Secretory PLA_2 ($sPLA_2$) family:					
I	A.	Cobra, Krait	13-15	PC>PE	AA release, neuronal apoptosis, edema, anticoagulant, cardiotoxicity, neurotoxicity, myotoxicity, hepatotoxicity, lethality, cytotoxicity, inhibition of platelet aggregation
	B.	Porcine pancreas, Human pancreas/ lung/ kidney/spleen/ liver/ovary/ brain	13-15	PG > PE, PS >> PC	Eicosanoid formation, cell contraction, proliferation & migration, diet induced obesity, hepatic steatosis, diabetes, neuronal apoptosis, dry eye disease, hyperlipidemia
II	A.	Rattle snakes, vipers, human synovial fluid/ lung/ spleen/ bone marrow/Platelets/placenta/serum/thymus/chondrocytes/keratinocytes	14	PG, PE, PS >> PC	Acute phase protein, AA release, anticoagulant, pro-inflammatory, cell proliferation & migration, skin abnormality, neuronal apoptosis, alzheimer's disease multiple sclerosis
	B.	Gaboon viper	13-15	-	-
	C.	Rat/Mouse testes/ brain/ pancreas, pseudogene in humans	15	PG, PE>>PC	-
	D.	Rat, mouse, human lymphoid organ/ colon/ pancreas/ thymus/ spleen/skin/lung/ovary/endothelium/ eosinophils/keratinocytes	14	PG, PE > PC	AA release, atherogenic, anticoagulant, degranulation of mast cells, asthma, chronic obstructive pulmonary disease

	E.	Rat, mouse, humanbrain/heart/lung/placenta/thyroid gland/smooth muscle/ uterus	14-15	PG > PE > PC	Atherogenic, ulcerative colitis, chronic rhinosinisitis
	F.	Rat, mouse, humankidney/liver/placenta/testis/ synovial fluid/ thymus/endothelium	16-17	PG,PE>> PC	AA release, atopic dermatitis, colorectal cancer
III		Bees, Lizards, humans heart/kidney/liver/ leukocytes/ placenta /skeletal muscle/ epithelial cells	15-18;55	PG>PC	Inflammatory, atherogenic, cancer, AA release, anaphylactic, dermatitis, induces axonopathy
V		Rat/mouse/ human heart/lung/ placenta/ airway epithelium/ chondrocytes/ eye/ fibroblasts/ pancreas/neutrophils /macrophages/	14	PG > PE, PC > PS	AA release, atherogenic, anticoagulant, phagocytosis, airway injury, myocardial injury, degranulation of mast cells, sepsis, chronic hepatitis
IX		Marine snail	14	-	-
X		Mice, human gut/lung/testis spleen/thymus/airway epithelium and endothelium/macrophages/ keratinocytes/neutrophils/ intestine	14	PC > PE, PS	Inflammatory, pro-/ anti-atherogenic, asthma, cancer, skin abnormality, myocardial injury, arthritis, sepsis
XI	A/B	Green rice shoots (PLA2-1)/ (PLA2-2)	12.4/12.9	-	-
XII	A	Mice, human heart/ brain/ gut/ kidney/liver/pancreas/placenta/ lung /skeletal muscle	19	PG > PS >> PC	Kuhnt-Junius degeneration, malignant glioma
	B	Human gut/kidney/ liver / heart/ skeletal muscle	19	Inactive	Acute pancreatitis

XIII		Parvovirus	<10	-	-
XIV		Symbiotic fungus/bacteria	13-19	-	-
Cytosolic PLA2 (cPLA2) family:					
IV	A (cPLA2α)	Rat kidney, Human U937 cells/platelets /neutrophils	85	PC, PE, PI; high *sn*-2 AA specificity	Cancer, inflammation, Intestinal ulceration, psoriasis, acute lung injury, rheumatoid arthritis, anaphylaxis, brain injury, bone resorption
	B (cPLA2β)	Human brain/heart/ liver/pancreas	114	PC, PE; low *sn*-2 AA specificity	-
	C (cPLA2γ)	Human heart/skeletal muscle	61	PC; low *sn*- 2, AA Specificity	-
	D (cPLA2δ)	Human keratinocyte/ murine placenta	91	PC, PE; *sn*-2 LA specificity	-
	E (cPLA2ε)	Human, murine thyroid/ heart/ skeletal muscle/testis	95	PC, PE; *sn*-2 AA & LA specificity	-
	F (cPLA2ζ)	Human, murine thyroid/stomach/ spleen	95	PC, PE; *sn*-2 AA & LA specificity	-
Intracellular calcium independent PLA2 (iPLA2) family:					
VI	A1(iPLA2)	P388D1 macrophages, CHO cells	85	-	Diabetes, Wallerian degeneration
	A2(iPLA2β)	Human B-lymphocytes, Testis	88		
	B (iPLA2γ)	Human heart/skeletalmuscle/placent	90	-	-

		a/kidney/liver/ brain			
	C (iPLA2δ)	Human/murine neurons	146	-	Oxidant induced cell injury
	D(iPLA2ε), E(iPLA2ζ), F (iPLA2η)	Human	52,55, 27	-	-
VII	A (PAFAH Or Lp-PLA2)	Human/mouse/porcine/bovine Plasma	45	PC & PE; *sn*-2 acetyl group specific	Atherogenic, inflammation, generation of ROS, acute respiratory distresssyndrome, marker of coronary heart disease
	B (PAFAH II)	Human/bovine kidney/liver	40	PC & PE; *sn*-2 acetyl group specific	-
VIII	A (PAFAH 1b α1) and B (PAFAH 1b α2)	Human brain	26	PE; PAF specific	Miller-Deicker lissencephaly
Lysosomal PLA2 (LPLA2) family:					
XV	-	Human/murine/bovine lysosomes	45(deglycosylated)	No head group &*sn*-2 specificity	Phospholipidosis, atherosclerosis, complement activation
Adipose specific PLA2 (AdPLA2) family:					
XVI	-	Human adipocyte	18	-	Obesity, metabolic syndrome

Abbreviations: AA-arachidonic acid; LA-linoleic acid; PC-phosphatidyl choline; PE-phosphatidyl ethanolamine; PG-phosphatidyl glyceerol; PS-phosphatidyl serine; PI – phosphatidyl inositol; PAF- platelet activating factor; ROS-reactive oxygen species. (Table was adapted and revised from 21-40)

***Secretory phospholipase A_2* (sPLA$_2$)**

sPLA$_2$ is largest family of PLA$_2$ enzymes, comprising more than one third of the PLA$_2$ super family groups. The sPLA$_2$, purified and characterized from cobra venom (sPLA$_2$groupIA or sPLA$_2$IA) was the first enzyme reported to exhibit PLA$_2$ activity. The enzyme was subsequently characterized from viper venom (sPLA$_2$IIA) and mammalian pancreatic juice (sPLA$_2$IB). The first mammalian non-pancreatic sPLA$_2$ was purified, sequenced and cloned from the synovial fluid of arthritic patients (sPLA$_2$IIA) and was tagged as inflammatory PLA$_2$ (54-55). Since then sPLA$_2$IIA has become the major target in regulating the chronic inflammatory diseases and snake venom induced complications. Consequent research has lead to the discovery of several sPLA$_2$ subgroups. Currently, sPLA$_2$ family includes 10 major groups (Group I - III, V and IX - XIV) and 18 subgroups from a wide spectrum of organisms (mammals, reptiles, molluscs, insects, plants and bacteria). These are all low molecular weight proteins (13-19 kDa, except human sPLA$_2$III which is 55 kDa), requiring mili molar concentration of calcium and with an unusual number (6-8) of disulphide linkages. Individual sPLA$_2$s exhibit unique tissue and cellular localizations and specific enzymatic properties. In order of their discovery, human sPLA$_2$s are grouped and numbered as sPLA$_2$IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB.

Mechanism of sPLA$_2$ catalysis

Catalytic mechanism of sPLA$_2$ can be divided into four steps:

(i) Binding of calcium and substrate,

(ii) Generation of base mediated attack on bound substrate

(iii) Formation and collapse of tetrahedral intermediate, and

(iv) Release of product.

The calcium ion present in the active site of sPLA$_2$ is coordinated by two oxygen atoms of the carboxylate group of Asp 49; three oxygen atoms from the calcium binding loop and two conserved water molecules. Catalysis begins with binding of substrate (phospholipid) to the enzyme. A proton is transferred from water molecule to the His 48 of sPLA$_2$ and the resulting hydroxyl group attacks the electrophilic carbonyl carbon of the ester bond at *sn*-2 position of the phospholipid. Subsequently, the proton on His 48 is transferred back to the phospholipid ester oxygen which results in the cleavage of ester bond and product release. Three water molecules move into the active site to release the product.

Significance of sPLA$_2$ IIA

Amongst this large group of PLA$_2$s, sPLA$_2$IIA appears to be the rate limiting enzyme in chronic inflammatory conditions and hence the preferred target for the development of effective anti-inflammatory drugs. This is because

i) sPLA$_2$IIA is present both in humans and snake venoms and is responsible for the adverse inflammatory reactions in various clinical conditions and in snake venom induced pathological events. Hence targeting this enzyme has a dual beneficial effect.

ii) Several other studies showed that sPLA$_2$ and/or sPLA$_2$ products activatecPLA$_2$ [40-42]. In human astrocytoma cell line 1321N1 [43-44], murine bone marrow derived mast cells, rat renal mesangial cells, human mast cells and neutrophils [45-47] sPLA$_2$ induce cPLA$_2$ activity and eicosanoid production. In mast cells and neutrophils, sPLA$_2$IIA can activate cPLA$_2$ through mitogen activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK) ½ [48].

iii) sPLA$_2$IIA mediates cytokine induced delayed AA release and ionophore induced immediate AA release, which is generally employed for the production of pro-inflammatory lipid mediators [49].

iv) sPLA$_2$IIA, IID and V preferably cleave phospholipids with charged head groups such as PE and PS which are buried inside [50-51]. Consequently,sPLA$_2$X and V can efficiently hydrolyze PC rich phospholipids from the outer leaflet of the plasma membrane and release AA under resting conditions.

v) Due to its preference for anionic substrates, sPLA$_2$IIA can also act on apoptotic cells or micro-vesicles shed from activated cells, contributing tothe progression of inflammatory condition [52].

vi) Apart from sPLA$_2$ IIA, sPLA$_2$IB, sPLA$_2$V and sPLA$_2$X are gaining attention as mediators of inflammation (Table 4). sPLA$_2$IB is involved in diet induced hyperlipidemia, obesity, diabetes and atherosclerosis, whereas, sPLA$_2$V has a prominent role in allergic airway inflammation, lung injury and atherosclerosis. Similarly, expression of sPLA$_2$X is directly associated with cell proliferation and metastasis of lung, colon and breast cancer. However, the *in vitro* assay systems for these new sPLA$_2$s are not well established and hinder the inhibitor screening process. Since sPLA$_2$I, II, V and X belong to one structural class, inhibitor of sPLA$_2$IIA might be

effective in targeting other $sPLA_2$s also.

Role of $sPLA_2$IIA in inflammation

Highly elevated concentration of $sPLA_2$IIA is found in patients suffering with inflammatory diseases such as septic shock and bacterial infections [53-54], liver cirrhosis [55], acute pancreatitis [56], multiple organ failure [57], trauma [54], rheumatoid arthritis [58], and ulcerative colitis [59], neurological disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [60-62]. Nevalainen *et al.,* (2005) showed that in the serum of patients suffering from septic infections, $sPLA_2$IIA is markedly elevated [53]. The free fatty acids and lysophospholipids released by the action of $sPLA_2$IIA affect the function and properties of vascular endothelial cells, smooth muscle cells and macrophages. Global registry of acute coronary events demonstrated that plasma $sPLA_2$IIA activity is an independent predictor of death in patients with acute coronary syndromes [64-69].

The other product of $sPLA_2$IIA, lysophospholipid, participates in cell signaling, phospholipid remodeling and membrane perturbation. Lysophospholipid activates polymorphonuclear leukocytes (PMNLs) and makes them permeable through monolayer of endothelial cells [70]. They are involved in the stimulation of histamine secretion by mast cells, act as growth factors (lysophosphatidic acid, LPA), and induce tissue damage, proliferation of cancer cells and tumor metastasis. They are also precursors for a powerful inflammatory mediator, PAF.

Free radicals, $sPLA_2$ IIA and inflammation

Oxidant or free radicals generation is a part of the normal metabolism of many types of cells and is critical for the cell homeostasis. A free radical can be defined as any molecular species capable of independent existence that contain an unpaired electron in an atomic orbital. Free radicals include reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$), hydroperoxyl radicals ($H_2O\cdot$), peroxide radicals (ROOH), peroxyl radicals ($ROO\cdot$), alkoxyl radicals ($RO\cdot$), hypochiorous acid ($HOCl^-$), and reactive nitrogen species (NOS) such as nitric oxide ($NO\cdot$), nitrogen dioxide ($NO_2\cdot$) and peroxynitrite ($ONOO\cdot$)(Figure 5) [71].

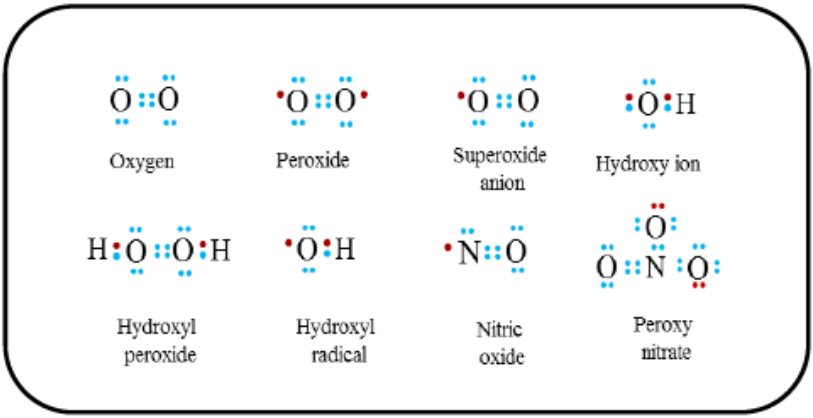

Fig 5: Electronic structures of common reactive species *(Adapted from Held, 2012)*

Metabolism of arachidonic acid by LOX and COX pathway is one of the major sources for the generation of free radicals. Free radicals are produced as highly reactive intermediates or as biproducts during the conversion of arachidonic acid into stable eicosanoids. Major radical species produced through COX and LOX pathway include hydroxyl radicals, alkyl radicals, peroxyl radicals, hydroperoxyl radicals, superoxide radicals, toxic aldehydes such as malondialdehyde (MDA). These free radicals are advantageous in the biological system as they assist in the destruction of invading pathogens. They are also involved in several physiological functions such as cell proliferation, migration, adhesion and survival. However, when there is an overproduction of free radicals or when the antioxidant defence systems are destabilized, these radicals can alter the homeostasis. They induce undesirable oxidation causing membrane damage, protein modification and DNA damage which ultimately leads to cell death. During chronic inflammatory diseases increased levels of pro-inflammatory lipid mediators along with free radicals augment the condition by several folds [72-74]. Free radicals have been implicated in several inflammatory disease conditions such as asthma, atherosclerosis, arthritis, cardiovascular disease, cancer, diabetes, inflammatory bowel disease, ischemia/reperfusion injury, liver disorders, neurological disorders, pancreatitis etc,. [71-72, 75-76].

The free radicals generated through AA metabolism in turn activate $sPLA_2IIA$ and enhance membrane lipid peroxidation. Increased membrane lipid peroxidation destabilizes membranes and alters calcium homeostasis. Also, increased calcium mobilization stimulates phospholipid hydrolysis through calcium dependent $sPLA_2IIA$ activation. $sPLA_2IIA$ in turn activates

the downstream reactions and generates a pool of pro-inflammatory eicosanoids and free radicals which further worsen the situation and results in chronic inflammation. Hence, regulation of AA pathway enzyme ($sPLA_2IIA$) and free radical production is an important strategy to control chronic inflammatory disorders.

Snake Venom $sPLA_2$

$sPLA_2$s are not restricted to mammals only but are also found in snakes, bees and lizards. While mammalian $sPLA_2$s are generally non-toxic, snakes and insects contain both toxic and non-toxic $sPLA_2$s. Mortality due to snake bite is indeed an important yet neglected public health hazard in many tropical and subtropical countries and the lethal effect is mainly attributed to $sPLA_2$s present in snake venom [77]. $sPLA_2$ present in the venom of elapidae (eg., *Naja naja*) and hydrophidae (eg., *Pelamis platurus*) family snakes belongs to group IA ($sPLA_2IA$), venom of viperidae (eg., *Vipera russelli*) family snakes contain group IIA $sPLA_2$ ($sPLA_2IIA$) and bee and lizard venom $sPLA_2$s represent group III ($sPLA_2III$).

These venom $sPLA_2$s can act locally as well as systemically and induce variety of pharmacological effects such as pre- and post-synaptic neurotoxicity, hemostatic alterations, edema induction, myonecrosis, hemorrhage, hypotension, cytotoxicity, apoptosis and cardiotoxicity [78-79]. The inflammatory events triggered by snake venom $sPLA_2$s are initially attributed to their catalytic activity which releases AA and lysophospholipid. However, later it was confirmed that these $sPLA_2$s contain an additional site, known as pharmacological site, which is distinct from the catalytic site. Snake venom $sPLA_2$s interact with certain membrane receptors with high affinity through this pharmacological site. This interaction targets the enzyme to a specific organ/tissue. Upon binding to the target, $sPLA_2$ induce its pharmacological effects either dependent or independent of catalytic activity [32]. Hence it is important to target both the sites for the effective management of snake bite complications. Because of their abundance, simple purification procedures and a high degree of structural similarities compared with human $sPLA_2$, snake venom $sPLA_2$s are widely used to understand the mechanism of action of human inflammatory $sPLA_2$ and in the design/development of new anti-inflammatory drugs [80, 81].

Targeting inflammation and current anti-inflammatory approaches

Large body of literature suggests that AA and its metabolites are involved in the multi-factorial inflammatory condition either directly or indirectly. Hence, suppression of eicosanoid production by specifically targeting key

enzymes such as LOX and/or COX has long been considered as a therapeutic strategy. Identification of salicylate as the active compound in the willow bark and its subsequent synthesis opened up a new class of drugs called non-steroidal anti-inflammatory drugs (NSAIDs) [82]. These NSAIDs are currently the best selling drugs in the market.

