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REVIEW ARTICLE

Novel Insights in the Metabolic Syndrome-induced Oxidative Stress and Inflammation-mediated Atherosclerosis

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ARTICLE HISTORY

Received: June 09, 2017
Revised: September 09, 2017
Accepted: September 28, 2017

DOI:
10.2174/1573403X13666171009112250

Abstract: ***Context*****:** Atherosclerosis is a progressive pathological process and a leading cause of mortality worldwide. Clinical research and epidemiological studies state that atherosclerosis is caused by an amalgamation of metabolic and inflammatory deregulation involving three important pathological events including Endothelial Dysfunction (ED), Foam Cell Formation (FCF), and Vascular Smooth Muscle Cells (VSMCs) proliferation and migration.

Objectives**:** Research in recent years has identified Metabolic Syndrome (MS), which involves factors such as obesity, insulin resistance, dyslipidemia and diabetes, to be responsible for the pathophysiology of atherosclerosis. These factors elevate oxidative stress and inflammation-induced key signalling molecules and various microRNAs (miRs). In present study, we have reviewed recently identified molecular targets in the pathophysiology of atherosclerosis.

Methods**:** Scientific literature obtained from databases such as university library, PubMed and Google along with evidences from published experimental work in relevant journals has been summarized in this review article.

Results**:** The molecular events and cell signalling implicated in atherogenic processes of ED, FCF and VSMCs hyperplasia are sequential and progressive, and involve cross talks at many levels. Specific molecules such as transcription factors, inflammatory cytokines and chemokines and miRs have been identified playing crucial role in most of the events leading to atherosclerosis.

Conclusion**:** Studies associated with MS induced oxidative stress- and inflammation- mediated signalling pathways along with critical miRs help in better understanding of the pathophysiology of atherosclerosis. Several key molecules discussed in this review could be potent target for the prevention and treatment of atherosclerosis.

Keywords: Metabolic syndrome, obesity, oxidative stress, inflammation, cardiovascular diseases, atherosclerosis.

1. INTRODUCTION

Atherosclerosis is the leading cause of mortality and morbidity worldwide [1]. A recent update given by American Heart Association (AHA) in 2015 states, that globally, Cardiovascular Diseases (CVDs) account for approximately 17.3 million deaths each year [2]. Atherosclerosis, a focal disease, occurs at the sites of disturbed blood flow specifically at arterial branch points and bifurcations. Atherosclerosis was earlier believed to be solely associated with excessive lipid accumulation. However, recent research advances identify it as a multifactorial and multigenic inflammatory condition of the arterial wall. The increased prevalence of several risk factors, identified as major contributors towards atherosclerotic plaque formation, includes hyperglycemia (HG), dyslipidemia and hyperinsulinemia grouped together as Metabolic Syndrome (MS) along with high blood pressure, cigarette smoking, poor diet and insufficient physical activity. These factors pose risks of atherosclerosis regardless of the ethnicity, geography and gender. A cascade of biochemical and molecular events initiated by these factors may progressively lead to inflammation, Endothelial Dysfunction (ED), Foam Cell Formation (FCF), Vascular Smooth Muscle Cells (VSMCs) proliferation and extracellular matrix remodelling, which are important parts of pathogenesis of atherosclerosis [3].

Recent studies suggest that during MS, persistent presence of various atherogenic molecules instigates a hypoxic environment along with oxidative stress. These changes together contribute towards enhanced ROS production in the cells, which leads to the activation and augmentation of various inflammatory pathways. Among these pathways, activation of NALP3 inflammasome complex and subsequent release of interleukin-1 beta (IL-1β) and IL-18 has also been implicated in atherosclerotic plaque development [4]. Further, Endoplasmic Reticulum (ER) stress, linked with a

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1573-403X/18 $58.00+.00

number of pathophysiological conditions, has also been implicated in atherosclerosis [5]. ER is an early response site during cellular stress that results in Unfolded Protein Response (UPR) [6]. In the presence of persistent atherogenic stimuli, the aggravated ER stress is quite prevalent. However, the detailed mechanism of ER stress in atherosclerosis remains unexplored.