Though treatments with these non-specific inhibitors of COX - 1/2 quickly resolve inflammation, fever and pain, their long term use during chronic disease conditions is associated with severe side effects such as gastrointestinal injury, hypercalemia, interstitial nephritis, proteinuria and acute renal dysfunction due to the suppression of housekeeping eicosanoids which are produced from COX-1 [83]. These undesirable effects of NSAIDs prompted researchers to look for an alternative and shifted the interest towards the development of COX-2 specific drugs called coxibs [84-85].Coxibs were initially found to be potent anti-inflammatory drugs due to their lack of/reduced gastrointestinal and bleeding complications [86-87]. However, long term use of coxibs was later found to be associated with increased cardiovascular risk. This is mainly because of imbalance in antithrombotic and vasodilatory PGI_2, a COX- 2 product and pro-thrombotic TXA_2 which is a COX-1 product [88-91]. Hence, these drugs are either withdrawn from the market (e.g. Rofecoxib or Vioxx in 2004; Valdecoxib and Parecoxib in 2005) or their use has been restricted (e.g. Celecoxib).

In addition to COX pathway, attention is also given to 5-LOX which produces LTs, which are involved in the pathogenesis of asthma and other allergic conditions. The discovery of 5-LOX specific inhibitors was not much successful due to their poor bioavailability. Zileuton is the only 5-LOX specific inhibitor available in the market to treat asthma with high restrictions due to its liver toxicity [92]. This strategy of selective inhibition of either COX or LOX enzyme met with a setback with the finding that inhibition of either the pathway results in enhanced production of other enzyme products. That is, inhibition of COX pathway leads to increased production of LTs through LOX pathway and vice versa [41]. For example, aspirin-like drugs effectively block PG production from AA. However, this blocking of COX products results in a shift in the AA pathway towards increased production of LOX products such as LTs which results in edema, bronchospasm and gastrointestinal problems [93]. In a recent study by Chong He *et al.*, (2012), when indomethacin was used to block PG synthesis, the level of LTB_4 (2.2 times), 5-HETE (1.8 times) and 12-HETE markedly elevated compared to control. Similarly, when zileuton (a 5-LOX specific inhibitor) was used to block LT synthesis, there is an increase in the level of PGE_2 (3.3 times), TXB_2 (7.5 times) and PGD_2 [94]. Production

of LTB_4 was stimulated in human osteoarthritic synovial explants incubated with naproxen, a COX inhibitor [95]. Intake of NSAIDs stimulated the formation of LTs and caused side effects such as aspirin triggered asthma in patients [96]. Likewise, production of PGE_2 and TXB_2 from 5-LOX-/- mice stimulated with A23187 was greater compared to control [97]. The present anti-inflammatory approaches are listed in table 5

Table 5: Present anti-inflammatory treatment approaches

Treatment approach	Description
Steroids	Like the glucocorticoid cortisol.
Non-steroidal anti-inflammatory drugs (NSAIDs)	The NSAIDs achieve their effects by blocking the activity of cyclooxygenase (COX). NSAIDs also inhibit clotting. They do this by interfering with the synthesis of thromboxane A_2 in platelets.
COX-1 and COX-2	COX-2, which is involved in the pain produced by inflammation. COX-2 inhibitors are effective against inflammation and seem to avoid damage to the GI tract. But, unfortunately, they increase the risk of blood clots - which can cause heart attacks and strokes -because they do not block the synthesis of thromboxane A_2 by platelets (which contain only COX-1).
Therapeutic Proteins	Recombinant DNA and monoclonal antibody technology have produced some new therapies against damaging inflammation. An IL-1 antagonist that binds and inactivates the IL-1 receptor. It binds TNF-α preventing it from carrying out its many inflammatory actions. Potent but carries a severe risk of allowing infections to develop.

PLA_2 inhibitors as anti-inflammatory drugs

Though arachidonic acid can be released by the action of various enzymes, PLA_2 appears to be the main enzyme responsible for its release. Inhibition of $sPLA_2$ not only blocks eicosanoid production, but also prevents the synthesis of lysophospholipids and thus production of PAF (Figure 2). Accordingly, inhibition of PLA_2 could result in suppression of several important classes of pro-inflammatory lipids like PGs, LTs, lysophosphatidate and PAF. Hence the use of PLA_2 inhibitors has been considered an attractive therapeutic strategy in the treatment of inflammation related diseases and tissue injury (113). Presence of$sPLA_2$ ($sPLA_2$IA and IIA) in the snake venom and research findings supporting their toxic effects in bitten victims made

$sPLA_2$ inhibition even more attractive. A large number of synthetic and natural molecules, including various classes of phytochemicals, endogenous proteins/metabolites and molecules from plants and marine organisms have been screened for $sPLA_2$ inhibition [21, 26, 61-62, 99-105]. However, even after decades of research, there is not even a single $sPLA_2$ specific inhibitor available in the market to treat chronic inflammatory disorders.

Two highly potent and selective inhibitors of $sPLA_2$, namely varespladib (LY315920), varespladib methyl (LY333013) and darapladib (SB-480848) exhibited excellent anti-inflammatory effects in several pre-clinical studies [106-107]. However, varespladib methyl previously reached Phase II clinical trials by Lilly and Shionogi for the treatment of severe sepsis, rheumatoid arthritis and bronchial asthma [108] recently terminated from the phase III VISTA-16 (Vascular Inflammation Suppression to Treat Acute Coronary Syndrome for 16 Weeks) clinical trial, where it was administered to treat patients with cardiovascular diseases [109-110]. Darapladib had failed to meet endpoints of phase III in a trial of 16,000 patients with acute coronary syndrome. Further, darapladib failed in clinical trial with 13,000 patients to reduce the risk of coronary heart disease death [111-112].

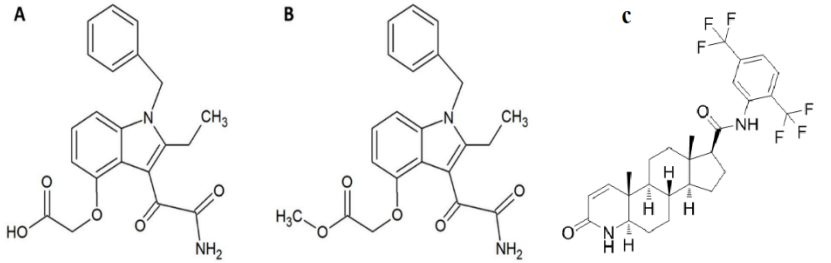

Fig 6: Structure of PLA_2 inhibitors A) varespladib,B) varespladib methyl and C) darapladib

Natural products as a source of drugs

Natural products are being used as a source of therapeutically effective medicines from time immemorial. It is estimated that approximately 70% of the world's population rely almost entirely on plant based medication [116]. Natural products offer greater structural diversity than the products obtained using standard combinatorial chemistry and hence can be used as lead structures against a wide range of targets [117]. Nearly 50% of drugs approved since 1994 are based on natural products. Thirteen natural product based drugs have been approved from 2005 to 2007 and 5 among these are the first members of new classes of drugs [118].

Currently, more than 100 natural products derived compounds are undergoing clinical trials and similar numbers of compounds are under pre-clinical studies. Paclitaxel (against ovarian & breast cancer), arteether (anti-malarial), galanthamine (to treat Alzheimer's disease), tiotropium (to treat chronic obstructive pulmonarydisease), vinblastine and vincristine (anticancer) are some of the plant-derived drugscurrently available in the market. Morphine-6-glucuronide (pain killer), exatecan (anti-cancer), vinflunine (anti-cancer) are all in phase III clinical trials while calanolide A (anti-HIV), forskolin (to treat glaucoma), guggulsterone (antihyperlipidemic) are few representative plant based drugs currently in phaseI/II clinical trials [239-241].

Along with their use as drugs to treat many disorders, natural products also assist in the discovery of many physiological aspects. The role of sodium-potassium ATPase was revealed by digoxin from *Digitalis purpurea*; action of endogenous opioids on receptors was discovered by morphine; different types of acetylcholine receptors were explored with the help of muscarine, nicotine and tubocurarine [119-120]. In similar lines, there are several natural molecules affecting the AA pathway enzymes/mediators. Curcumin and Epigallocatechin-3-gallate (EGCG) targets 5-LOX, COX-1, mPGES-1, and NF-κB signaling [121-122].

Along with phytochemicals, molecules from marine source and few metabolites produced in our body also inhibit various enzymes of AA pathway and exhibit strong anti-inflammatory activities. The antioxidant property of these biomolecules and the mechanisms by which they inhibit AA pathway enzymes has been studied and reviewed by several research groups and is summarized in table 6.

Table 6: Natural molecules interfering with AA cascade enzymes/mediators

S. No.	Molecule	Inhibition of AA pathway enzymes		Antioxidant activity (IC_{50})	*In vivo* activity (IC_{50})
		sPLA$_2$ source (IC_{50})	**LOX and COX (IC_{50})**		
Alkaloids					
1	Aristolochic acid	HSF (85 µM), human neutrophil, human platelet, porcine pancreas, *N. naja*, *T. flavoviridis*, *V. russelii*, *D. r.pulchella*	Inhibits LTB_4& 5- HETE synthesis	-	Inhibits *V. russelii* &*T. flavovirid* is PLA_2 induced mouse paw edema
2	Ajmaline	*N. naja*	Inhibits LOX & COX activity	-	Inhibits carrageenan-induced rat paw edema
Terpenes					
3	Ursolic acid	HSF (12 µM), HPF (12µM), *V. russelii* (12 µM),*N. naja* (18 µM)	Inhibits COX-2 activity (130 µM) & expression	-	Inhibits *V. russelii* &*N. naja*, HPF, HSF PLA_2 induced mouse paw edema
4	Oleanolic acid	HSF (3.49 µM), *V. russelii* (3.08µM), HPF (6.74 µM), *N. naja* (7.78µM)	Inhibits 5-LOX, COX-1 (28.6µM) & COX-2 (63.1µM)activities & COX-1 induction	Scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits iNOS induction and *V. russelii* PLA_2 induced mouse paw edema
5	Retinoic acid	HSF	Inhibits COX-2 expression & PGE_2	-	-
			Production		
6	Retinol acetate	HSF	Inhibits COX-2 expression & PGE_2 Production	-	-
7	Masticadienolic acid	Porcine pancreas (0.016 mole fraction), HSF (0.05 mole fraction), Bee venom (0.01 molefraction)	Inhibits 5-LOX activity	-	-

8	Glycyrrhetinic acid	HSF (30µM)	Inhibits COX-1 induction		Scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits iNOS induction
9	Glycyrrhizin	HSF	Inhibits PGE_2 production		Inhibits H_2O_2 & hydroxyl radicals NO production;	-
10	Betulinic acid	Bovine pancreas	Inhibits COX-1 & COX-2 activities		-	Inhibits carrageenan induced mouse paw edema & TPA induced mouse ear edema
Polyphenols						
11	Ferulic acid	*C. d. cumanensis*(3.930 mM)	Inhibits COX-2 expression	Exhibits trolox equivalent antioxidant activity		-
12	Caffeic acid	*C. d. cumanensis*(1.4 mM)	Inhibits 5-LOX activity (3.7 µM)	Exhibits trolox equivalent antioxidant activity		-
13	Ellagic acid	HSF, HPF, *V. russelii*, *N. naja*	Inhibits COX-1 & iNOS induction	Inhibits tyrosinase & xanthin oxidase, scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals		Inhibits $sPLA_2$ induced mouse paw edema
14	Protocatechuic Acid	Human recombinant PLA_2-V (3.3 mM)	Inhibits COX-2 activity	-		Inhibits iNOS expression
15	Quercetin	HSF, human plasma, *N. mossambica*, C. d. terrificus, *N. naja*, *V. resselii* (2 µM)	Inhibits 5- & 12- LOX, & COX activities, TXB2 & PGE_2 production, COX-2 expression	Inhibits lysosomal enzyme release from PMNLs, elevates cellular GSH content; NO production & lipid peroxidation; scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals		Reduce histamine & eosinophil peroxidase levels & neutrophil/ eosinophil infiltration, eNOS activity, IL-1β, IL-6, TNF-α production, iNOS expression & NF-κB activation
16	Myricetin	Porcine pancreas, C. atrox	Inhibits 5- & 12- LOX,	Scavenges hydroxyl radical		Inhibits iNOS expression

			& COX activities, & COX-2 expression		& NF-κB activation
17	Rutin	*V. russelii* (250 µM), *C. atrox* (250 µM), HSF, *N. naja*, porcine pancreas	Inhibits COX-2 activity & expression	Scavenges hydroxyl radicals; reduces PMNLs chemotaxis	Inhibits carrageenan induced rat paw edema; reduce histamine and eosinophil peroxidase levels & neutrophil /eosinophil infiltration; inhibits TNF-α production & iNOS expression
18	Morin	HSF, C. d. cascavella	Inhibits 5 & 12 LOX, COX-2 activities & expression	Inhibits NO production; scavenges hydroxyl radical	Inhibits IKK-β activation
19	Apigenin	Porcine pancreas, C. atrox	Inhibits COX activity & expression, platelet aggregation & human 15-LOX (4 µM) activity	Inhibits NO production; scavenges hydroxyl radical	Inhibits IL-6, IL-8, IL-4, IL-13 production, NF- κB activation & iNOS expression
20	Baicalein	$sPLA_2$IIA (14.2 µM)	Inhibits 5-LOX activity & LTC4 production	Decrease NO production	Inhibits IL-1β, IL-6, IL- 8, TNF-α production
21	Baicalin	$sPLA_2$IIA (12.9 µM)	Inhibits PGE_2 production	Decrease NO production	Inhibits LTB4, IL-1β, IL-6, TNF-α, IFN-γ, MCP-1, MIP-1 production
22	Wogonin	$sPLA_2$IIA (10.8 µM)	Inhibits COX-2 activity &expression, 12-LOX activity	Inhibits NO production	Inhibits IL-1β, IL-6, IL- 8, TNF-α production, NF-κB activation &iNOS expression
23	Catechin	Porcine pancreas, C. atrox	Inhibits COX activity	Elevates cellular GSH content;	Inhibits NO & TNF

				scavenges hydroxyl radicals; increases SOD, catalase & GST activity	production in LPS-stimulated primary macrophages
24	Epigallocatechingallate (EGCG)	*C. d. cumanensis* (380 µM)	Inhibits 5-LOX & COX activities, COX-2/PGE_2production & COX-2 expression	Decrease NO production; scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits TNF-α, IL-6, IL- 8, VEGF production, iNOS expression & NF- κB activation
25	Hesperetin	Porcine pancreas, C. *atrox*	Inhibits COX-2 expression	Exhibits trolox Equivalent antioxidant activity	Inhibits TNF-α Production
26	Naringin	C. d. cascavella	Inhibits LOX & COX activity	Inhibits ROS production	Inhibits NF-κB activation
27	Cyanidin	Human recombinant PLA_2-V	Inhibits COX-2 expression	Exhibits trolox equivalent antioxidant activity	-
28	Peonidin	Human recombinant PLA_2-V	Inhibits COX-2 expression	-	-
29	Delphinidin	Human recombinant PLA_2-V	Inhibit COX-2 expression	Exhibits trolox equivalent antioxidant activity	-
30	Genistein	HSF (5.75 µM), HPF (9.03 µM), *V. russelii* (11.75 µM), N. naja (11.12 µM)	Inhibits 5-LOX (125 µM), soy LOX (107 µM) & COX-1activities, LTC4 production (80 nM), COX-2 activity & expression	Inhibits NO production & lipid peroxidation; scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits IL-1β, IL-6, TNF-α production, iNOS expression & NF-κB activation; inhibits V. russelii PLA_2 induced mouse paw edema
31	Herpalycin 2	*B. pirajai* (PrTX-III) (11.34 µg/ml)	Inhibits AA induced platelet aggregation	-	-
32	Tectoridin	*V. russelii*& Bovine pancreas	Inhibits PGE2 production & COX-2 Expression	-	-

33	Ochnaflavone	Rat platelet (3.25 µM),HPF (3 µM), Porcine pancreas (20 µM)	Inhibits 5-LOX activity	Scavenges H2O2	Inhibits iNOS expression
34	Ginkgetin	HSF (0.9 µM), HPF, ratplatelet, porcine pancreas	Inhibits COX-2 expression, COX-1 & 5-LOX activities, PGE_2, PGD_2 (0.75µM) & LTC_4 (0.33µM) generation	Decrease NO production (2 µM), scavenges H_2O_2	Inhibits TPA induced mouse ear edema; iNOS & IL-1β expression; TNF-α synthesis
35	Bilobetin	HSF (1.2 µM), HPF, ratplatelet, porcine pancreas	Inhibits COX-2 expression	Inhibits iNOS expression & NO production (2.7 µM); scavenges H_2O_2	Inhibits TNFα synthesis
36	Papyriflavonol A	Human $sPLA_2$IIA (3.9 µM), $sPLA_2$V (4.5 µM), $sPLA_2$X (225 µM),porcine pancreas (76.9 µM)	Inhibits 5-LOX (7 µM) activity & LTC_4 production (0.65 µM)	-	Inhibits Passive Cutaneous Anaphylaxis (PCA) reaction in rats
37	Resveratrol	D. r. pulchella (43.4 µM)	Inhibits LOX, COX-1/2 activities, COX-2 expression	Decreases MPO activity; inhibits lipid peroxidation; elevates cellular GSH content; scavenges HOCl, DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits iNOS expression & NF-κB activation
38	Polydatin (Polygonin)	$sPLA_2$IIA	Inhibits PGE_2 production & COX-2 expression	Inhibits NO production	Inhibits TNF-α production, iNOS expression & NF-κB Activation
39	1,3,5-trihydroxy benzene (Phloroglucinol)	*C. adamanteus*	Inhibits COX activity & TXA2 production	Scavenges DPPH, H_2O_2& hydroxyl radicals	Inhibits ERK/p38 phosphorylation
40	1,3-dihydroxy benzene(Resorcinol)	*C. adamanteus*	Suppress COX-2 promoter activity	-	-
41	Propylgallate	*C. d. cumanensis* (1.84 mM)	Inhibits PGE*2*	Reduces ROS generation	Inhibits TNF-α production,

			production & COX-2 expression		iNOS& ICAM-1 expression
42	Curcumin (Diarylheptanoid)	*V. russelii*& bovine pancreas	Inhibits COX-1 (50 µM), COX-2 (> 100 µM) & 5-LOX (0.7 – 30 µM) activities	Decreases MPO activity & lipid peroxidation; scavenges DPPH, superoxide anion, hydroxyl & peroxyl radicals	Inhibits TNF-α production, NF-κB activation &iNOS expression
43	Rosmarinic acid	*B. jararacussu* (BthTXII &BthTXI), B. pirajai (PrTX-I)	Inhibits 5-LOX activity	-	-
Fatty acid					
44	Linoleic acid	Porcine pancreas	Inhibits COX-1/2 Activities	-	-
Endogenous antioxidants					
45	α-lipoic acid	HPF (0.75 µM), Human serum (0.8 µM), *V. russelii* (3 µM), *N. naja* (3 µM)	Inhibits PGE_2 production	-	Inhibits *V. russelii* PLA_2 induced mouse paw edema
46	Vitamin E	*D. r. pulchella*, *B. jararacussu* (BthA-I- PLA_2)	Inhibits COX-2 transcription, synthesis & activity, LTB_4 production, 5-LOX translocation	Inhibits lipid peroxidation & intracellular Ca^{2+} influx	-
Marine bioactives					
47	Avarol	HSF (158 µM)	Inhibits LTB_4 (0.6 µM) and TXB_2 (1.4 µM) release from A23187 stimulated rat peritoneal leukocytes	-	Inhibits carrageenan induced mouse paw edema, TPA induced mouse ear edema & superoxide generation
48	Bolinaquinone	Porcine pancreas (0.4 µM),	Inhibits synthesis &	Inhibits neutrophil degranulation, NO	Inhibits TPA induced

		HSF (0.2 µM), bee venom (0.1 µM)	release of LTB_4 (2.1 µM), PGE_2 production, 5-LOX activity (1.3 µM), COX-2 expression	& superoxide production	mouse ear edema, carrageenan induced mouse paw edema, neutrophil infiltration,IL-1β &iNOS expression
49	Cacospongionolide B	Porcine pancreas (4 µM), HSF (4.3 µM)	Inhibits 5-LOX & COX-1 activity	Inhibits NF-κB activation	Inhibits TPA induced mouse ear edema
50	Epitaondiol	HSF (63.4 µM)	Inhibits LTB_4 (30.1 µM) & TXB_2 (3.8 µM) production	Inhibits MPO activity (17.8 µg/ear)	Inhibits TPA induced mouse ear edema (20.7 µg/ear)
51	Fucoidan	Myotoxic PLA_2s from crotaline snake venom, human $sPLA_2$IIA (45 µg/ml	Inhibits PGE_2 production & COX-2 expression	Inhibits NO production	Inhibits IL-1β & TNF-α production, iNOS& MCP-1 expression; suppresses NF-κB activation
52	Ircinin	N. naja (5.5 µM), HSF (3.1 µM), Bee venom (3.5 µM), rat air pouch PLA_2 (0.2 µM)	Inhibits LTB_4 release (1.3 µM) & LTB4synthesis (15.7 µM)	Inhibits superoxide generation (40.1 µM) & MPO activity	Inhibits TPA induced mouse ear edema (51 µg/ear)
53	Manoalide	N. naja (1.9 µM), bee venom (0.1 µM), HSF (3.9 µM)	Inhibits 5-LOX (0.3 µM) & COX-2 activity, LTB_4& LTC_4 production	-	Inhibits PLC &iNOS activity
54	Petrosaspongiolide M	HSF (1.6 µM), bee venom (0.6 µM)	Inhibition 5-LOX activity & PGE_2 production	Inhibits NO production	Inhibits iNOS activity, TNF-α production & NF-κB activation
55	Pseudopterosin A	Pancreatic PLA_2 (3 µM)	Inhibits PGE_2& LTC_4 synthesis	-	Inhibits PMA induced mouse ear inflammation
56	Scalaradial	*N. naja* (3.9 µM), porcine	Inhibits LTB_4 release	Inhibits superoxide generation,	Inhibits TPA induced

		pancreas (2.4 µM), HSF (0.5 µM), bee venom (3.6 µM), Rat air pouch $sPLA_2$ (3.2 µM)	(0.1 µM) & LTB_4synthesis (1.4 µM)	neutrophil degranulation & MPO activity	mouse ear edema & PMA induced AA release in the mouse peritoneal macrophage
57	Stypotriol Triacetate	HSF	Inhibits eicosanoid production	Inhibits MPO activity	Inhibits TPA inducedmouse ear edema
58	Variabilin	Porcine pancreas (20 µM), HSF (6.9 µM), bee venom (26.4 µM), Rat air pouch $sPLA_2$ (15.1 µM)	Inhibits LTB_4 production & neutrophil degranulation	Inhibits superoxide production	Inhibits TPA induced mouse ear edema, carrageenan inducedmouse paw edema.
Miscellaneous molecules					
59	Pinitol (Cyclic polyol)	*N. naja* (1.92 mM)	Inhibits COX-2 expression	-	Inhibits NF-κB activation
60	Nordihydroguaiaretic acid (NDGA)	Porcine pancreas	Inhibits 5-LOX activity (28 µM)	-	-

Abbreviations: HSF – human synovial fluid; HPF – human pleural fluid; MCP-1 - monocyte chemoattractant protein-1; DPPH – 2,2-diphenyl-1-picrylhydrazyl; TPA - 12-O-tetradecanoylphorbol-13-acetate; PMA - Phorbol 12-myristate 13-acetate; NO – nitric oxide; MPO – myeloperoxidase; iNOS – inducible nitric oxide synthase; eNOS – endothelial NOS; NF-κB – nuclear factor kappa B: ROS – reactive oxygen species; SOD – superoxide dismutase; ICAM – intracellular adhesion molecule; PMNL – polymorphonuclear leukocyte; GSH - glutathione.

Conclusion

Inflammation is a sequence of complex reactions elicited by the host immune system in order to ward off various infectious agents. Arrays of bioactive molecules are produced in this event by various cells of the body which mediate multiple reactions. Eicosanoids which comprise prostaglandins, prostacyclins, thromboxanes, leukotrienes and lipoxins are major lipid mediators amongst them. Arachidonic acid (AA) which is produced by the action of secretory phospholipaseA_2IIA (sPLA_2IIA) at the *sn*-2 position of membrane phospholipid is the major precursor for the synthesis of eicosanoids. The downstream enzymes, namely 5-lipoxygenase (5-LOX) and cyclooxygenase (COX-1/2) further act on AA and generates eicosanoids. Numerous species of free radicals are also produced in this cascade of events either as intermediates or as by products which further worsen the situation. Hence, inhibition of these sPLA_2IIA by antioxidants is an important strategy in the regulation of eicosanoids production and subsequent regulation of chronic inflammatory disorders. Natural molecules are useful tool in this regard due to reduced side effects compared to synthetic molecules.

During oxidative stress many reactive oxygen species and oxidized lipids stimulated PLA_2 enzyme activity. On the other hand, several antioxidants isolated from medicinal plants, which are capable of neutralizing the reactive oxygen species and showed anti-inflammatory properties by inhibiting the PLA_2 enzyme. To combat the effects of free radicals generated during our metabolic process organisms has developed many powerful endogenous antioxidants. Since free radicals and oxidized lipids stimulate PLA_2 activity the role of endogenous antioxidants in the regulation of PLA_2 is very important in understanding this complex inflammatory reaction.

Till date many sPLA_2 inhibitors are characterized by researchers, which can be useful to treat acute and chronic inflammatory diseases. Among these, some of the typical compounds reported as "classical" PLA_2 inhibitors include anti-malarial drugs, aminoglycosides, alcohols and polyamines. These molecules generally do not inhibit PLA_2 parse, but act by blunting PLA_2 interaction with its substrate or even calcium (123-124). Therefore, the lack of specificity of such compounds indicates that they should not be used as PLA_2 inhibitors.

Further, potent molecules such as oleanolic acid, quercetin, genistein, ginkgetin, vitamin E, manoalide and bolinaquinone inhibit sPLA_2, LOX and COX-1/2 activities at low micromolar concentration. However, their entry into the market as an effective anti-inflammatory drug is not much successful. This is mainly because of their cytotoxic nature and/or problems associated during

formulations. For example, manoalide irreversibly inactivates several $sPLA_2$s by forming covalent modification with lysine residue [125-126]. It also has inhibitory effect on 5-LOX, COX-2, PLC, iNOS expression and calcium channels [127-130]. Manoalide was taken to phase I clinical trial by Allergan Pharmaceuticals as a topical antipsoriatic. However, it was discontinued due to formulation problems. In addition, several of the natural molecules tested in vitro against AA pathway enzymes might not be effective in *in vivo* assay systems. Disappointingly, some of these natural $sPLA_2$ inhibitors have side effects. For example aristolochic acid is carcinogenic (131). Hence, there is still need for an effective anti-inflammatory molecule with fewer or no side effects.

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Chapter - 3

Mistakes in Handling Specimen's Collection and Its Rectification

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Introduction

Elimination of medical errors is a mandatory step for all pathologists. Errors occurring in surgical and clinical (tissue, blood and body fluids- urine CSF, seminal fluids, peritoneal fluids, plural fluids, pericardial fluids and synovial fluids) pathology involve specimen defects, specimen labeling, processing, diagnosis, and reporting defects. Occurrence of these errors is generally found during pre-laboratory, laboratory, and post-laboratory phases. In 1999, National Academy of Medicine published the paper *"**To Err is Human: Building a Safer Health System**"* [1], which defines medical error as the incapability to complete a planned action or the use of a wrong plan to attain a goal on a broad aspect.

The sections in the pathological specimen include hematology, urine, body fluids, parasitology and microbiology, general and specialized biochemistry, hormonology, serology, coagulation, and flow cytometry (FCM).

The pathology testing process starts with the requisition from the hospital's departments or through private practitioners. For inpatients, the process starts with the laboratory clerk in responsible to the collection of data related to patients' identification data including their name, age, sex, file number, and physician's name and register them in the hospital information system (HIS). If the requisition is not emergent, a trained laboratory staff collects the blood samples from all hospital departments next morning. In emergent cases, the staff collects the blood samples and immediately transports it to the laboratory. For outpatients, the physicians in private practice fill out the appropriate requisition form which is sort of as a referral form to the laboratory. After that the clerk registers the patient's identification data into the HIS.

Once the specimens are transported to the laboratory, a technologist

evaluates the samples for misidentification errors, inappropriate container, improper labeling, inadequacy of sample collection, and inadequacy of sample/anticoagulant ratio volume. Any probable errors found are immediately reported to the ordering department followed by a request for a new proper sample. The initial steps constitute analytic procedures which includes specimen preparation, including preparing suitable samples for like centrifugation, aliquoting, and sorting specimens. Further tests are performed in different laboratory section. Depending on the type of tests, the result data is entered to HIS both manually and automatically. Further, the test results are observed by medical staff for evaluation, interpretation, and appropriate action. If any result is labeled clinically questionable by the trained technicians, the quality manger checks the testing process and usually the specimen is retrieved or in some circumstances asks for a new sample. The quality manger records the errors on a special roster and the date, time, source of the test, type and detail, and signature are recorded. ***The laboratory error definition that has been incorporated in the International Organization for Standardization (ISO) Technical Report 22367 is defined as a defect occurring at any part of the laboratory cycle, from ordering test to reporting, interpreting, and reacting to results*** [1].

Meier describes the pathology production process in 12 steps. The production process begins with the [2]**-**

1. Correct identification of the patients' samples
2. Selection of tissue specimens and blood, body fluids
3. Labeling and transport
4. Accession
5. Receipt and sampling of all types of pathological specimens
6. Fixing, embedding, and cutting section
7. Mounting, staining the slides, and labeling them
8. Delivering them to surgical pathologists
9. Examining, collating, and interpreting slides
10. Examining the possibilities of ancillary tests or other information
11. Composition of reports,
12. Finally the receipt of reports and interpretation of the report

Therefore, the clinical pathology report is a complex task with multiple steps in which there is a possibility of error.

There are basically four types of errors distributed according to the

processing step in the laboratory-

1. Defective identification
2. Defective specimen
3. Defective interpretation
4. Defective report

There are basically three phases in laboratory error [2]**-**

These defects are categorized in three phases, ***pre-analytic phase, analytic phase and post-analytic phase.***

- Pre-analytic phase includes defective identification (samples, body fluids and tissue specimen) and defective specimen (loss of the specimen, erroneous in measurement or gross description, floaters, inadequate sampling, and the absence of indication of ancillary studies when necessary).
- Analytic phase includes errors in classification, false negative or positives
- Post-analytic phase includes the defective reports (erroneous or missing non-diagnostic information, error in dictation or typing, report delivery and errors related to computer or format, transmission and upload error).

Errors in Pre-Analytical Phase

Recommendations for specimen collection and handling

The processes of sample handling are challenging to control, but they impact the quality of the specimen. Hence certain guidelines should be followed in order to avoid errors in clinical pathology.

1. Patient Identification

This process involves the patient to be identified with due respect to his privacy and his related medical records data. Patient's identification is a necessary step at the time of specimen collection with at least two acceptable patient-specific identifiers such as Full name, identification number, dates of birth, photo on government issued ID card, and other specific personal identifiers [4-8].

2. Proper Labeling [7,8]

- Specimen should be labeled in the presence of the patient with atleast two patient-specific identifiers (full patient name, identification

number, Date of Birth, additional identifiers such as patient's gender, requisition number, ordering physician, specimen source (e.g. skin, blood and body fluids etc.).

- Correct positioning of the label on the collection container is important for eg; the label should not be pasted on to the container lid (in whole or part).
- Overlapping of the label and over-writing on the vials should be avoided.
- All subsequent labeling of patient samples (blocks and slides) must follow same patient-specific identifying process.

3. Barcoding and/or Radio Frequency Identification(RFID) [9,10]

- All parameters should be used for standard specimen labeling. The unique specimen bar code or RFID label must be consistent across all applications such as specimen container, requisition label, cassette and slide labels.
- Bar coding and/or RFID documentation must be validated and maintained.
- Automatic identification scanning equipment is authenticated for accurateness and resistant to chemicals used for anatomic pathology handing.
- The barcode or RFID tracking system must have intelligent location capabilities if used for specimen chain of custody tracking.

4. Completion of requisition

- **Patient identifier** [11,12]
  - ❖ Written procedures on how to properly complete a pathology requisition must be made available to all health care workers.
  - ❖ Written or electronic request for patient testing should be obtained from authorized person.
  - ❖ Patient identifiers should be included on the requisition / test order- patient's name, unique identifier i.e. health record or master index number, date of Birth or gender.

- **Specimen name/type/site** [11,12]
    - ❖ Name and address or other suitable identifiers of the authorized person requesting the test.
    - ❖ Name and address or other suitable identifier for the individual responsible for receiving the test results
    - ❖ Name and address of the laboratory submitting the specimen
    - ❖ Test and or tests to be performed
    - ❖ Procedure performed
    - ❖ Specimen site – if more than one specimen is collected during a single procedure; each specimen should be individually identified by anatomic site and or specimen type
    - ❖ Date and time of procedure or specimen collection
- **Quality assurance** [17]
    - ❖ A policy and procedure for identification of labeling discrepancies must be made available.
    - ❖ Appropriate labeling changes on the rejection specimen should only be done by the originator.
    - ❖ Label and requisition must be a match.
    - ❖ Policy and procedure to handles specimen acceptance and rejection should be proper
    - ❖ The information on the specimen and the requisition form should be same.
    - ❖ The parameters for specimens which are deemed acceptable: Identification of the patient sample (labeling), completion of the requisition to include all required demographic and clinical data, specimen container to be used, type and volume of fixation, transport packing, temperature and method and additional specialized instructions.

5. Accessioning [18]

- Each specimen container received must be compared to the requisition to ensure correct match of at least 2 patient-specific identifiers:
    - ❖ Full patient name
    - ❖ Assigned identification number e.g. health record / master index

- ❖ number
- ❖ Date of Birth

- Additional requisition information to be checked:
  - ❖ Number of specimen containers
  - ❖ Type of specimens submitted
  - ❖ Complete clinical history
  - ❖ Name of requesting physician to return report to
  - ❖ Collection data related to fixation (section D)

6. Handling prior to Gross Examination [8,13,14,19]

- There should be sufficient storage space for surgical specimens in an orderly fashion after accessioning, and prior to gross examination such as space should be provided for containers and accompanying paperwork/request slips as well as the storage area should be clean, free of clutter, and well ventilated.
- Site specific documentation which mentions sterile procedures, sample collection into specialized media, requisition, referral lab, labeling procedure for sub – specimens, completion for further testing i.e. microbiology or pathology, holding and transport instructions for specialized testing (i.e. refrigerate), specimen cross contamination must be available to all staff handling the specimens
- Fresh specimens for immediate gross examination should be kept in their labeled containers at room temperature. The fresh specimen should be kept in its labeled container and refrigerated until it can be examined. Written procedure should be followed to prevent cross contamination
- Sealed containers should be used to avoid spillage, loss of fixative, loss of specimen, and to prevent drying of the specimen prior to gross examination.
- An appropriate room temperature should be maintained, so that specimens are neither frozen nor damaged by excessive heat with appropriate ventilation for adequate air movement, without buildup of fixative or other noxious vapors.
- If insufficient fixative is present for the specimen received in the laboratory additional fixative should be added.

7. Patient preparation:

Many tests require patient's preparation in some specific way to ensure

fruitful results. Highest quality results depends on the specimen quality submitted for analysis

- **Fasting requirements:** Fasting specimen is preferred for majority of test(s) performed on serum, plasma or whole blood because fat particles present in non-fasting specimens interfere with analytical procedures.

  ***Fasting can be defined as no consumption of food or beverage, other than water, for 8-12 hours**.*

- **Provocation tests:** Some tests require the patient to ingest few substances for example Glucose Tolerance Tests requires the patient to drink a solution containing glucose. Blood specimens are obtained before and after the drink to measure the concentration of glucose in plasma or serum [20].

8. Order of Draw:

Cross-contamination of additives between tubes could be avoided through blood collection tubes drawn in specific order and to maintain sterility when blood cultures are drawn. The recommended order is as follows:

Clear (Discard) Tube - For special coagulation tests such as platelet function tests or clotting factor assays it is generally recommended that a discard tube must be drawn prior to drawing a Light Blue (Sodium Citrate) tube.

Although, it is not necessary for routine coagulation test. But when drawing coagulation tests using a butterfly, a discard tube must be drawn first only to prime the line [20].