Despite being a highly investigated disease process, various aspects of atherosclerosis are still unclear, including the role of various atherogenic molecules and molecular mechanisms of initiation of atherosclerosis. In past one decade, a number of microRNAs (miRs), the 18-22 nucleotides long, non-coding RNAs, have been identified as critical regulatory molecules in various inflammatory pathologies, including atherosclerosis [7]. The miRs are named based on their sequential identification, for example if last published miR is miR-22, the next newly identified miR will be named miR-23. Additionally, the naming also depends on the organisms in which these miRs are identified, for example in hsa-miR-22 the prefix hsa stands for *Homo sapiens*. Further, miRs are synthesized along with regular gene transcription and subsequently undergo cleavage to form precursor miR. The precursor miRs are further processed by DROSHA and DICER enzymes into mature miRs, which directly bind to 3' UTRs of its targeted mRNA and cause its degradation or inhibition of translation. The nature and complementarity of a given miR sequence determine the level of silencing of target genes. The high complementarity sequence of miRs to 3' UTRs of its target mRNA causes degradation and as complementarity decreases the level of silencing also decreases. The miRs have a diversified function where one miR may simultaneously regulate approximately 100 different mRNAs, and a single mRNA may be regulated by many miRs [8]. However, it also depends on the cell type and cellular environment. Cigarette smoking is one of the prominent cardiovascular risk factors that induces several miRs, however, their role in atherosclerosis is not well understood. Further studies in this field are needed to highlight the role of cigarette smoke-induced miRs in the pathogenesis of atherosclerosis. Studies indicate that the levels of miRs are modulated by oxidative stress and various inflammatory stimuli during major atherosclerotic events *viz*; ED, FCF and VSMCs proliferation and migration [9] (Table **1**). These evidences indicate that atherosclerosis is indeed multifactorial and multigenic pathology. Here in, we have discussed latest studies and findings to consolidate and enhance the understanding of atherosclerosis pathophysiology, especially the major events including ED, FCF and VSMCs proliferation with emphasis on inflammation and oxidative stress-mediated signalling pathways as well as roles played by miRs.

2. ENDOTHELIAL DYSFUNCTION AND ATHEROSCLEROSIS

ECs normally maintain the balance between vascular constriction and dilation, antithrombotic and anti-inflammatory conditions through elaborate molecular cascades. Constant exposure of ECs to oxidized apo-lipoprotein B/ LDLs and latter's accumulation in sub-endothelial space of arterial walls creates an inflammatory microenvironment [10, 11]. Additionally, several factors such as dyslipidemia, diabetes mellitus, and hypertension have been identified to cause injury to ECs lining, affecting their functions including barrier function and contraction dilation cycle, which result in ED, a hallmark of atherosclerosis initiation [12]. ED is

Table 1. Summary of miRs and their target genes altered during atherosclerosis.

Atherosclerotic Events	Affected miRs	Target Gene and Effect of miRs	Outcome on Atherosclerosis Progression
ED	miR-138	↓ S100A1	Increase
	miR-92a	↑ICAM-1, ↑MCP-1, ↑NF-κB, ↓KLF-2, ↓KLF-4, ↓eNOS	Increase
	miR-146-5p	↓TRAF-6, ↓IRAK1	Decrease
	miR-19b-3p, miR-221-3p, miR-222-3p	↓ PGC-1α	Increase
FCF	miR-134a	↑LPL	Increase
	miR-155	↓HBP1	Increase
	miR-33	↓ PGC-1α	Increase
	miR-223	↓TLR4/ NF-κB, ↑PI3K/Akt	Decrease
VSMCs proliferation and migration	miR-559	↓TGF-β2	Decrease
	miR-34a	↓Notch-1	Decrease
	miR-135b-5p	↑HIF-1α	Increase
	miR-499a-3p	↓MEF2C	Increase

further characterized by reduced activity of endothelial nitric oxide synthase (eNOS), overexpression of adhesion molecules, activation of different inflammatory signals and secretion of cytokines. ED, if not repaired, paves the way for the development of atherosclerosis in the environment of oxidative stress as discussed below.