Vials cap color coding are used for different type of tests-

- ❖ Blood Cultures (1 Blue and 1 Purple bottle)
- ❖ Light Blue (Sodium Citrate)-coagulation test (1:9).
- ❖ Gold (SST Serum Separator)-chemistry,
- ❖ Red (Clot Activator)-chemistry
- ❖ Dark Blue (Navy/Clot Activator)-chemistry
- ❖ Green (Heparin/Lithium)-chemistry
- ❖ Lavender (Purple/K3EDTA)-hematology, HbA1C
- ❖ Pink (K2EDTA)-hematology, HbA1C
- ❖ Grey (Potassium oxalate/sodium fluoride)-glucose

9. Specimen Quality

Specimen quality is an essential component for gaining accurate results.

Few considerations must be followed while drawing, storing and transporting blood specimens [20]-

- **Hemolysis:** This is the process of the lysis of the red blood cells (RBC) membrane, causing the release of their components into the surrounding fluid. It is visually detected by showing a pink to red tinge in serum of plasma. Certain reasons for the occurrence of hemolysis can be autoimmune hemolytic anemia, transfusion reaction or improper specimen collection, specimen processing, or specimen transport.

Recommendations to prevent hemolysis:

- After collections, the technician should gently invert specimen tubes with clot activator several number of times to ensure the distribution of the clot activator homogenously within the sample, and allow the specimen to clot for 30 minutes in a vertical position. Those Serum tubes which are without clot activator should be allowed to clot for 60 minutes in a vertical position.
- Specimens should not be centrifuged at higher speed or for longer than necessary. It should only be done if they will not be delivered to the laboratory within 4 hours of collection.
- Choice of the collection needle gauge size should be dependent on the patient's physical characteristics and the amount of blood drawn. 20-22 gauge needles work best for routine collections.
- Collection needle that is too small or too large should be avoided. Small-bore needle may results in a large vacuum force applied to the blood which can cause shear stress on the RBCs leading to their rupture. Large bore needle could result in faster and forceful flow of blood through the needle which could again cause rupturing of RBCs.
- Prolonged tourniquet time could also cause the interstitial fluid to leak into the tissue and cause hemolysis.
- Cleansing the venipunture site with alcohol and its usage without drying may also cause hemolysis.
- The needle should not be removed from the vein with the vacuum tube still engaged.

- Exposure to excessive heat or cold can cause also cause hemolysis.

    - **Inadequate Draw/Quantity Not Sufficient (QNS):** Hematology and coagulation test(s) require blood sample upto the line marked on vials therefore; the ratio of anticoagulant to blood is specific for the volume of specimen. Short-draws could not be used for coagulation test(s) as they could result in RBC crenation, reduced MCV and hematocrit, and alteration to WBC morphology, platelet, and total WBC count.

    - **Clotted Specimens:** All hematology, coagulation, and other whole blood specimens drawn in tubes with anti-coagulate must be free of clots. Clots, large or small, will lead to erroneous results for these tests.

Errors in Analytical Phase

10. Lab Sample/Source:

- **Whole blood**

    - Whole blood is drawn into tubes with anticoagulant.
    - After the tube is filled, it must be 5-6 times gently inverted to ensure adequate mixing.
    - Yellow top tubes for genetic testing and those for microbiology must be distinguishable, they must not be interchangeable.
    - Navy Blue (EDTA) should not be confused with dark Blue which contains no additive.
    - Sodium Citrate tubes must be filled to the required volume of the tube in order to maintain the appropriate concentration of citrate anticoagulant in the plasma to be tested.
    - Red top tube should be used for whole blood specimen.

- **Plasma and Platelet Poor Plasma:**

  Specimens should be drawn into tubes that contain anticoagulant.

    - After the tube is filled, it must be 5-6 times gently inverted to ensure adequate mixing.
    - Some specimens may require immediate centrifugation, separation and freezing. Other may need to be double centrifuged to completely remove platelets.
    - Centrifugation must be carried out for 10 minutes at 3000 rpm.
    - Plasma should be transferred into an appropriate transport tube.

- ❖ Specimens should be labelled as "plasma" after it has been separated.

- **Serum:**
  - ❖ Specimens should be drawn into tubes that does not contain any anticoagulant.
  - ❖ Red top or serum separator tube should be used for collecting blood for obtaining serum and allowed to clot (about 30 minutes).
  - ❖ Centrifugation must be carried out for 10 minutes at 3000 rpm.
  - ❖ Serum must then be transferred into a properly labeled plastic transport.
  - ❖ Gel or serum separator tubes must not be used for drug levels as the drug may be absorbed into the gel.
  - ❖ Hemolyzed, lipemic, or icteric specimens may cause erroneous results

- **Urine:**
  - ❖ Urine specimens are collected in screw-capped sterilized container, plastic urine container.
  - ❖ All urine specimens should be collected as clean catch urine specimens.
  - ❖ The first voided morning specimen is preferred.
  - ❖ Secure caps should be used to prevent leakage.

- **Fecal (Stool):**
  - ❖ Random collections require various preservative media if it is not delivered immediately.

- **Body Fluid (peritoneal fluid, cerebrospinal fluid, pleural fluid etc.):**
  - ❖ Specimens should be collected in four sterile EDTA tubes, labeled as 1, 2, 3, and 4 in the order in which they are withdrawn.
  - ❖ The tubes are usually disbursed for analysis as follows: Tube 1 is for chemistry and serology tests hence this tube should never be used for microbiology since it is most likely to contain skin contaminants. Tube 2 is for Microbiological testing. Tube 3 is for cell counts and tube 4 is for miscellaneous or referral test request.

- ❖ Specimens must be transported to the laboratory within 1 hour of collection in order to prevent deterioration of cells and glucose.

- **Synovial Fluid:**
  - ❖ Normal synovial fluid does not form clot but diseased joint form a clot as they contain fibrinogen. Therefore, both anticoagulant and non-anticoagulant specimens should be collected.
  - ❖ EDTA tubes are recommended specimen containers: for hematology cell counts, differentials and viscosity.
  - ❖ Fluids should be added to the tube immediately after collection to avoid clot formation.
  - ❖ Tubes should be gently inverted 5-10 times to ensure adequate mixing of the fluid and the anticoagulant in case of other tubes.
  - ❖ Free sterile tube or container must be used for microbiology testing and crystal examination.

Other recommendations regarding blood specifics in clinical pathology testing [20]:

- **ABO Group & Rh Type and ABO Typing:**
  - ❖ ABO Typing: A, B, AB, O
  - ❖ Rh typing: Rh positive/Rh negative
  - ❖ Stability: Room temperature- 24 hours; Refrigerated- 10 days; Frozen- Unacceptable
  - ❖ Transport and Storage: Refrigerated
- **Antibody Detection, RBC**
  - ❖ Panel identification should be performed on all positive specimens.
  - ❖ Stability: Room temperature- 24 hours; Refrigerated- 10 days; Frozen- Unacceptable
  - ❖ Transport and Storage: Refrigerated
- **Antibody Titer**
  - ❖ Antibody identification must be performed, at an additional charge, prior to performing this test.
  - ❖ Stability: Room temperature- 24 hours; Refrigerated- 10 days; Frozen- Unacceptable

- ❖ Transport and Storage: Refrigerated

- **Direct Coombs (Anti-Human Globulin)**
  - ❖ Stability: Room temperature- 24 hours; Refrigerated- 10 days; Frozen- Unacceptable
  - ❖ Transport and Storage: Refrigerated

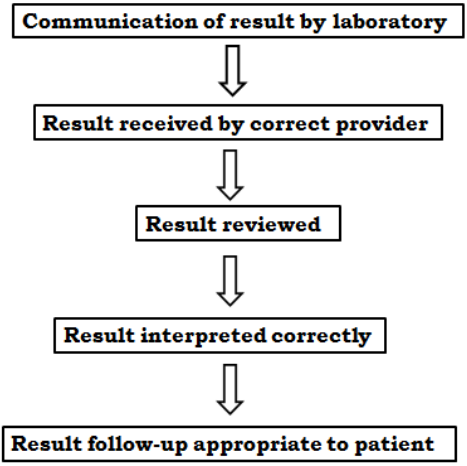
- **Elution & Antibody Identification, RBC**
  - ❖ Stability: Room temperature- 24 hours; Refrigerated- 10 days; Frozen- Unacceptable
  - ❖ Transport and Storage: Refrigerated

- **Type and Cross match**
  - ❖ It includes ABO and Rh type, Antibody screen
  - ❖ Cross matched blood can be held for a patient for 3 days. After that time it should be released without notification to the physician since patient sample used for cross match or antibody screen is used only if it is less than 3 days old.
  - ❖ If it is known that the patient has not been recently transfused or pregnant, this can be extended to 14 days, for pre-surgical patients.

Post analytical error

Errors that occur after the analysis is complete but remains confined within the laboratory itself under its control. This phase includes result reporting, critical value notification, manual transcription of results and subsequent data entry, and analysis [21]. The post-analytic phase has been thought to be less prone to error than the pre-analytical phase because of the adoption of advanced technology and automated results. It is difficult to detect post-analytical errors of any type and hence it also makes it difficult to directly link laboratory errors and patient safety. This process involves variety of professions and settings [21]. Since it is dependent upon different variables, it is hard to pinpoint a single reason or explanation for the occurrence of the error.

Post-analytical process involves following steps



Failures in communication of test results:

- Tests performed but not reported
- Patient identification error in results reporting
- Delay in reporting
- Incorrect calculation
- Critical result notification failed or delayed
- Results not sent because of communication failures

Failure modes in electronic receipt of results

- Logic errors in interface and results routing
- Provider record issues
- Electronic health record (EHR) system settings
- System maintenance–related errors

Recommendations related to reduce post analytic errors [22]

1. All test results before release must be evaluated only after the comparison of the results with reference intervals and/or critical results, patient diagnosis and previous test results (if available); and confirmation of test results.
2. Reference intervals or relevant limits according to age and gender must be present next to each test result for clinical decision-making.

3. It is recommended that the present test should be compared with predetermined result because any difference between successive results indicates significant change in the patient's clinical condition, or problem with the sample.
4. A delta check should be used as an integral part of the laboratory. The difference from the previous result is calculated as a delta percent change and compared with RCV limits. If the delta check exceeds the laboratory-specified limits include:
    - Reviewing clinical data which includes clinical diagnosis, therapeutic interventions, contacting a physician);
    - Retesting the current and previous
    - Checking for the presence of haemolysis, lipemia, icterus, clot or error in tube labelling of the previous and current sample
    - If all previous actions do not provide a valid explanation for such a difference, the analytical system must be re-checked for proper functioning.
5. When results exceed the upper limit of the analytical measurement range, automatic dilution by the analyzer should be used if possible.
6. Laboratory personnel with master's degrees or specialization in medical biochemistry and laboratory medicine should be appointed as they have the necessary competencies to confirm test results and decide whether to release them after review and any additional procedures.
7. The laboratory should record policies and procedures about releasing reports
8. Automated or semi-automated selection and reporting of test results is the recommended procedure.
9. Each laboratory should compile a list of laboratory tests for which critical limits should be defined.
10. Critical results have to be reported within 30 minutes of confirmation
11. The laboratory must have a documented procedure for identifying, collecting, marking, accessing, storing and safely disposing of biological samples.
12. Monitoring of quality indicators in daily laboratory work is recommended.
13. Monitoring and periodical analyses of all incorrect laboratory test

reports released for any reason, as well as monitoring the reasons for corrections of laboratory test report are recommended.

Limitations and Challenges to Quality-Improvement Measures [21]

The advances made by practitioners in improving test results quality due to improved testing procedures, advance laboratory information technology, automated analytical process, and improve patient safety are counterpoise by certain hurdles inherent to the delivery of safe laboratory services. Some of these challenges and limitations are as follows:

i. Conversion of laboratory services into ever larger organizational units and outsourcing of laboratory services make the total testing process more difficult which often compromise on certain pre- and post-analytical points of procedure, such as prolonged time for specimen transportation, compromised specimen integrity, difficulties in communication with health care professionals. These result in spewing out of analytical results; this undermines the testing procedures by enhancing the number of, and risk of, errors in laboratory medicine.
ii. Economic pressure experienced by many health care organizations leads to downsizing of laboratory staff which has dramatically risen workloads and drastically affected personal productivity. Although, errors and patient safety problems confronted due to this reason are not immediately evident but become evident in the long term. Unsafe procedures and processes accumulate over time and result in adverse events.
iii. To reduce such errors, some laboratories have developed facilities to provide near-patient testing and point-of-care-testing (POCT), for instance, levels of blood gases and serum electrolytes. Such alternatives could reduce pre-analytical factors such as specimen collection, collection in appropriate vials, and maintenance of the integrity of specimens.

Changes adopted towards quality improvement [23]

Despite the limitations and challenges confronted by the laboratories, quality improvement is the key to bringing about patient safety. Changes in the following areas are desirable to achieve improvement in laboratory services.

(i) Change the work Culture

The work culture is an integral part of an institution. Every staff member should wash his or her hands in the prescribed manner and patient

identification should be appropriate before specimen collection. To internalize the safety culture, each new health care professional should be thoroughly trained about the safest ways to provide care.

(ii) Establish System-Wide Transparency

The transparency of the laboratory system is the key holder of the accuracy of the total testing process; a high level of transparency depends upon a system-based approach that proclaims that errors are the result of flawed systems on the whole and not the laboratory personnel. This approach inspires staff to interact more positively to identify weaknesses in processes. Staff members must also be rewarded for errors acknowledgements, hazards identification, and initializing examination of ways to prevent future adverse events.

(iii) Develop Multidisciplinary Teams

Collaborative input from health care and laboratory professional's result in improvement in medical facilities and the introduction of new and complex tests. All members must acknowledge the experience and contributions of other team members also. It is significant to develop a team that consists of pathologists, medical technologists, cytotechnologists, histotechnology's, health care professionals such as physicians and nurses, and other types of laboratory professionals, all of whom work together to provide optimal patient care.

(iv) Introduce Information Technology to Advance Patient Safety

Current information systems have an unexploited capacity for real-time care delivery and retrospective care analysis to provide the data that will bring about variations in all facets of health care. Laboratory information systems must be combined with other clinical systems to harness the capacity of health care and laboratory professionals to recognize and to mitigate risks before these risks become confirmed safety issues.

(v) Support National and International Agencies for Patient Safety

All laboratories should involve in national and international safety programs by providing copies of guidelines to laboratory professionals, health care professionals, and patients. Also, all laboratories should voluntarily obtain accreditation by national or international regulatory agencies.

(vi) Take a "Patients First" Approach

In order to provide high-quality facilities, laboratory staff should consider any problem from the viewpoint of patients. To accomplish this, laboratory

staff should allow patients to provide reviews on their understanding and satisfaction with laboratory facilities, as well as hospital infection prevention and patient safety. The “patients first” approach yields safer care and higher levels of patient satisfaction.

Conclusion

Since more than half of laboratory errors occur during the pre-analytical process, adequate preparation and awareness of the intervening factors that can affect laboratory results are critical for reducing laboratory errors. It is important to remember that all three phases of laboratory experiments must be closely monitored in order to maximize the consistency of the findings.

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Chapter - 4

Nanao Herbal Medicines

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Abstract

Herbal medicine has been used for thousands of years. It is estimated that 80% of world population rely on traditional herbal medicine for primary health care. In recent years, herbal remedies have been considered as dietary supplement for disease prevention and as alternative/complementary medicine. With the rising utilisation of herbal products, safety and efficacy of herbal medicine have become a public health concern. Phyto-therapeutics need a scientific approach to deliver the components in a sustained manner to increase patient compliance and avoid repeated administration. This can be achieved by designing novel drug delivery systems (NDDS) for herbal constituents. NDDSs not only reduce the repeated administration to overcome non-compliance, but also help to increase the therapeutic value by reducing toxicity and increasing the bioavailability. One such novel approach is nanotechnology. Nano-sized drug delivery systems of herbal drugs have a potential future for enhancing the activity and overcoming problems associated with plant medicines.

Introduction

Herbal medicines are being used to cure the diseases from ancient times. Infact, herbal remedies are gaining more importance than any other medicinal discipline as they are more advantageous and safer. Moreover, they have the quality of targeting treatment on the human body. Herbal drugs consist of thousands of constituents that work together and fight against various diseases. The usage of herbal medicines has been increased due to their therapeutic activity and lesser side effects. Formulating herbal medicine is not a simple task; it should be checked for various factors like quality, efficacy and reproducible therapeutic effect. Standardised herbal products should contain well defined constituents are required for reliable clinical

trials and to produce beneficial therapeutic effects as the pharmacological properties of herbal formulation depends on the phytochemical constituents present in the formulation.

The problems generally associated with the herbal remedies are less bioavailability and need of repeated administration. These problems can be lowered by delivering the components in a sustained manner which avoids repeated administration and increases patient's compliance. Even most of the herbal drugs are poorly soluble in water due to their hydrophobic nature which results in reduced bioavailability and enhanced systemic clearance. In order to overcome the problems like decreased bioavailability and increased systemic clearance, need of much dose and repeated administration evolves which is the major limitation for the herbal drug usage. On other side, traditional medical treatment faces a biggest challenge that drugs used for the treatment of diseases affecting wider areas in the body including healthy tissues. Hence, this type of treatment requires continuous observation which results in slow progress of treatment and it has serious side effects too. So, there has been a need to find out a new method to overcome all these difficulties. Development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of market/bioactive compounds and other major constituents, is a major challenge to scientists. These limitations of plant origin drugs can be overcome by formulating them with suitable novel drug delivery system [1].

Among all the novel drug delivery systems, nanotechnology is considered as the significant one. It shows great promise for providing us in the near future with many breakthroughs that will change the direction of technological advances in a wide range of applications. Nanotechnology is the one of the novel drug delivery system which aims to develop devices and dosage forms in the range of 1-100nm. The word nano is derived from the Greek language which means "dwarf". A nanometer is calculated as one billionth of meter or it is equal to length of 10 hydrogen atoms placed side by side or equal to 1/80000 of thickness of human hair[2]. Nanotechnology refers to engineering and manufacturing of materials at the molecular level which commonly refers to the size range of 100nm. Nanotechnology is developing as a very essential field in the medicinal and pharmaceutical sectors by playing an important role in enhancing quality of life of human beings. It has many applications in the medical sector which includes both diagnosis and therapeutic. However, therapeutic uses are considered as the main application of technology in the health sector.

Fast growing nanotechnologies serves as a good source for the

production of innovative novel herbal drugs. It combines the methods of pharmaceutics for drug formulation, biochemistry, molecular biology, and process and technology. Among all the novel drug delivery systems, nanotechnology has wide relevance for application because of its advantage in management and accurate analysis. Also, the applications of nacreous with protected resources such as artificial biological carbohydrates, polders, and libidos have yielded desirable results. The nanomaterials can significantly enhance the pharmacokinetics and therapeutic index of plant origin drugs [1]. Development of nanodosage forms offers many advantages like enhancement of solubility and bioavailability, pharmacological activity, stability and protection from toxicity, degradation and targeted delivery to a particular cell or tissue etc.

To emphasize more clearly, "nanotechnology" is the engineering and manufacturing of materials at the nano size and these nanosized herbal medicine drugs are proved to be having high possibility for the movement that can overcome various difficulties connected with plant medicine by their special characteristics like typical tininess and forbidden discharge of the medicine. In this drug delivery system, nanotechnology helps in carrying optimum amount of drug to their site of action by bypassing the barriers like acidic pH of stomach and first pass metabolism. Hence, the means of "herbal medicine" in the nanocarriers will extend its possibility to cure a variety of chronic diseases.

Nanotechnology can also be useful in detecting various plant diseases. The researchers are using nanotechnology for detecting plant disease at an early stage so that the food is protected from the possible damage. Therefore advent of nanotechnology is considered to be the biggest engineering innovation since the industrial revolution [2]. Proponents of this new technology promised to re-engineer the man-made world, molecule by molecule, sparking a wave of novel revolutionary commercial products from machines to medicine [2].

Nano-drug delivery system has the following advantages

- Delivers adequate amount of drug at site of action
- Small particle size of nanomedicine enhances the dissolution in the blood.
- Permeation through the barriers is also more due to smaller particle size
- Side effects can also be reduced.

Even though nanotechnology has various advantages, it is also important to mention the disadvantages of nanotechnology in several medicinal areas. Clinical researchers have mentioned the following disadvantages.

- High cost
- Difficulty of scaling up processes
- Easy inhalability of nano particles which leads to serious lung diseases and other health issues that further results in changes in homeostasis or even death.

Examples of nanoherbal medicines:

The fact that nanoparticles provide more effective drug delivery system than other conventional drug delivery systems is proved by the following examples

1. In a study, the effect of synthesized silver nanoparticles together with plant products and commercially available drugs were evaluated against biofilm inhibition and its biochemical composition of clinical isolates of Candida tropicalis and Staphylococcus aureus. The silver nanoparticles were synthesized from Lactobacillus acidophilus 01 strain and tested with aqueous extracts of Aloe vera, ginger (Zingiber officinale), garlic (Allium sativum), tulsi (Ocimum tenuiflorum), oils (e.g., coconut oil), antifungal drugs (e.g., fluconazole and itraconazole) for C. tropicalis and antibacterial antibiotics (e.g., tetracycline and chloramphenicol) for S. aureus. The synthesized silver nanoparticles, together with plant products and commercially available drugs, showed a maximum inhibitory effect against both the tested clinical isolates [3].
2. Biotechnological (or nanotechnological) approaches provide attractive possibilities for the large scale production of artemisinin. Hairy root cultures of A. annua can be generated by infection of roots with Agrobacterium rhizogens. Hairy roots grow quickly, reach high densities and can produce significant amounts of secondary metabolites like artemisinin, By nanotechnology, genetically modified plants deliver considerably higher amounts of artemisinin than wild-type plants. With the implementation of sophisticated biotechnological (or nanotechnological) production techniques, it will be possible to meet the high demand of artemisinin for malaria treatment and hopefully in the future for cancer chemotherapy as well [4]

3. Using nanotechnology for "phytotherapy", the researchers (at Kaohsiung's Medical University, Graduate School of Biochemistry) ensured that a new herbal medicine compound was able to enter cancer cells without damaging the healthy cells of the human body. They explained that the new drug is produced by extracting cancer fighting ingredients of Chinese herbs such as milk veteh root, sealwort, cassia twigs and liquorice root and uses nanotechnology to reduce these ingredients to their smallest size, enabling them to enter cancerous cells without damaging healthy cells. The researchers said that this new drug has four advantages such as it only kill cancerous cells; has rapid medicinal effects; does not harm other organs and can be ingested orally without a physician's supervision. When taken together with western medicine, this drug is also able to lower the dosage and reduce resistance to cancer drugs, thereby enhancing overall efficiency. However, this medicine has not been found to be effective for all forms of cancer. During the experiments, this drug was reportedly found to be effective in alleviating symptoms of pulmonary cancer, breast cancer, bone cancer, liver cancer, tongue cancer, primary or metastatic lymphoma, cervical cancer, ovarian cancer, brain cancer and skin cancer [5].
4. Camptothecin is a plant alkaloid derived from the Chinese tree Camptotheca acuminate. The alkaloid camptothecin caused DNA damage by specifically targeting DNA topoisomerase, effectively devastating a broad spectrum of tumours Although the antitumour activity of camptothecin has been intensively studied for nearly fifty years, recent advances in drug delivery systems of camptothecin have considerably improved this drug's efficiency due to development in nano-sized dosage forms of camptothecin derived drugs [6].
5. Transdermal gel was formulated from optimized nano transferosomes of diclofenac diethylamine and curcumin for providing sustained and targeted effects.
6. pH sensitive nanoparticles of curcumin- celecoxib combination were formulated as a potential therapy for ulcerative colitis.
7. Curcumin solid lipid particles were prepared with a high loading capacity and chemical stability for the treatment of oral mucosal infections.
8. Curcumin loaded hydrogel nanoparticles of hydroxyl propyl methyl

cellulose and polyvinyl pyrrolidone were successfully formulated and exhibited a significant improvement in anti-malarial action.

9. Biosynthesis of silver nanoparticles was demonstrated by using leaf extract of Mukin scabrella, it exhibited significant anti-microbial activity against MDR-GNB nosocomial pathogens.
10. Fluorescent silver nanoparticles were synthesized using leaf extract of Artemesia annua and they have shown antibacterial and fluorescent activity.
11. Gold nanoparticles were synthesized by using gall extract of Pistacia integerrima and they are known to show significant anti-fungal and anti-nociceptive activity.
12. Nickel nanoparticles were synthesized from Aegle mermelos correa leaf extract and it was known to show anti-inflammatory activity.
13. Magnetic nanoparticles were used to coadminister the drugs like curcumin and temozolomide and this formulation is known to show anti-cancerous activity.
14. Invitro efficacy against the complications of diabetes mellitus and the invivo toxicity was evaluated by using Syzygium cumini seed plant extract and of polymeric nanoparticles containing ASC. It showed high inhibitory activity against LDL particles.
15. Pessimmon leaf extract was formulated as an self-nano emulsifying drug delivery system (SNEDDS) formulation shows increased solubility and bioavailability
16. Sefsol and linoleic acid optimized SNEDDS formulation showed protective effect against paracetamol induced hepatotoxicity.
17. Zedory turmeric oil extracted from rhizome of Curcuma zedoria and was formulated as SNEDDS in order to improve the bioavailability, stability and aqueous dispersion activities.
18. Oleanolic acid was efficiently encapsulated in methoxy poly (ethylene glycol) with poly (lactic acid) and Mpeg – POLY- (lactic – coglycolic acid) as nano formulation for cancer treatment.
19. Polycaprolactone / polyvinylpyrrolidone nano-fiber mat was formulated with the extract of bark of Centella asiatica to improve wound healing activity in rat model.

Other examples of nano-herbal medicines are tabulated as follows

S. No	Active ingredients	Method of preparation	Biological activity
1	Curcuminoids	Micro-emulsion technique	Anti-cancer and Anti-oxidant activities
2	Glycyrrhizin acid	Rotary-evaporated film ultra-sonification method	Anti-inflammatory and Anti-hypertensive activities
3	Flavonoids and lignans taxel	Nano-suspension method	Hepatoprotective, anti-oxidant activities
4	Taxel	Emulsion solvent evaporation method	Anti-cancer activity
5	Artemisinin	Self assembly procedure	Anti-cancer activity
6	Camptothecin	Dialysis method	Anti-cancer activity
7	Berberin	Emulsion and Ionic gelation method	Anti-cancer activity
8	Curcumin	Wet-milling technique	Anticancer activity
9	Paclitaxel	Nanoprecipitation	Anticancer activity
10	Berberin	Emulsion, Ionic gelation	Anticancer activity
11	Camptothecin	Encapsulated with hydrophobically modified glycol	Anticancer activity
12	Ginkgo biloba	Combination of Dry and wet processes. (Gas-phase and liquid-phase grinding)	Treatment of Alzheimer's disease and Dementia
13	Triptolide	Nano encapsulation	Anti-arthritis activity
14	Salvia miltiorrhiza	Phospholipid complex loaded	Anti-hyperlipidaemic activity
15	Quercetin	Gelatin and chitosan loaded	Anti-oxidant activity
16	Breviscapine	Lipid encapsulation	Cardio-protective activity
17	Naringenin	Nano precipitation	Antioxidant, Anti-inflammatory activities
18	Dodder	Nano precipitation	Antioxidant activity
19	Silymarins	Cold homogenization	Hepatoprotective activity
20	Genistein	Nano emulsion and chitosanmicrosphere	Antioxidant activity
21	Centella asiatica	Ionic gelation	Anxiolytic activity
22	Annual mugwort	Hydrophilic encapsulation	Antimalarial activity
23	Curcumin	Encapsulation with sonication	Anti-inflammatory activity

24	Cysteine residues and proteins	Nanosuspension	Anti-microbial activity
25	Amines, carboxylic acids, aldehydes and ketone	Nanosuspension	Anticancer and antioxidant activity
26	Amides and phenolics	Nanosuspension	Anti-cytotoxic and antibacterial activity
27	Amines, amides, phenolic and alcoholic compounds	Nanosuspension	Anti-fungal activity
28	Amines, amides, phenolic and alcoholic compounds	Nanosuspension	Anti-inflammatory and mosquitolarvicidal activity
29	Curcumin and temozolomide	Nanosuspension with sonication	Anticancer and antitumor activity
30	Gallic, chlorogenic ellagic acids, catechin, epicatechin, quarcetin etc	Emulsification/evaporative solvent technique	Anti-fungal activity
31	Thujone, camphor, Pinenes	Hot melt ultrasonication	Antioxidant activity
32	Flavonoids (Quercetin and Kaempferol)	Self nanoemulsion	Antioxidant activity
33	Quercetin	Self nanoemulsion	Anti-liver toxicity
34	Essential oil	Self nanoemulsion	Improves oral bioavailability thereby increase drug loading and decreases inert oil requirement
35	Realgar, frankincense and myrrh oil, musk, and bezoar	Ball milling and solvent evaporation method	Anti-tumour activity
36	5,7-Dihydroxy-6-methyl-8-prenylflavanone; 5,7-dihydroxy-6-methyl-8-prenyl-4_-methoxy-flavanone; 5,7-dihydroxy-6-prenylflavanone; and5-hydroxy-7- methoxy-6-prenylflavanone	Nanoemulsion	Anti-inflammatory activity
37	Oleanolic acid	Ring opening polymerization followed by nanoprecipitation method	Anti-cytotoxic activity
38	Alcoholic, phenolic	Nano-fiber fabrication	Antibacterial activity

	compounds and carboxylic acids	throughelectro spinning	
39	Alcoholic, phenolic compounds and carboxylic acids	Electrospinning	Anti-cytotoxic and antibacterial activity

Preparation of nanocarriers:

The techniques employed for the preparation of nanocarriers are

1. **High pressure homogenisation method:**

- This technique is a reliable method for the large scale production of nanostructured lipid carriers.
- In this method, lipid is pushed through a very high shear stress with a pressure of 100-2000 bar which results in disruption of particles to nanometer range.

2. **Complex coacervation method:**

- It is a spontaneous phase separation method which seperates two liquid phases
- In colloidal systems, the interaction of two oppositely charged polyelectrolytes occurs upon mixing in an aqueous solution.
- Example: Coacervation or ionic gelation method has been focused for the preparation of nanoparticles using biodegradable hydrophilic polymers such as chitosan, sodium alginate, and gelatin. This method has been used for the preparation of chitosan nanoparticles[7].

3. **Co-precipitation method:**

- It is nothing but modified complex coacervation which is used to prepare nanoscale coreshell particles.
- This method has been reported to increase the dispersion stability of poorly water soluble drugs.

4. **Salting out method:**

- This method is based on the concept that solubility of non electrolyte in water is reduced by the addition of an electrolyte
- Example: Nanospheres are formulate by salting out method, initially in a solvent, polymer and drug are dissolved which is consequently containing the salting out agent (electrolytes), most commonly, this technique uses for heat sensitive substances [7].

5. **Nano-precipitation method:**

- This method is also known as solvent displacement method.
- It is based on interfacial deposition of a polymer after displacement of a semi-polar solvent miscible with water from a lipophilic solution which results in the decrease of interfacial tension between two phases; thereby the surface area is increased with a formation of small droplets of organic solvent without any mechanical stirring.
- Example: For most of the poorly soluble drugs, nano-precipitation method is well suited. By adjusting preparation parameters, nano-sphere size and drug release can be controlled effectively [7].

6. **Solvent emulsification-diffusion method:**

- This method is generally performed using organic solvents like methyl acetate, ethyl acetate, benzyl alcohol, butyl acetate etc which are partially miscible with water.
- In this method, emulsion is diluted with water to allow diffusion of the solvent in to the continuous phases. SLC and NLC are formed due to lipid precipitation and finally the solvent is eliminated by vacuum distillation or lyophilisation.
- In other words, this method involves the preparation of an o/w emulsion, oil phase contains polymer in presence of organic solvent and aqueous phase contain stabilizer which are emulsified using a high shear mixer, followed by adding up of water to provoke the diffusion of organic solvent, thus consequential in development of nanoparticles. Example: Breviscapine liposomes for CVS disease, cyclosporine-loaded sodium alginate glycolate technique, and doxorubicin-loaded nanosphere or nano-capsules [7].

7. **Supercritical fluid methods:**

- It is the method which is used to extract and separate substances using supercritical fluid as a solvent.
- The temperature and pressure of the supercritical fluid are higher than the critical point.
- This method can be used to prepare sub-micrometre and nanosized formulations.
- Carbon dioxide and water are the most commonly used super critical fluids.

- Supercritical fluid extraction of emulsion has been prepared through solid lipid nanoparticles using supercritical Co2. This technique uses supercritical fluid for removing the solvent from o/ w emulsion. The supercritical anti-solvent precipitation can serve as a substitute for supercritical fluid extraction of emulsions [7].

8. Self assembly methods:

- These are the processes where the atoms or molecules organize themselves in to nano structures by physical or chemical reactions

9. Emulsion-Solvent Evaporation Method:

- This method includes two steps. The first step is emulsification of the polymer solution into an aqueous phase and the second step involves evaporation of polymer solvent inducing polymer precipitation as nanospheres.
- These nanospheres are collected by a process called ultracentrifugation and washed using distilled water inorder to remove stabilizer residue or any free drug and these nanospheres are lyophilized for storage.
- This method is further modified and is known as high pressure emulsification and solvent evaporation method. The primary step in this method is the preparation of a emulsion which is then subjected to homogenization under high pressure and finally followed by overall stirring to remove organic solvent. The size can be controlled by adjusting the stirring rate, type and amount of dispersing agent, viscosity of organic and aqueous phases and temperature. However this method can be applied to liposoluble drugs and limitation are imposed by the scale up issue. Polymers used in this method are polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), Poly (caprolactone)\ (PCL), Poly (β-hydroxybutyrate) (PHB) [8].

10. Double Emulsion and Evaporation Method:

- Solvent and evaporation method have the limitation of poor entrapment of hydrophilic drugs. To overcome this difficulty, double emulsion and evaporation method is employed where the hydrophilic drug is encapsulated by the addition of aqueous drug solutions to organic polymer solution under vigorous stirring which results in the formation of water in oil emulsions. The resultant water in oil emulsion is then added in to second aqueous phase

under vigorous stirring to form water in oil in water emulsion. The emulsion is then subjected to evaporation for the removal of solvent and finally the nano particles are isolated by using centrifugation method at high speed. The formed nanoparticles must be thoroughly washed before subjecting it to lyophilisation.

- In this method, the following factors like the amount of hydrophilic drug to be incorporated, the amount of stabilizer used, the concentration of the polymer used and the volume of aqueous phase affects the characterization of nanoparticles [9].

Types of Nanopharmaceuticals:

The following are the types of nanopharmaceuticals

1. Polymeric nanoparticles:

These are the particles that are in the size range of 1 to 1000nm which can be loaded with active compounds. These are the type of particles that draw large attention and are easily made. These are mostly made from biodegradable polymers and they can increase time of circulation and the stability of drug. These are generally non-toxic as they are made from biodegradable polymers. Moreover, in addition of being non-toxic, these particles offer other advantages like controlled drug release, biocompatibility. These particles are more suitable for scale-up methods. Poly (lactide-co-glycolic acid) (PLGA) and poly (lactic acid) (PLA) are the most widely used polymers. Other substances like sugars, proteins, such as albumin, gelatine nanoparticles and many other naturally occurring molecules can also be used as polymers [8].

i. Eg i: Researcher developed triptolide-loaded poly (DL-lactic acid) nanoparticles which helps to overcome the problems like poor solubility and toxicity of triptolide by using biocompatible and biodegradable polymers, poly (DL-lactic acid).these particles were uniform in size, spherical in shape with smooth surface [10].

ii. A researcher synthesized a new biodegradable and self- assembling polymer, methoxy poly (ethylene glycol)- palmitate, for curcumin delivery to cancer cells. The system comprised of methoxy poly (ethylene glycol) as hydrophilic part, palmitic acid as hydrophobic part and curcumin was present in the core of polymer micelle. The prepared micellar nanocarriers were spherical in shape [11].

iii. Researcher developed hydrophobically modified glycol chitosan nanoparticles-encapsulated camptothecin for tumor targeting better

stability and for the prevention of the hydrolysis of Camptothecin in physiological condition. In this procedure, the hydrophobic 5β-cholanic acid moiety was chemically conjugated with hydrophilic glycol chitosan backbone and camptothecin was encapsulated for intravenous administration [12].

iv. Other drug called hypericin which is a highly lipophilic agent is insoluble in physiologically acceptable media, as a result, systemic administration of the drug gets decreased and restricts its diagnostic applications. To overcome these problems, an injectable suspension of polymeric nanoparticles with hypericine was developed using biodegradable and biocompatible synthetic polymers such as polylactic acid (PLA) or polylactic-co-glycolic acid (PLGA) with improved photodetection and photodynamic therapy for the early diagnosis of cancer.

v. Researcher developed nanoparticles of *Cuscuta chinensis* by nanosuspension method for improvement in the absorption of poor water soluble constituent, quercetin [13].

vi. In a study, researchers studied a biopolymeric delivery system for controlled release of catechin. The antioxidant activity of catechin is decreased dramatically when it is introduced in an alkaline environment. In order to protect catechin, chitosan encapsulated catechin particles were developed [14].

vii. In a study, the researcher developed chitosan nanoparticles for the extract of *Ziziphus mauritiana* and checked the effect on its immunomodulatory activity [15].