2.1. Mechanisms of Oxidative Stress and Inflammation - Induced Vascular Endothelial Dysfunction

Modified lipoproteins and cigarette smoke independently generate excessive ROS in ECs through NADPH oxidase (NOX), a membrane-bound enzyme complex, specifically NOX 1, 2 and 5 [13]. Increased oxidative stress creates an imbalance in EC homeostasis, and induces overexpression of inflammatory cytokines such as IL-1β, IL-6 and TNFα [14], and adhesion markers such as ICAM-1 and VCAM-1. The increased expression of adhesion markers has also been reported during diabetes [15, 16]. Metabolic stress and inflammation caused by elevated blood glucose seem to involve following mechanisms that may cause ED. HG-mediated activation of p90 ribosomal S6 kinase (p90RSK) could form a complex with Extracellular-signal-regulated kinase-5 (Erk-5). Erk-5, a signalling intermediate and a transcription factor, is known to regulate the EC homeostasis [17]. The complex formation with p90RSK inhibits nuclear translocation and transcriptional activity of Erk-5 [16]. Inhibition of Erk-5 has been reported to suppress anti-inflammatory genes that may contribute to ED [16, 18]. In stress condition, decreased activity of Erk-5 also leads to augmented ROS production in ECs [19], which could lead to increased inflammation and stress *via* NF-κB pathway (Fig. **1**).

Elevated ROS level in human intestinal microvascular ECs and human oesophageal microvascular ECs has been shown to induce nuclear translocation of NF-κB/p65, upregulation of COX2 and prostaglandin E2 (PGE2), and overexpression of ICAM-1 and VCAM-1 [20, 21]. These evidences indicate that ROS is a crucial player in ED through modulating traditional inflammatory cascade of NF-κB as well as Erk-5. Recently, a novel inflammatory pathway Thioredoxin interacting protein (Txnip)-induced NALP3 inflammasome activation has been identified during acute ischemia in ECs [22]. Txnip- induced inflammation leads to caspase-1 activation and secretion of IL-1β and IL-18 in ECs which results in the precipitation of inflammatory ischemia in the cells of arterial walls [4, 22] (Fig. **1**).

In the ECs several other inflammatory pathways are also triggered in response to circulating fatty acids such as palmitic acid. These molecules cause excessive production

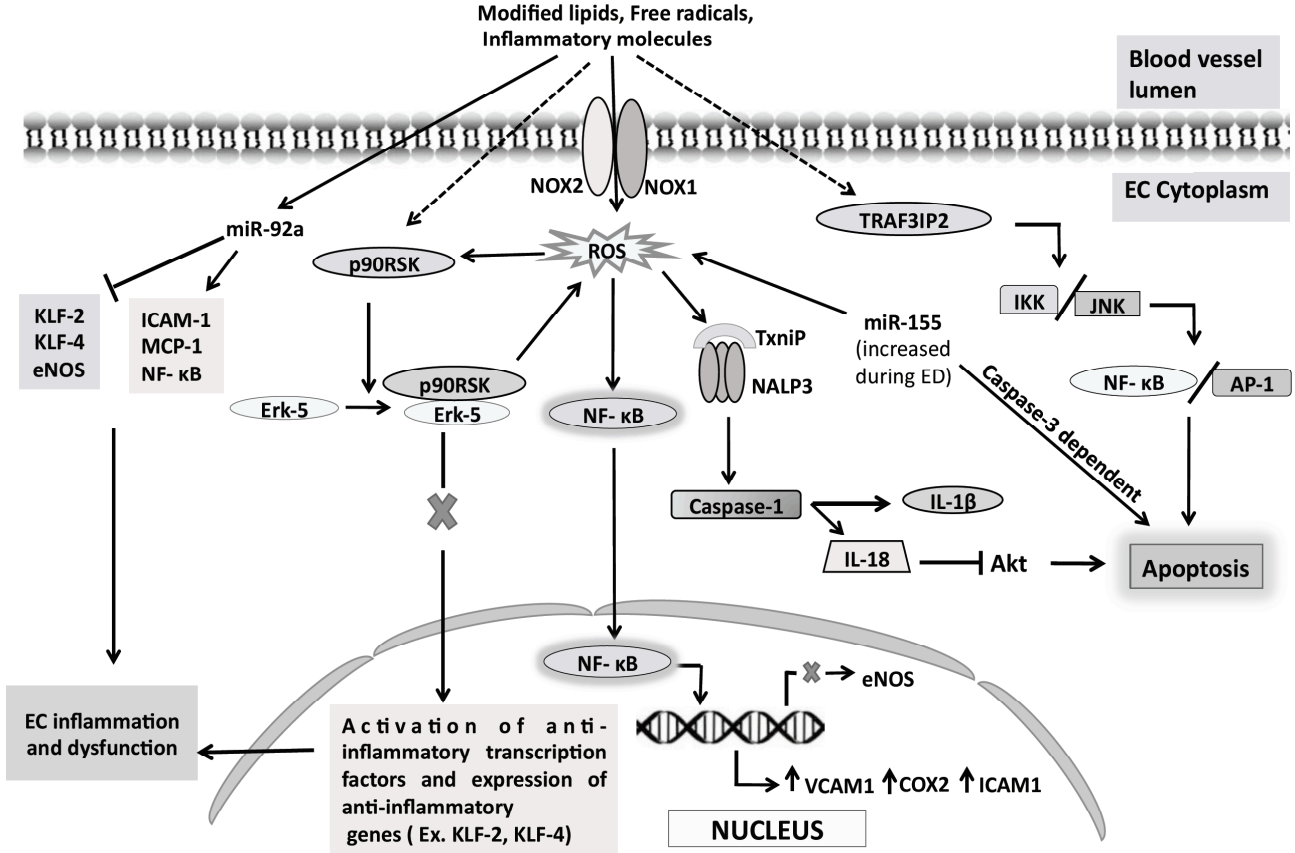

Fig. (1). Schematic representation of mechanisms of dysfunction and apoptosis in ECs in the presence of different atherogenic stimuli. Presence of atherogenic stimuli induces ROS, TRAF3IP2, p90RSK and over expression of various miRs. Induction of ROS *via* NOX leads to NF-κB activation that increases expression of inflammatory markers and decreases eNOS. Overexpression of miR-92a further increase expression of inflammatory genes and decreases expression of anti-inflammatory genes. Increased ROS also induces p90RSK that inactivate Erk-5 by confining it to cytosol, thereby inhibiting the expression of anti-inflammatory genes. ROS further induces NALP3/caspase-1/IL-18 pathway leading to apoptosis *via* TRAF3IP2/JNK pathway and overexpression of miR-155.

of extracellular ATP followed by an increased activation of purinergic receptors P2X7 and P2X4 [23]. The purinergic receptors are known to activate inflammation cascade that further causes ROS generation, which stimulate redox sensitive kinases including p38 mitogen-activated protein kinase (MAPK). The redox sensitive signalling increases the expression of inflammatory factors such as IL-6, IL-8, ICAM-1 and VCAM-1 along with reduced level of eNOS which precipitate ED [23]. Further, atherogenic molecules also induce ER stress that activate MAPK and inhibit eNOS which in turn activate NADPH oxidase-mediated ROS generation in ECs leading to ED [24]. Inhibition of ER stress in ECs has been shown to restore vasoconstriction and vasodilation property of the blood vessels [25], suggesting regulatory role of ER stress in ED.