2. Solid lipid nanoparticles:

These are the nanoparticles that are spherical in shape and composed of lipids which include triglycerides, diglycerides, monoglycerides, fatty acids and steroids. These nanoparticles consist of solid lipid core matrix. In other words, these are referred as spherical structures containing a lipid core which is surrounded with surfactants. The surfactants are used to stabilize the lipid core and these particles are capable of solubilising lipophilic drugs. These can be used for the hydrophilic drugs and other fictionalizations processes. SLN share other types of nano-carriers, their common advantages like their to encapsulate non polar insoluble drugs in its polymeric core, shielding the drug from the outside environment – which could be sometimes harsh – and as consequence increases the drug stability and reduces its toxicity to the body. Other advantages include not only the ability to functionalize the SLN

surface with markers and targeting devices in order to enhance the targeting process, but also their ability to produce a sustained effect and stability. The advantage over other types of delivery systems in that they are easier to prepare, cheaper and much easier for scale-up productions [8].

3. Magnetic nanoparticles:

These are the nanoparticles consisting of magnetic materials and can be manipulated using magnetic fields. High quality iron oxide magnetic nanoparticles are synthesized by thermal decomposition, co-precipitation and micelle formation, hydrothermal and laser pyrolysis techniques.

4. Metal and inorganic nanoparticles:

Inorganic nanoparticles are very useful because of their properties as they are found in various kinds of forms including magnetic, metallic, and ceramic and in various shapes, sizes, and designs. Due to tunable properties, inorganic nanoparticles are being widely used in the medicinal formulation and medical applications as they can be effectively used for formulating blocks for drug delivery systems. Various functional groups are used in these nanoparticles to conjugate with ligands, antibodies etc. However, there are disadvantages of the inorganic particles for therapeutic and diagnostic effects. When they interact with biosystems, they cause toxicity.

5. Quantum dots:

These are often described as artificial atoms that exhibit discrete energy levels and their band gap can be modulated by varying the size. These are also defined as the particles with physical dimensions smaller then exciton Bohr radius.

6. Polymeric micelles:

These are self assembly nanoconstructs of amphiphilic copolymers with core shell structures and are used as carriers for delivery of drugs and nucleic acids. Micelles are usually spherical shaped structures having a size range of 5-25nm. This micelle is nothing but the amphiphilic aggregates. The core of the micelle is usually hydrophobic in nature if they aggregate in polar media and if the micelle aggregate in non-polar media, they do form inverted micellar system with hydrophilic core.

Micelles are recorded as the perfect carriers for drugs and are more useful than any other types of nanoparticles. If any drug formulation faces the problem of poor solubility, micelles come in to mind first to overcome the problem of solubility. The understanding of solubilisation power of micelles is not recent, it has been proven from long time specifically as

detergent. Hence, micelles are being used as drug carriers and as solubilising agents. However, these micelles has other disadvantages like these particles cannot be used to overcome other problems accompanying natural active compounds such as toxicity, stability issues and fast clearing rate. However, some of these advantages can be reduced by the preparation of polymeric micelles. Polymeric micelles help to overcome the disadvantages of micelles such as stability issues. These are more stable particles with longer shelf life and stay longer in the body [8].

7. Liposomes:

Liposomes were discovered by the British haematologist Alec Bangham in 1961 and its resemblance to the cell membrane attracted immediate attention. The name liposome was derived from the Greek language in which "lipo" means fat and "soma" means body, which strongly describes the liposomes nature. These are nothing but the spherical vesicles that are composed of lipid bi-layer made mainly of lipids. Whereas in some cases, other constituents are added along with lipids in order to modify their physical and chemical properties.

Liposomes are prepared easily by disturbing the lipid film in aqueous medium. This disturbance occurs may be as a result of force produced by a large shear via various techniques such as sonication. Liposomes vary from micelles in a way that they are composed of bi-layer lipid membrane whereas micelles are composed of monolayered lipid vesicles.

8. Dendrimers:

These are highly ordered, branched polymeric molecules which are also known as arborols and cascade molecules. These are synthesized by divergent method and convergent method.

Identification and characterization of nano herbal medicine:

The following are the advanced techniques useful for the identification and characterization of nano herbal medicines

1. High performance Liquid chromatography (HPLC):

Both the Preparative and analytical HPLC are widely used in pharmaceutical industry for the purpose of isolation and purification of herbal compounds. Vasicine, which is the major bioactive alkaloid of *Adhatoda vasica*, was estimated by HPLC in two polyherbal drug formulations - *Shereeshadi Kashaya* and Yastyadivati, and its content was found to be 18.1 mg/100 g in *Shereeshadi Kashaya* and 0.7 mg/100g in Yastyadivati [16].

Standardization of the *Triphala* (an antioxidant-rich herbal formulation) which is a mixture of *Emblica officinalis*, *Terminalia chebula* and *Terminalia belerica* in equal proportions has been reported by HPLC method by using the RP18 column with an acidic mobile phase [17].

The combination of HPLC and LC/MS the most powerful technique for the quality control of herbal medicine like licorice [18].

2. High performance thin layer chromatography (HPTLC):

HPTLC is used for both qualitative and quantitative phytochemical analysis of herbal drugs and formulations. By using this technique several samples using a small quantity of mobile phase can be analysed simultaneously. The active constituents like Gallic acid, rutin and quercetin of Terminalia chebula were estimated by HPTLC method.

HPTLC technique was used for determination of two biomarkers present in Ashwagandha like Withaferin A and β sitosterol d-glucoside [19] simultaneously.

Glycoside (Jamboline), Tannin, Ellagic Acid and Gallic Acid present in the mother tincture of Syzygium Jambolanum was quantitatively evaluated in terms of stability, repeatability, accuracy and calibration by HPTLC [20].

The HPTLC method employed for the analysis of diosgenin [21] gives accurate, faster and cost effective quantitative control

3. UPLC:

Ultra performance liquid chromatography (UPLC) was employed to evaluate decocting-induced chemical transformations and chemical consistency between traditional and dispensing granule decoctions [22, 23].

4. Liquid chromatography-Mass spectroscopy (LC-MS):

LC-MS is considered as method of choice in many stages of development of drug. The properties of Amino glycosides like the high solubility of drug in water, low plasma protein binding, and more than 90percent of aminoglycosides were excreted through the kidney were determined by the Liquid Chromatography-Mass Spectroscopy analysis.

Further this technique helps in analysis of amino glycosides in plasma samples with ion pairing chromatography [24]. The pharmacokinetic studies of Chinese medicinal herbs were also determined using LC-MS.

5. Gas chromatography - mass spectroscopy (GC-MS):

It is the system which is useful for the identification of large number of

components present in natural and biological systems. For example, in a polyherbal formulation (Megni) containing nine ingredients mainly Myristica fragrans, Eucalyptus globulus, Gaultheria procumbens and Mentha piperita, each ingredient is identified and quantified was analyzed by GC-MS method [25].

Thirty-five volatile compounds were separated and identified [26].

6. Capillary Electrophoresis:

Herbal drug is evaluated in terms of specificity, sensitivity and precision by using the method called capillary electrophoresis. Various studies related to herbal medicines have been reported and major studies were reported on two types of active constituents like alkaloids and flavonoids. Furthermore, the time taken for the analysis of herbal products by capillary electrophoresis method was two times shorter than taken by HPLC and 100 fold less than analysis done by solvent consumption. The hyphenated CE instruments, such as CE-diode array detection, CE-MS and CE-NMR, have been utilized [27].

Evaluation of nanoparticles

1. X-ray powder diffraction (XRD):

It is a rapid systematic method which is used for phase detection of the crystalline material and can endow with information on unit cell measurement and atomic spacing [28]. The X-ray is produced by a cathode ray tube that is potable to fabricate monochromatic radiation, collimated to on purpose, and projected toward the sample.

2. Thermogravimetric analysis/differential thermal analyzer:

Thermogravimetric analysis (TGA) is a thermal analysis method which deals with the changes in weight in a substance in a proscribed environment as a utility of temperature and time [29]. It is appropriate to use this method for the evaluation of all types of solid materials, including both organic and inorganic materials. This technique is also known as Differential thermal analysis. It is a calorimetric technique that analyzes the temperature, and heat surge related to thermal transitions in a substance [30]. This enables stage transitions to be resolute (e.g., melting point, glass transition temperature, crystallization). Thermogravimetric analysis (TGA) is a type of evaluation technique performed on samples that determines changes in weight, change in temperature, Particle size, poly dispersity index. The particle size and polydispersity index of materials can be analyzed by a dynamic light scattering method at a set angle and optimized temperature. This method is used to reveal the surface charge and physical stability of the formulation.

3. Transmission electron microscopy (TEM):

Transmission electron microscopy is useful to investigate the structural surface and shape of carriers and the formulation. To evaluate the sample through this technique, at first the samples should be diluted with distilled water, later place a drop of sample on a 200 mesh carbon film covered with copper grid and further stains the sample with a suitable staining solution. Finally dry the sample and analyze the shape of sample.

4. Dynamic light scattering:

Dynamic light scattering method is the fastest method used for the determination of the particle size. This method is commonly employed for the size determination in colloidal particles in nano and submicron range particles. The dynamic light scattering can also be used for the determination of particle size distribution.

5. Nuclear magnetic resonance:

Nuclear magnetic resonance (NMR) is used for the estimation of both the qualitative nature and size of nanoparticle measurement. NMR can also provide data about the physicochemical state of the constituents present inside the nanoparticles.

6. Determination of encapsulation efficiency and recovery:

The study aims to determine the efficiency of the drug to encapsulate into the carrier. In this, the sample was diluted with an organic solvent and sonicated in an ultrasonic bath for 30 min to extract drug. The extracted mixture was centrifuged at suitable rpm for 10 min and analyzed by HPLC or UV.

7. Stability studies:

The formulated nanodrug must be tested for the their stability period. The common and conventional techniques by which stability of nanoparticle can be analyzed are as follows:

a. Transmission electron microscopy (TEM)

b. **UV-visible spectroscopy (UV-Vis) and c. Zeta potential**

a. Transmission electron microscopy:

The image of TEM provides the information about the particle distribution and size distribution of nanoparticles over the proposed shelf-life period.

b. UV-visible spectroscopy:

In this method, a sample is placed between the light source and the photodetector [31]. UV-visible light beam intensity is calculated before and after the transitory through the sample. These measurements of intensity of beam of UV light before and after transitory of sample are compared at every wavelength to specify the sample's wavelength-dependent spectrum. The data is then plotted as absorbance as a function of wavelength.

c. Zeta potential:

Zeta potential is an assessment of the electric charge on the surface of nanoparticle, quantifying the charges. When a nanoparticle has a web surface charge, the charge is a screen by the concentration of ions of contradictory charges close to the nanoparticle surface [32]. These layers of oppositely charged ions move with the nanoparticle and collectively with the layer. The magnitude of the zeta potential provides in sequence about particle stability. The higher the magnitude of potential exhibit amplified electrostatic revulsion and therefore amplified stability.

8. Surface plasmon resonance:

This is based on the fact that every nanoparticle has its unique resonance absorption wavelength. The resonance condition is established when the frequency of light photons matches the natural frequency of surface electrons, oscillating against the restoring force of positive nuclei[33]. At the nanometer scale, particles put on view property are not inherent in individual atoms or to those in the bulk substance. The optical properties of nanoparticles are distinctly reliant on particle size and interpretable medium. When the nanoparticles move toward each other, they agglomerate owing to pH change and finally, UV can be used to learn the agglomeration of the particle.

9. Atomic force microscopy:

By using this method can create a topological map of a sample and which is mainly based on the forces between the tip and the surface of the sample[34]. It is one of the most promising tools to obtain an ultra high resolution of the particles.

Toxicity of Nanoparticles:

Due to the growing use of nanotechnology in high-tech industries is likely to affect human as the human beings are being exposed to intentionally generate engineered nanoparticles. Nanotechnology is also being employed in the field of medical sciences when trying to achieve a

personalized medicine. However, the most attractive properties of nanoparticles in the field of medicine such as small size, chemical composition, structure, shape and large surface area etc may contribute to the toxicological profile of nanoparticles in biological systems. In fact, as small the size of particle, the more the surface area they have per unit mass and this property of smaller particle size makes nanoparticles very reactive in the cellular environment.

Therefore, the intrinsic toxicity of the particle surface will be enhanced. Various body organs such as respiratory organs, blood, central nervous system (CNS), gastrointestinal (GI) tract and skin have been shown to be targeted by nanoparticles. A typical urban atmosphere contains approximately 107 particles/cm^3 of air that is less than 300nm in diameter. For example, Carbon in elemental form is a major component of these particles and the size of these particles is a determinant of their ability to cause systemic cardiovascular effects. Indeed, fine and ultrafine particulate matter (from 0.1 to 2.5 mm in mass median aerodynamic diameter) that can more easily access the vasculature via inhalation are linked to cardiovascular dysfunctions, particularly in subjects with pre-existing vascular diseases [2].

Recent advances:

Mesoporous nanoparticles: The scientists are using nanotechnology for plant research to be called as "phytotherapy". The new breakthrough solution is the use of "mesoporous" nanoparticles. These mesoporous nanoparticles can introduce the gene and activate the gene at the same time, in a very precise and controlled manner and without any toxic after effects. These mesoporous nanoparticles are coated with chemicals and by using these mesoporous nanoparticles as carriers, the genes will be delivered in to the plants. The chemical coating of these nanoparticles induces the plants to swallow them, effectively ingesting them inside the plant cell walls, where the genes could be inserted.

Plant viruses

Recently, nanotechnology has employed plant viruses for drug delivery in cancer. Plant viruses are added to the long list of types of nanoparticles which are being investigated as next generation formulations for nanotech cancer therapy.

For example, from North Carolina State University, "Nanoparticle 'smart bomb' targets drug delivery to cancer cells". Researchers (at North Carolina State University) are succeeded in modifying a common plant virus to deliver drugs only to specific target cells inside the human body, without

affecting the surrounding tissue. Each tiny "smart bomb" is one thousands of times smaller than the width of a human hair. These smart bombs could lead to more effective chemotherapy treatments with very minimal or absolutely no side effects. The researchers observed that the virus is appealing in both its ability to survive outside of a plant host and its built-in "cargo space" of 17 nm, and it can be used to carry chemotherapy drugs to tumour cells directly. The researchers attached small proteins to the virus on its exterior surface, called "signal peptides", that cause the virus to 'seek out' the target cells, such as cancer cells. These "signal peptides" helps the virus to enter the cancer cell by serving as "pass words", where it releases its cargo. Viruses that lack a lipid envelope (i.e., they consist of a genome surrounded by a protein capsid and other protein structures) are provided with molecularly precise container of known structure and organization to which targeting molecules can be attached. The encapsidation of various nanoparticles up to 17 nm in diameter by the 36 nm diameter "Red Clover Necrotic Mosaic Virus" (RCNMV) has been reported. This plant virus has a genome consisting of two single strand RNA molecules. The two genomic RNA molecules form a complex that binds the viral capsid protein and initiates the assembly of the virion. A small RNA molecule that mimics the site on the second genomic RNA required to initiate virion assembly can be tethered to various nanoparticles and then serve to initiate virion assembly, forming uniform virus-like particles about 33 nm in diameter- slightly smaller than the native virus particles- that encapsidate the nanoparticle within the protein shell. The relatively small size of the virus-like particles is an advantage because particles in the 30 nm range can be delivered directly to the cell nucleus via the nuclear pore complex. The virus-like particles are also sturdy enough to facilitate purification Calcium serves as the key to keep the virus' cargo enclosed, the virus cargo gets opened in the environment where calcium levels are low. When the virus is in the bloodstream, virus cargo is in enclosed state due to higher levels of calcium in the blood. After virus entering in to the cell, the cargo gets opened due to lower levels of calcium and delivering the cancer drugs only to the targeted cells [1].

Out of different nanoparticles which are used as carriers of drug, the plant virus becomes most successful as drug carriers by possessing properties like high stability, ease of manufacture, ability to target cells and ability to carry therapeutic cargo. Another factor that makes the virus cargo more unique is the toughness of its shell, when the virus is in a closed state, nothing will leak out from its interior and when it does open, it opens slowly, which means that the virus has time to enter the cell nucleus before

deploying its cargo, thereby increasing the drug's efficacy. Researchers believe that these plant virus cargos will reduce the side effects of common chemotherapy treatments, while increasing the effectiveness of the treatment.

Future prospective:

"Herbal remedy" in the nano- carriers will increase its potential for the treatment of various chronic diseases and health benefits. Many successful examples with experienced evidences are present among us in the direction of nano research. Herbal remedies are also prosperous resources of advantageous compounds holding antioxidants and constituents that can be made use in purposeful foods. Collaborative research among the traditional "Herbal remedies" and newer approaches of modern drug delivery system, i.e., "Nanotechnology" has established the attractive therapies to the pharmaceutical in near future that will enhance health of people.

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Chapter - 5

Osteoporosis: A Current Update on Globally Epidemic Asymptomatic Disorder

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Abstract

Osteoporosis, a silent bone disease until the fracture occurs, characterized by low bone mass and deterioration of microstructure of bone with consequent increase in fracture susceptibility and bone fragility. Osteoporosis is more common in women, Caucasians, and older people (55 to 65 years). Globally, over 200 million people are suffering with osteoporosis. In India, more than 50 million people are suffering with osteoporosis or low bone mass. Osteoporosis induced fractures are more common in women (one in 3 women) and men (one in 12 men) and contributes to morbidity and mortality. Approximately 60,000 hip fractures, 50,000 forearm fractures and 40,000 symptomatic vertebral fractures occur in the United Kingdom per year. Incidence of osteoporosis increases with age, from 2% at the age of 50 to 25% at the age of 80 years in women. Prevalence of osteoporosis induced fractures is one in two women and one in five men at the age of 50 years. Moreover, it increased the disability-adjusted lifespan, healthcare cost and decreased quality of life. Fracture risk algorithms after combining bone mineral density and clinical risk factors are widely employed in clinical practice to identify the high-risk individuals for therapy. This timeline article reviews the diagnosis, management and pathogenesis of primary and secondary osteoporosis. In spite of this, we also focus on the emerging therapies, the future of novel treatment paradigm and precision medicine against osteoporosis.