Oxidative biomolecules such as oxLDLs and lysolipids (components of oxLDL and minimally modified lipoproteins) are known to induce apoptosis in ECs of primary coronary artery through the recruitment of TRAF3 interacting protein 2 (TRAF3IP2), an adapter protein which is an important regulator of inflammatory and autoimmune response [26]. OxLDL-mediated oxidative stress induces TRAF3IP2 expression in the ECs through the generation of super oxides and hydrogen peroxides [27]. The activated TRAF3IP2 further activates downstream IKK/NF-κB and c-Jun N-terminal kinases (JNK) cascades which propel the ECs towards apoptosis [26] (Fig. **1**). Further, inhibition of mammalian target of rapamycin (mTOR) by oxLDL-induced oxidative stress has been implicated in cardiovascular complications [28, 29] through increased level of autophagy-related 13 (AR-13) proteins. AR-13, a mammalian autophagy factor, forms an autophagy complex under dephosphorylated state and induces autophagy and apoptosis. Oxidative stress has been shown to inhibit mTOR, which dephosphorylates AR-13 leading to autophagy and apoptosis in vascular ECs leading to ED [30].

Collectively, the preceding discussion suggests that metabolic stress-derived oxidative agents such as HG, fatty acids and oxLDL may cause dysregulation of critical pathways including Erk-5-mediated EC homeostasis, NALP3 inflammasome-mediated EC inflammation, and mTOR- and TRAF3IP2-mediated EC apoptosis. These pathways, although unrelated, could potentially cause ED which helps in the development of atherosclerosis. Therefore, targeting these novel molecules and pathways could be important in decreasing the incidence of ED and atherosclerosis.

2.2. Stress-sensitive miRs and their Role During Endothelial Dysfunction

Although identified recently, miRs have been implicated as important regulators of cellular pathophysiology *e.g*. miR-138 has been shown to decrease the activity of eNOS *via* downregulation of S100A1 in the presence of inflammatory cytokines such as TNF-α, Angiotensin II and Endothelin-1 [31]. S100A1, an important Ca^{2+} binding protein and a critical factor for eNOS activity, gets downregulated by miR-138. Another athero-miR, miR-92a has been found highly expressed in atherosclerotic plaques as well as in oxLDL condition *in vitro* [32]. It has been implicated in EC activation and inflammation by increasing the expression of ICAM-1, MCP-1 and NF-κB on one hand, and by decreasing the expression of Krüppel-like Factor 2 (KLF2), KLF4 and eNOS on the other (Fig. **1**) [32, 33]. These alterations are crucial in elevating inflammation and pathophysiology of ED.

The presence of inflammatory molecules such as LPS and TNF-α reduces miR-146b-5p expression which in turn downregulates the expression of TRAF-6 and IRAK1, two direct signal transducers of TLR4. In addition, miR-146b-5p also inhibits the expression of adhesion molecules and cytokines in ECs [34-36]. Therefore, its reduced level during low-grade inflammation or bacterial infection would mean that expression of TLR-4- mediated signal transducers TRAF-6 and IRAK-1 would increase leading to increased inflammation in ECs. Indeed, decreased level of miR-146b-5p has been linked with increased inflammation and ED in ECs [37]. Further, inflammatory cytokines TNF-α and IFN-γ are reported to cause overexpression of a set of miRs *e.g*. miR-19b-3p, miR-221-3p and miR-222-3p, which then downregulate the peroxisome proliferator activated receptor γ co-activator 1 alpha (PGC-1α) in the intimal layer of atherosclerotic blood vessels [38]. PGC-1α is a central regulator of cellular energy and mitochondria biogenesis. Thus, its downregulation causes mitochondrial dysfunction leading to excessive ROS generation that contributes to ED through induction of apoptosis in ECs [38]. Furthermore, miR-155 has been implicated in ED by downregulating nitric oxide level in ECs and inducing apoptosis *via* caspase-3 activation (Fig. **1**) [39].

These recent studies have enhanced the understanding of the functions of these miRs and implicated them in atherosclerosis pathophysiology suggesting them as important targets in the prevention and/or treatment of atherosclerosis.

3. FOAM CELL FORMATION AND ATHEROSCLEROSIS

In dyslipidemia patients ED paves the way for immune cells, monocytes and neutrophils, to transvasate into the sub-endothelial space [40]. Neutrophils are the first to infiltrate the sub-endothelial space followed by monocytes, where they contribute to an inflammatory microenvironment [40]. Various endogenous molecules released by neutrophils, monocytes and macrophages, including cytokines such as TNF-α, IL-1, IL-6 and IL-18, chemokines and growth factors, play a contributory role in establishing inflammatory environment [41, 42]. Due to polarizing nature of these molecules, monocytes differentiate into macrophages and secrete numerous cytokines, chemokines and free radicals which augments inflammation [43, 44]. Further, due to their scavenging nature the macrophages uptake, and/or synthesize and accumulate excessive oxidized lipids and get transformed into foam cells. It is likely that the uptake and *de-novo* synthesis of lipids go hand in hand as the macrophage's sterol sensing system does not recognize the accumulated modified lipids, which is interpreted as deficiency of sterols. This perceived deficiency may be fulfilled by uptake of excessive modified lipids through upregulated scavenger receptors and also possibly by synthesizing new lipid molecules *de-novo*. In macrophages, sterol regulatory element-binding protein **(**SREBP) cleavage-activating protein (SCAP) re-

mains bound to SREBP and keeps it inactive. Upon sensing low sterols, SCAP escorts SREBP to undergo site-specific cleavage followed by its translocation into the nucleus where SREBP regulates the transcription of lipid synthetic enzymes. In this manner SCAP/SREBP system usually regulates lipid homeostasis. SREBP is stimulated by various endogenous and exogenous biomolecules [45]. During oxidative stress or accumulation of modified lipids, this system fails to recognize already amassed modified lipid as native sterols, resulting in SREBP activation which could trigger *de-novo* lipid synthesis. In this manner, unregulated excessive lipids accumulation converts monocyte-derived macrophages (MDMs) into foam cells. As one of the critical players in atherosclerosis, foam cells have been one of the prime interests of investigators to control and regulate atherosclerotic plaque formation. Altered lipid homeostasis especially *de-novo* lipid synthesis in MDMs during MS could be a novel molecular target for atherosclerosis prevention and therapy.

3.1. Modified LDLs, the Major Contributors in Dysregulated Inflammatory Pathways in FCF

In metabolic stress-mediated chronic inflammatory condition, excessive and persistent presence of free radicals and oxidizing agents provides an oxidative environment, which oxidizes and modifies the circulating lipid molecules, especially LDLs. OxLDL is a strong stimulus that activates both ECs and monocytes, which consequently express myriads of inflammatory proteins and receptors. OxLDLs also cause ER stress which further increases the expression of CD36 through upregulation of inositol-requiring kinase/endonuclease-1 (IRE-1), an ER stress responsive transmembrane protein and activation of transcription factor-6 (ATF-6) [46]. Further, lipoprotein lipases enhance CD36-mediated lipoprotein uptake by monocytes and MDM. CD36 through the recruitment of Na^+/K^+ ATPase-Lyn complex activates Lyn and mitogen-activated protein kinase kinase kinase 2 (MEKK2) [47, 48] (Fig. **2**), which in turn promote the migration and colonization of monocytes in sub-endothelial space. Lyn also phosphorylates JNK1/2 MAP kinase which helps in modified LDL internalization that favors FCF [49].

In obese individuals with MS, the circulating adipokines such as visfatin promote the internalization of oxLDL by MDMs [50]. This phenomenon is attributed to PI3K- and ERK-dependent signalling which in turn augment the expression of scavenger receptor-A and CD36, and downregulate the cholesterol efflux proteins such as ATP-binding cassette transporters subfamily A member 1 (ABCA1) and subfamily G member 1 (ABCG1) [51]. These receptors, exces-