Introduction

Osteoporosis is a metabolic bone disorder which increases a risk of fracture due to low mineral density of bone leading to decreased bone strength, mineralization and impaired microstructure of bone (1-2). Osteoporosis is an undiagnosed disorder until the manifestation of fractures such as proximal humerus hip, pelvis, wrist, and spine resulting in hospitalization (3-5). In 1993, International consensus defined two important manifestations of osteoporosis that is microarchitecture and bone mass and in the following years, WHO produced a diagnostic criteria for osteoporosis of healthy young women by using SD scores for bone mineral density (BMD). BMD T-score for osteopenia is between 1-2.5 and for osteoporosis is 2.5 or less (6). More than 200 million osteoporosis cases have been reported globally (2, 5). Up to 30% women are suffering with osteoporosis in Europe and the USA and 30% men (2, 7). Although, osteoporosis is more common in post-menopausal women with 40% incidence in Europe and the United States while in the Indian women, it varies from 25% to 65% (2, 8). Menopause and advancing age are two crucial factors that increased the resorption rate than absorption, consequently enhancing the risk of fracture (5, 9). The hip fracture epidemiology is well defined among other fractures which occurs exponentially as a function of age (after the age 0f 80 years), approximately 2.7 million hip fracture have been reported in 2010 globally and geographical variation is also high with hip fracture (Incidence differences is more than 10-fold) (5). Historically, incidence of hip fracture has frequently occurred in people of European ancestry (especially in northern Europe) while lowest incidence has been reported in eastern Asian populations (10). At the age of 50 years, probability of having hip fracture in men and women is 3.5% and 14.6% respectively in the rest of lives (2, 11)(Tuzun *et al*., 2012; Whitmarsh *et al*., 2019). In the past few decades, divergent secular trends have been found to be associated with hip fracture, it has been increasing in Asian countries and decreasing in North America (12). Factors that contributed to these trends are obesity, birth period cohort effects, lifestyle, urbanization, and consequences of screening (13). Approximately 30% hip fracture and 20% vertebral fractures occur in men (5, 14). Several lines of evidence showed that vertebral fracture is clinically not recognized but still has importance as a marker of bone fragility which indicated about the increased risk of other types of fracture including hip fracture (15). Variation in incidence and prevalence of vertebral fracture has been reported in the whole world while higher variation was observed in Asia and North America (16). However, prevalence of forearm fracture is likely to be low in males in contrast to females and higher risk of the development of hip and vertebral fracture in men with

forearm fracture. Further, low BMD has been reported in men with distal forearm fracture with respect to age matched control subjects (17-18). Therefore, management of this disease and associated consequences is very important to improve the quality of life and reduce health care costs which will also be helpful in decreasing hospitalizations, medical visits, and nursing home admission.

Etiology

Bone strength is integration of two important features of bone that is bone density and bone quality (19). Several factors are defined that also increase the risk of osteoporosis-induced fractures.

(1) Osteoporosis - Bone density and Bone quality: Bone, an immensely adaptive material that changes its composition and structure over times of overuse or disuse (20). WHO (world health organization) provided a criteria for assessment of bone status and consequent risk of fracture which is defined by T-score, number of standard deviations (SDs) after analyzing the result of patient's test which may be falls below (negative T-score) or above (positive T-score) with respect to the young normal reference mean (6). Bone mineral density (BMD) is defined by T and Z-score (number of SD of a patients BMD when differs from average BMD of their sex and age) (21). Although, clinically it is defined as a proxy for overall bone strength, expressed as gm (grams) of mineral / cm^2 (square centimeter) or gm / cc (cubic centimeter) (5, 22). A majority of epidemiological studies have been demonstrated that risk of fracture is increased when BMD declines (9, 23). A longitudinal cohort study on women of Europe has been reported that the risk of vertebral fracture increased (by a factor of 1.5 per 0.5 gm/cm^2) after decreasing spinal BMD value. BMD is generally measured by using dual energy X-ray absorptiometry (DXA) to correlate with risk of fracture. Reduction of SD by one promote the risk of fracture by 1·5–2 times, and by 2·5 times when hip fractures are correlated with hip BMD (9, 24). Most of the fractures in individual occurs with BMD T-score which does not meet the criteria that defines the osteoporosis (-2·5 or less), this is considered as a limitation of BMD. BMD shows low sensitivity for when used alone for diagnosis of osteoporosis (9, 25). Bone quality which reflects the combination of both bone strength and BMD is another useful concept. Bone strength is defined by structural and material properties of bone which affects overall bone quality. Microarchitecture (eg, cortical thickness / porosity, trabecular thickness and connectivity) and geometry (shape and size) are the two components that define the structural property of the bone. Collagen composition (Cross-linking and types), damage accumulation (like

microfractures) and mineralization (crystal size and mineral-to-matrix ratio) defines the material property of the bone. The bone turnover rate affects these components of bone strength (26-27). Alteration in the bone remodeling affects these properties resulting in increased risk of fracture in aged women (2, 28). In spite of this, deficiency of estrogen hormone due to menopause leading to loss of bone turnover and bone which culminates with increased propensity of fracture (26, 28). In addition of BMD, several other clinical risk factors (like sex, age, and previous fracture) can be employed for assessment of fracture risk with or without BMD. Several tools have been developed for the assessment of fracture to determine absolute fracture risk from these clinical factors (Table-1).

Table 1: Risk assessment prediction tool

	Risk factor - Input	**Outcomes**	**Specific Features**
Fracture risk assessment tool	Sex, age, body-mass index, previous fragility fracture, family history of hip fracture, alcohol consumption of ≥3 units/day, use of glucocorticoid ≥3 months, secondary osteoporosis, rheumatoid arthritis, current cigarette smoking, femoral neck BMD or T score (optional)	10 year major osteoporotic fracture (clinical forearm, hip, vertebrae, proximal humerus) ;10 year hip fracture	9 international prospective cohorts, Meta analysis of risk factors, Population specific calibration; includes competing mortality
Garvan Fracture Risk Calculator	Sex, age, risk fractures after age of 50 years (none, 0, 1, 2, ≥3), history of falls in 12 months (none, 0, 1, 2, ≥3), femoral neck BMD of T-score (optional), weight	5/10 year osteoporotic fracture (hip, wrist, metacarpal, clinical vertebrae, clavicle, pelvis, patella, scapula, humerus, proximal tibia, distal, femur and sternum, 5/10 year hip fracture	Dubbo epidemiological study (858 Australian men and 1358 women) include dose-response for number of previous fracture
Q-fracture scores-2016	Sex, age, weight, height, alcohol, SLE, rheumatoid arthritis, COPD, diabetes, previous fracture,	1/10 year osteoporotic fracture (clinical spine, distal forearm, hip or	357 practice in wales and England (> 1 million men and >1 million women) includes

	history of falls, HRT, cardiovascular disease, chronic liver & kidney disease, antidepressant or anti-convulsant use, epilepsy, endocrine problems, Parkinson disease, epilepsy	humerus fracture), 1/10 year hip fracture	smoking (4 level), alcohol (5 level), type of diabetes, BMD is not an input variable

COPD: Chronic obstructive pulmonary disease

SLE: Systemic lupus erythematous

HRT: Hormone replacement therapy

Adapted from Compston *et al*., 2019

(2) Osteoporosis - Dietary factors: Skeleton contains up to 99% of calcium found in the body (29). Several lines of evidence reported a relationship between calcium intake and BMD ranges from weak, non-significant and positive association (30-31). Results of these studies were based on small sample size, as well as they have not included factors like sex and age. A majority of other studies reported a modest relationship of dietary calcium and BMD and shown that it decreases the rate of cortical bone loss (31-32). Dietary calcium intake (580 mg/day) is inversely proportional to age while direct relationship with femoral neck BMD and physical activity index (PAI) in women and femoral neck BMD and lumbar spine in men (31-32). Hence, adequate amount of dietary calcium intake and maintaining a physical activity may reduce the chances for the development of osteoporosis. Deficiency of vitamin-D is common among the elder people, especially related to the households and geriatric patients. Vitamin-D deficiency causes mineralization defects, high bone turnover, bone loss, hip, and other fractures and hyperparathyroidism (33-34). The low serum concentration of 25-hydroxy (OH) vitamin D were found in geriatric patients in Ireland and the United Kingdom (35-36). Low serum concentration of 25-(OH) D were also observed in patients with Parkinson and Alzheimer's disease in Japan (37-38), as well as in patients with hip fractures in different countries(37-38). High prevalence of vitamin-D deficiency was observed in healthy adults (14%) and independent elder people (39%) in France (35, 39). Deficiency of vitamin-D was also observed in patients with hip fracture in Australia (35, 40). A significant Vitamin-D deficiency that affects BMD at hip has been reported in urban Indians (41-42). Rural populations in India use too many phytates that affects the absorption of calcium resulting in calcium deficiency (41, 43). Numerous drugs are also interfering with absorption of calcium in the human

body including anticonvulsants, diuretics, antiinflammatory drugs, corticosteroids, nonsteroidal asthma medications with corticosteroids, immunosuppressive medications, and a number of antibiotics (21, 44).

(3) Osteoporosis - Hereditary / Genic factors: Race is one of the important factors for BMD and risk of fracture. A majority of studies reported that incidence rate of fracture more frequently occurred in women in contrast to men. In Asian and white people, higher rates of osteoporosis induced fracture were observed in women in all age groups after age of 50 years (9, 37) while in Hispanic people, higher rates were observed in men in contrast to women with age group of 50-59 years and after age of 60 years, this gender specific effects were found to reverse (37). Men had higher rates in contrast to women in Negro populations up to age of 70 years while after this age, women had higher rates of osteoporosis induced fracture (9, 37). Incidence rate of hip fracture increases with age with respect to all race, ethnic groups and both genders (9, 45). A study conducted in the United States notified that the rate of increased risk of fracture was low in Asians compared with Hispanic and Non-Hispanic Black (45). Some studies reported a minimum hip fracture and highest mean BMD value for black women (19, 46-47). Longer period of hospitalization has been found to associate with black patients after hip fracture and non-ambulatory at discharge in contrast to white patients (48-49). (Kellie *et al*., (1990) (30) reported that mortality rate was higher in black women (9.3%) during hospitalization after hip fracture compared with white women (5%). Above studies suggested that age, gender, ethnicity and race affect the incidence of hip fracture. Body size, another factor that affects the propensity of fracture, A study led by Ensrud *et al*., (1997) (50) reported that aged women with small body size are at increased risk of hip fracture due to low hip BMD value. Women experiencing hip fracture by twice which have maternal history of hip fracture in contrast to women without maternal history (51).

(4) Osteoporosis - Behavioral factors: Diverse behavioral factors increase the risk of fracture which induces the development of osteoporosis and atraumatic fractures. Cigarette smoking, one of the key factors which increase the risk of hip fracture and bone loss in aged people due to decreased calcium efficiency of intestine (52-54). The risk of fracture is similar in smokers and non-smokers at age of 50 after age of 60 it is higher in smokers (17%) and 108% at 90 (Law and Hackshaw, 1997; Wu *et al*., 2016) and also reported that the risk of fracture was increases by 19% in smokers of England compared with non-smokers (12%) at the age of 8**5**. Intake of alcohol (>207 ml/per week) is another factor for risk of fracture (55). In spite of this, caffeine

is also promoting the risk of hip fracture and bone loss in older women with recessive homozygous (tt) variants of the Vitamin-D receptor (56). High consumption of coffee has been found to be associated with risk of fracture in Swedish women while consumption of tea was not (56-57).

(5) Osteoporosis - Clinical factors: Peak bone mass, a major determinant of the bone density in older individuals (58-59). The gaining of peak bone mass starts in *In Utero* while it is completed at the age of 40. Basically, low peak bone mass is correlated with propensity of fracture risk while detailed role of peak bone mass is still not explored (5, 19, 59). Reduction of bone mass and increased bone loss with age has been reported in women after cessation of ovarian function (in the first year) at menopause (60-61). Rapid bone loss and low bone mass has been reported in the postmenopausal women with low body weight, body mass index and % of body fat (60, 62). Low bone mass and bone loss plays a key role in the development of postmenopausal induced osteoporosis independently. Low serum concentration of estradiol (<5 pg/mL) high serum concentration of globulin (≥ 1 mg/dL -sex hormone binding protein) have been found to increase the risk of hip and vertebral fracture in women at age of 65 (63). Elder Women with family history of hyperthyroidism also have a high risk of hip fracture independent of BMD resulting in impairment of neuromuscular and bone strength (37). A number of studies also showed a relationship between increased risk of hip and vertebral fracture and previous fracture history at any sites (64-66). Above studies suggested that existing defects in microstructure of bone may influence the increased risk of fracture indecently of BMD. A woman (1in 5) with vertebral fracture develops a new vertebral fracture in subsequent years (64, 66). Impaired vision also increases the risk of hip fracture in aged white women in an independent manner (37). Poor grip strength linked with hand that may develop due to diabetic neuropathy, joint disorders, cognitive impairment also increases the risk of fragility fracture in an independent manner postmenopausal woman (51, 53).

(6) Osteoporosis - Medical factors: Secondary osteoporosis is associated with a different kinds medical disorder like hematologic disorders (eg, pernicious anemia and thalassemia), gastrointestinal diseases (eg, malabsorption syndromes and inflammatory bowel disease), and hypogonadal states (eg, amenorrhea), as well as due to medications (19). Glucocorticoids (most common medication) affects the quality and quantity of bone (Peel *et al*., 1995). A majority of studies reported that glucocorticoids treatment leads to bone defects mediated by trabecular perforation and increased bone turnover (67-68). In postmenopausal women, glucocorticoids increased the

risk of fracture sooner in contrast to women with initially higher BMD values (69-70). It is estimated that about 10% bone loss occurs in patients treated with glucocorticoids after the first year of therapy after that trends are 2%-3% each year (69, 71). Approximately 20% of patients had fragility fracture in the first year of treatment with glucocorticoids (72).

Postmenopausal Osteoporosis: The central role of estrogen deprivation in postmenopausal induced osteoporosis has been well documented. Deprivation of menopausal estrogen stimulates bone remodeling which culminates with bone loss because bone remodeling favors resorption over formation of bone (73-74). The level of estradiol is directly proportional to BMD, hence after menopause, low estradiol level is correlated with low BMD and increased risk of fracture. Decrease in the activity of aromatase enzyme in both men and women leading to decrease the level of estradiol which correspond to low BMD and higher risk of fracture. Reduction in circulating estrogen induces the recruitment of osteoclast at the active remodeling site which enhances the resorption depth and basic multicellular unit (BMU) activation frequency which culminates with acceleration of bone resorption process. In spite of this, deprivation of estrogen also negatively regulates the BMU by decreasing the lifespan of osteoblast. Alteration in RANKL/OPG system is the most common mechanism that occurs due to estrogen deprivation. Several lines of evidence reported that several cytokines (IL-11, IL-6, TNF and IL-1) and RANK/OPG system are activated due to estrogen deprivation (75-77) while conflicting results have been reported in human studies. Bone biopsies study reported the high mRNA level of RANKL/OPG in the bone (especially in proximal femur) of patients who have fracture with respect to controls with osteoarthritis (78). Pacifici *et al*., (79), demonstrated that the immune system is a key determinant in increased resorption after withdrawal of estrogen. Estrogen withdrawal also disturbs the ratio of mineralization; it promotes negative calcium balance by decreasing the absorption of calcium in the intestine while increasing its excretion in urine and also induces secondary hyperparathyroidism (80-81). In the one of setting, poor intake of calcium (<800-1000 mg/day) and deficiency of Vitamin-D (i.e., serum 25-OH vitamin D <20 ng/ml or <50 mmol/l) increases the level of parathyroid hormone (PTH) which further induces the bone loss and high bone turnover specifically in older women (82-83). Older individuals are more prone to bone impairment and enhanced risk of non-vertebral fracture with deficiency of calcium and vitamin-D (34). Continuous increase in PTH leads to increased bone resorption over formation and especially affecting the cortical sites (84). Several lines of evidence reported that enhanced levels of PTH increased the level of sclerostin which inhibits the canonical Wnt

signaling (85). Upregulation of insulin-like growth factor binding protein-4 and downregulation of IGF-1 has been found to be associated with secondary hyperparathyroidism due to insufficiency of calcium and vitamin-D.

Senile Osteoporosis: Bone mass declines in both men and women throughout adulthood after achievement of growth due to reduction in bone formation (2, 86). Due to lack of sex hormone after aging, the rate of bone remodeling is increases in gradual manner. The first affected site is trabecular bone due to the presence of a large number of remodeling surfaces / unit volume resulting in trabecular perforation and thinning and, finally, trabeculae loss (2, 86). Normal aging alters the ratio of bone formation and bone resorption (2). Several studies reported that BMD decreases with age (87-88). A recent study showed that a significant reduction in cortical thickness (13%) a BMD (38.1%) was observed in elderly women (2).

Pathogenesis - Osteoporosis: The pathogenesis of osteoporosis is multifactorial disorder with complex interplay of dietary, hormonal, genetic, lifestyle and physical factors. It may be either primary (idiopathic) or secondary and each condition are associated with low BMD. Inverse relationship has been found between BMD and increased risk of fracture. Two to threefold increase in incidence of fracture risk is observed for each SD reduction in BMD value (89). Several other factors affect the BMD in an independent manner including skeletal geometry, bone turnover, trabecular architecture, and propensity for falling and postural instability. Age at which initiation of loss begins, rate of bone loss and achievement of peak bone mass is key determinant of BMD.

(1) Peak bone mass: Approximately 80% of the peak bone mass is contributed by genetic factors and remaining one by influence of environmental factors including diet, age of puberty and exercise (5, 90-91). Numerous genes are associated with BMD including estrogen receptor, collagen 1α1, vitamin D receptor, IGF-1(IGF-1) binding protein and insulin-like growth factor 1 (92-93). Intrauterine development is also considered as a factor for peak bone mass achievement because of an association between peak BMD and birth weight, growth rate of child (94-95).

(2) Bone Loss: Loss of bone begins between 35 and 45 years of age in both sexes which is accelerated after postmenopausal and continued until the end of life in both sexes (5, 96). Bone loss associated with age that may be influenced by alcohol consumption, smoking, low body mass index, smoking, alcohol consumption, impaired metabolism of vitamin-D secondary hyperparathyroidism and physical inactivity (5, 90-91). Loss of estrogen

hormone (sex steroid) at menopause condition in women is a main contributor for the development of osteoporosis resulting in increased bone loss and bone turnover. A study led by Stepan *et al*., (97) demonstrated the role of sex steroid in the preservation of bone density in men as they observed the accelerated bone loss after castration. Hypogonadal has been found to be associated with 50% of men with hip fracture and 20% with symptomatic vertebral fracture (98). A number of studies have been demonstrated that serum oestradiol not testosterone is responsible for vertebral fracture and alteration in BMD in men (99-100). Although, oestradiol, a dominant sex hormone which regulates the resorption of bone in men (100-101). Several factors (genetic, intrauterine development and environmental factors) interact with each other to determine the peak bone mass. Age related decline in sex steroids affects the rate of bone loss in both indirect and direct manner.

(3) Secondary Osteoporosis: Another form of osteoporosis is secondary osteoporosis because of several factors including alcohol consumption, anticonvulsants, multiple myeloma, corticosteroids, hyperthyroidism, hypogonadism, and skeletal metastases. A majority of studies reported that approximately 55% of men and 30% of women with symptomatic vertebral fractures was due to secondary osteoporosis (102-104). Several lines of evidence also showed that increased risk of hip fracture is associated with secondary osteoporosis (105-107). Cushing syndrome accelerates the risk of bone loss due to prolonged medication with glucocorticoids (105, 108). Despite this, patients of rheumatoid arthritis also require prolonged glucocorticoids medication which leads to secondary osteoporosis and it is most common medication and found to be linked with drug induced osteoporosis (105). Decline in BMD has been reported within 3-6 month of glucocorticoids therapy initiation (105). Causes of secondary osteoporosis may also vary with respect to gender like excessive use of glucocorticoids, alcohol and hypogonadism induce the osteoporosis more likely in men in contrast to women (109-110). Increased risk of osteoporosis has been reported in men who is receiving androgen deprivation therapy (ADT)**,** 19.4% of patients with ADT treatment experienced fracture when compare with those (12.6%) who is not treated with ADT (110-111).

(4) Falls: Several lines of evidence showed that non-skeletal factor (increased risk of falls and physical frailty) also contributes to the risk of fracture other than skeletal factor and BMD (112-114). Nguyen *et al*., (114) reported that cumulative effects of high body sway and low BMD showed increased risk of fracture compared with either one alone. A majority of studies showed that the condition like dementia, strokes, vertigo, visual

impairment and alcohol abuse increased the risk of hip fracture (115-116).

Emerging concept – Pathogenesis of osteoporosis: Sex hormones and estrogen plays a key role in the development of osteoporosis (117). Indeed, several other mechanisms have been found to be associated with development of fragile bones. Increase in oxidative stress due to aging is one of central mechanism that leads to decrease in bone formation and osteoblast lifespan while increase in osteocyte and osteoblast apoptosis culminating with osteoporosis (118). Involutional related oxidative stress induce the translocation of mesenchymal cells into adipogenic lineage (at the expense of osteoblast lineage) by shifting β-catenin (principal effector molecule of Wnt signaling pathway) to the antioxidant FoxO-mediated pathway. Studies on mice showed that lack of FoxO transcription factor which acts again oxidative stress had similar bone loss which was observed in age related oxidative stress but at younger age (118-119) while mice with increased expression of FoxO displays enhanced bone mass (120). Oury *et al*., (121) reported the role of serotonin (hormone / Neurotransmitter) in regulation of bone mass. Serotonin hormone which is synthesized peripherally impedes the bone formation while centrally synthesized serotonin neurotransmitter promotes bone formation and impedes bone resorption while mechanism is not well defined (122-124). Leptin is a regulator of central serotonin which explains about central neural-regulation of skeletal homeostasis (125-126). FSH (follicle stimulating hormone) and TSH (thyroid stimulating hormone) may also play a key role in the regulation of bone remodeling through binding on receptors expressed on the bon cells (127-129). TSH impedes the bone turnover while FSH enhances the resorption by an independent manner on osteoclast through osteoblast (129-130). The different mechanisms of skeletal homeostasis associated with these factors are assessed in mice. Involvement of these factors in the development of menopausal and age-related osteoporosis is still not explored.

Screening / Diagnosis - Osteoporosis: The final consequences of osteoporosis is fracture especially hip, vertebral and distal forearm. Historically, low trauma fracture is the basis of diagnosis of osteoporosis. Most of the organizations recommend the BMD screening for all adults with a history of fracture after the age of 50 years (3-4, 131-132). BMD screening is recommended for women of 65 year of age and older, for younger women who are under the greater risk of fracture or equivalent condition in comparison with healthy Caucasian women of 65 years of age. The endocrine society defined criteria and recommends BMD screening for men with age of 70 years and older and men who have additional risk factors for the development of secondary osteoporosis with age of 50 to 59 years (133). Barr

et al., (134) have been demonstrated that screening reduces the risk of fracture in 25.9% of patients with age of 45 to 54 years were observed after 9.5 years mean follow up. The gold standard to measure the BMD is dual-energy X-ray absorptiometry (DXA) device especially in case of vertebral and hip fractures (3, 132, 135) and obtained T-score are used to predict the risk of fracture and BMD. Low value of BMD (negative BMD T-score) is an indicator of risk of fracture (Table-2).

Table 2: WHO criteria for osteoporosis

Diagnosis	T-Score
Normal	- 1 and Higher
Osteopenia	-1 and -2.5
Osteoporosis	- 2.5 or lower
Severe osteoporosis	- 2. or lower with one or more fragility fracture

The National Osteoporosis Foundation of America approved a screening of patients after 1-2 year of treatment initiation and two-3 years thereafter and other studies recommend a test in every 4 years (3, 136-137). The North American Menopause Society (NAMS) approved that screening is allowed after two to five years of menopause in women. NAMS also noted that repeated testing is not allowed in patients who are under osteoporosis treatment until the 1 to 2 years after the initiation of treatment (138). Researchers from University of Sheffield, UK developed tool for assessment of fracture risk called as Fracture Risk Assessment Tool (FRAX) on the basis of risk factors like race, age, gender, alcohol abuse, history of smoking, body mass index, smoking history, use of glucocorticoids, rheumatoid arthritis, secondary osteoporosis, prior family history of fracture prior as well as neck BMD measurements to see the 10-year probability of osteoporotic fracture like hip and other major one (132) and this tool can be used with other tool like DXA to predicts the appropriate osteoporosis patients for treatment. However, FRAX has a lot of limitations including it is not fit with total lumber spine and hip BMD, ethnic minorities and for age outside the range (40 to 90 years). In spite of this, it does not utilize the history of fall to determine the risk of fracture and finally, it does not indicate which patients have to treat.

Management – Osteoporosis: Clinicians should include identification and treatment of cardinal features of secondary osteoporosis including prevention of falls, lifestyle changes to reduce bone loss and treatment of osteoporosis. The Royal College of Physicians (RCP) defined a guideline for osteoporosis management on the basis of grade accessible for each

intervention (5, 22, 127). Grade-A recommendation is based on random control trials, Grade B recommendation is based on results obtained from controlled studies without epidemiological or randomization studies, Grade C recommendation is based on report of expert committee or recognized authorities of clinicians.

(1) Investigation – Secondary osteoporosis: Particular treatment for secondary cause of osteoporosis like hyperparathyroidism, hypogonadism and hyperthyroidism enhances bone density by 10%-20% (9, 139-141). Therefore, it is very crucial to focus on the physical testing, family history and appropriate investigation (Table-3). Measurement of 25-(OH) D and intact PTH may be helpful in excluding vitamin-D insufficiency and secondary hyperparathyroidism in patients with malabsorption, previous gastric resection, limited sunlight exposure and anticonvulsant treatment. If supplementation of vitamin-D & calcium is planned for treatment then measurement of PTH and serum 25-(OH) D are not useful as results are unconvincing for management. Tests for anti-endomyseal antibodies, investigations suggestive for malabsorption and individuals with undetermined osteoporosis should be carried out to exclude the coeliac disease.

(2) Lifestyle changes: Advice should be given to patients with osteoporosis and fractures to evaluate the lifestyle to reduce the bone loss including less use of alcohol and tobacco, eating a balanced diet (rich in calcium), to maintain regular exposure to light and physical activity. Only exercise comes under Grade-A which has a valuable effect on BMD while increased intake of calcium in the diet and reduction in tobacco abuse comes under Grade-B and has a valuable effect on BMD.

(3) Prevention of fall: Fall assessment should be done for patients having recurrent fall and history of fracture to recognize and modify the extrinsic (Loose carpets, trailing wires, and ill-fitting footwear) and intrinsic (musculoskeletal and neurological disease, poor vision, and medications) risk factors for falling. A randomized controlled trial on 301 elderly patients (>70 years), each with risk factor of falling proved that fall assessment is fruitful (142). Geriatric assessment with slight modification of risk factors decreases the rate of fall up to 35% over 12 months compared with those who are under the normal healthcare and social input. Close *et al*., (143) also reported the reduction (61%) in the rate of fall among the intervention group with respect to control groups. Although, both studies do not observe reduction in fracture incidence. Hip protectors are used with specially designed underwear to reduce the impact of force if falls cannot be secured in the patients. A study

performed on the elderly nursing home residents showed 50% reduction in hip fracture with the help of hip protector over 12 months (144). This designed underwear is Bulky and uncomfortable to wear, these are disadvantages of this garment (145). Parker *et al.*, (146) reported only 2.2% of patients with hip protector sustained the fracture of hip with respect to controls (6.2%).

Treatment – Osteoporosis: Treatments for silent disease can be classified into anabolic and antiresorptive agents. Antiresorptive agents increase BMD by 5%-10% by decreasing bone resorption after 1st year of treatment while anabolic agent increases BMD by 50% (147-148). There are many drugs which show a promising effect on BMD including raloxifene, oestrogen, bisphosphonates, calcium and vitamin D, calcitonin, and parathyroid hormone.

(1) Hormone Replacement Therapy (HRT): Several small controlled trials showed that HRT reduces rapid bone loss which occurs after menopause (5, 22). A study performed on 464 postmenopausal women (5-year randomized controlled trial) showed that HRT decreases the risk of non-vertebral fractures up to 71% (149-150). The beneficial effect of previous long-term HRT on bone density has been found to decrease progressively when treatment was stopped and may be completely lost up to the age of 75 years (151). A few studies carried out on elderly women with established osteoporosis showed that HRT increases spine bone density up to 5% (149, 152-153). Lufkin *et al.*, (153) also reported the decrease in incidence of vertebral fracture up to 60%. Meta-analyses of randomized trials showed that HRT also reduces the vertebral fractures by 30% (154) and 27% reduction in the non-vertebral fractures (149, 155).

(2) Bisphosphonates: Bisphosphonate or diphosphonate, an analogue of pyrophosphate and it is used to treat osteoporosis and similar diseases. It reduces bone resorption by decreasing osteoclast function and recruitment. Diphosphonate shows prolonged action in contrast to other drugs as they persist for a long time (many months) in the skeleton. Several lines of clinical studies reported that cyclical etidronate (Didronel PMO) increased the spine bone density up to 5% while decreasing the incidence of further vertebral fracture up to 60% in older women (75 years) with established osteoporosis (9, 156-158). An epidemiological study showed that cyclic etidronate also decreases the incidence of hip fractures up to 44% in women with age of 76 years (72). A study performed on 994 women with osteoporosis (between 45 and 80 years) showed that Fosamax (alendronate) increased bone density at the femoral neck and lumber spine up to 5.9% and 8.8% respectively (159). Another study showed that Fosamax significantly increased bone density of

spine, forearm, and femoral neck BMD while decreased the incidence of fractures up to 50% (Black *et al*, 1996). Cummings *et al.*, (63) reported that Fosamax reduces the vertebral deformation up to 44% in older women with low hip bone density. Several lines of studies demonstrated that Actonel (Risedronate) is effective in the treatment of postmenopausal induced osteoporosis (160-162). The study carried out in Europe, Australia and America demonstrated that Actonel significantly increased the BMD of femoral neck, lumbar spine, and femoral trochanter over three years by 1.6%–3.1%,5%–6%, and 3.3%–6.4% respectively compared with placebo group over 3 years (160-162). They also reported the reduction in non-vertebral fracture (33%–39%), as well as decrease in incidence of new vertebral fractures (41%–49%) decrease in non-vertebral fractures in older women treated with Actonel with respect to placebo group (160-162). These above data suggested that BMD measurements are critical in recognizing patients who would gain benefit from diphosphonate treatment.

(3) Raloxifene: Raloxifene (Evista), an oestrogen receptor modulator having agonist actions on the skeleton while it shows antagonistic effects on the endometrium and breast. A few studies showed that Raloxifene increased femoral neck and lumber spine BMD up to 2%-3%, decreased the risk of vertebral fractures up to 30%-50% and reduced the occurrence of breast cancers in women (31-80 years) with osteoporosis (5, 163-164). No evidence has been reported that raloxifene reduces the prevalence of non-vertebral fractures.

(4) Vitamin-D and Calcium: Supplementation of calcium may reduce the bone loss in aged women and men (Peacock *et al*., 2000) while there are no convincing available reports that it reduces the risk of fracture in osteoporosis patients. The outcomes associated vitamin-D supplementations are also inconsistent (34, 165). Combination of vitamin-D and calcium supplementation are more useful in the treatment of frail elderly patients with osteoporosis rather than alone use of these supplements. A French study who used the combination of elemental calcium (1.2 g) and vitamin-D3 (800 IU) daily and reported that this supplementation reduced the PTH levels, decrease the risk of hip fracture and increased femoral neck BMD up to 27% (166-167). Another study demonstrated that daily supplementation of vitamin D3 (700 IU) with elemental calcium (500 mg) had an adequate valuable effect on BMD and reduced the occurrence of non-vertebral fracture up to 54% (168).

(5) Calcitonin: Calcitonin, an antiresorptive agent having a rapid but short-term effect on the function of osteoclast. A few studies showed a small increase in BMD and decrease in vertebral fracture after treatment with

calcitonin in postmenopausal women with osteoporosis (110, 169-170). The Prevent Recurrence of Osteoporotic Fractures (PROOF) studied the effects of intranasal calcitonin up to 5 years in women with osteoporosis and reported the marginal increase in BMD. The 200 IU dose of calcitonin decreased the probability of new vertebral fractures up to 33% while reduction in fractures with 100 and 400 IU of calcitonin was found to be non-significant (171). Kanis *et al*.,(90) reported that intranasal (Miacalcic) and parenteral calcitonin (Calsynar) reduced the fracture by 57% with respect to placebo (66% for non-vertebral and 55% for vertebral). Short courses of calcitonin are helpful in the treatment of acute pain associated with osteoporotic fractures. The daily use of nasal salmon calcitonin (200 IU) for 28 days reduced the pain and promoted the earlier mobilization with respect to the placebo group in women with acute vertebral crush fractures (110, 172).

(6) Parathyroid Hormone (PTH): The physiological function of PTH is to maintain the normal level of calcium in blood circulation. PTH promotes both bone resorption and formation resulting in decrease or increase in BMD value which depends upon mode of administration (173-174). Elevation of PTH due to continuous infusion causes a greater resorption than formation resulting in bone loss. Transient increase of serum PTH due to daily injections leads to greater bone formation, as well as increase in BMD. Earlier studies based on human PTH showed a large increase in BMD value (174-175). Large interventional studies (trial) are become possible after the development of recombinant human PTH (rhPTH) Use of rhPTH leads to increase in BMD up to 9%–13% in the lumbar spine, as well as in the femoral neck (3%-6%) in postmenopausal women with osteoporosis with respect to placebo preparation (176). Reduction in new vertebral fractures (65%) and non-vertebral fractures (53%) has been also reported in postmenopausal women with osteoporosis.

Treatment of osteoporosis in men: A few studies have been examining the beneficial effect of calcium and vitamin-D, diphosphonate, testosterone and rhPTH. Beneficial effect of Calcitriol on BMD and incidence of fracture has been not observed in men (162, 177). Observational studies in men demonstrated that Intermittent cyclic etidronate increased the lumbar spine BMD up to 5%–10%, with slight increase in the hip BMD (178), as well as it shows similar effect on bone density in women and men while its effect on incidence of hip fracture in men is still not clear. Daily use of alendronate (10 mg) in men (between 31-87 years) with osteoporosis increased the lumber spine BMD up to 7.1% and femoral neck BMD by 2.5% with respect to controls (179). Similar increase in BMD has been reported in the patients with eugonadal and hypogonadal men after treatment with alendronate (180).

Reduction in occurrence of new vertebral fracture has been also reported in men with osteoporosis with respect to placebo (180-181). Supplementation of vitamin-D and calcium has been also shown to enhance BMD and reduce non-vertebral fractures in both men and women over 65 years (168, 182). Testosterone improves the bone mass in men with hypogonadal induced osteoporosis (139, 183). In spite of this, it may also increase spine bone mass in eugonadal men who have vertebral fractures. Testosterone significantly increases spine bone mass by 5% in eugonadal men with vertebral osteoporosis in six months, however no changes has been found to be associated with hip bone density (180, 184). Another study demonstrated that long-term corticosteroid treatment increased spine bone mass by 5% after twelve months after testosterone therapy, while no change has been found to be associated with control after 12 months observation (185). A study performed on rhPTH in men (30–68 years) increased lumber spine BMD up to 13.5% and femoral neck BMD by 2.9% over 18 months (186). Another study also observed an increase in BMD and decrease in vertebral fracture (50%) with rhPTH in men with osteoporosis (180).

Conclusion: Osteoporosis is a global concern causing approximately 8.2 million fractures each year. Fracture resulting due to osteoporosis is a major cause of mortality and morbidity in aged people. The pathogenesis associated with osteoporosis is complex and multifactorial, contributing to alteration in bone turnover. Alterations in sex hormones in later life is one of the key mechanisms for bone loss. In addition to this, oxidative stress and wnt signaling pathway are novel mechanisms which also contributes to the development of osteoporosis. Further, unveiling the role of wnt signaling pathway in skeletal homeostasis at the molecular level may open the window for therapeutic approaches in the near future. In spite of significant advances in the risk assessment of fracture and availability of various pharmacological therapy, still many high-risk individuals do not get appropriate investigations and treatments. Implementation of novel care systems and establishing secure and productive long-term treatment regimens to reductions in risk of fracture in future.

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