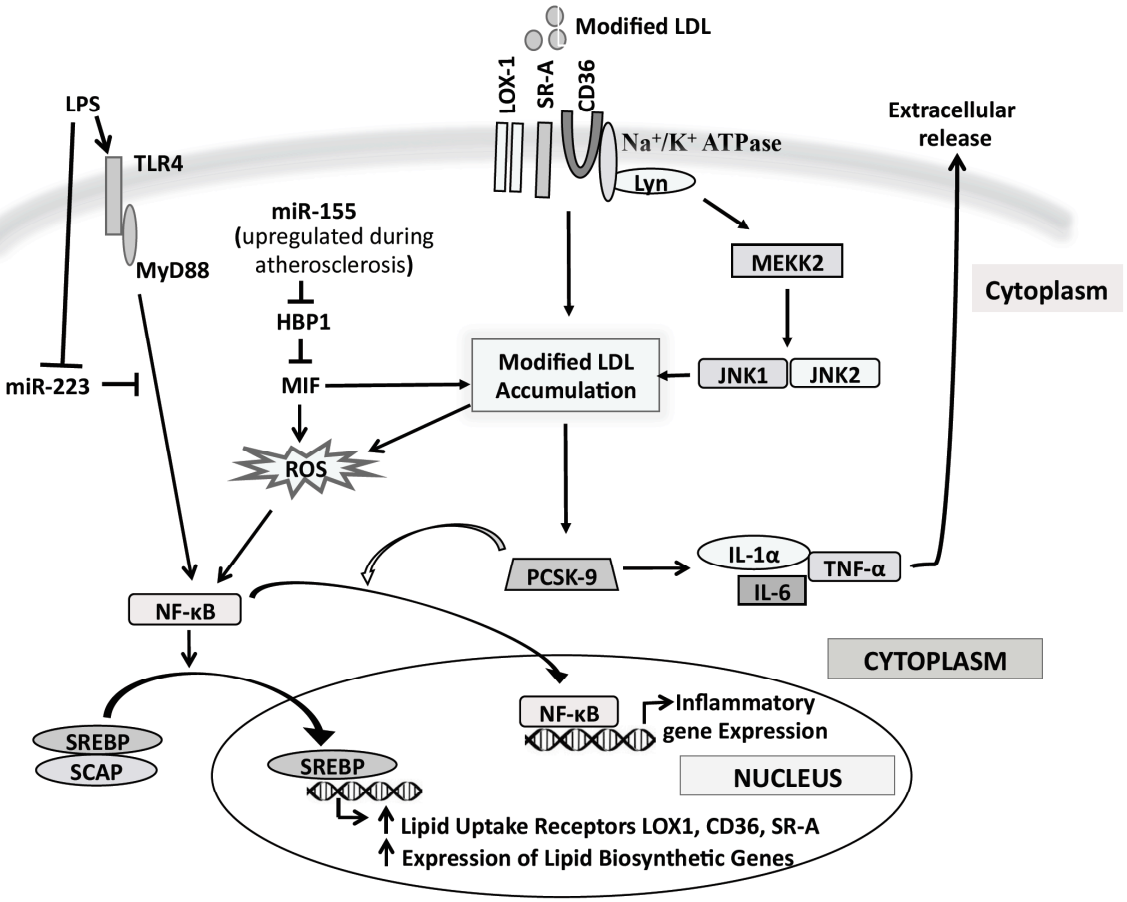

Fig. (2). Schematic representation of molecular mechanisms of foam cell formation in the presence of various atherogenic molecules. CD-36 associated with Na^+/K^+-ATPase and Lyn complex activates JNK pathway that increases lipid accumulation. Accumulated lipids induce PCSK-9 activation which mediates nuclear translocation of NF-κB and synthesis of cytokines, whereas decreased expression of miR-223 mediates TLR4/MyD88/NF-κB pathway activation, which facilitates nuclear translocation of SREBP. Modified lipids in macrophages positively regulate ROS production which is also induced by miR-155 by increasing MIF and downregulating HBP1.

sively expressed on the cell surface of monocytes and macrophages during metabolic dearrangement and oxidative stress, facilitate the uptake and accumulation of modified LDL and contribute to FCF by activating different pathways. One of these pathways of modified lipid uptake is through the downregulation of Angiopoietin-like 4 (ANGPTL4), a protein known to suppress lipoprotein uptake by inhibiting LPL activity [47]. However, targeting ANGPTL4 alone may not be sufficient as other receptors expressed on macrophage may play compensatory roles.

Hypoxia is a critical feature observed during plaque formation, and known to mediate increased glucose uptake in foam cells [52]. Excessive glucose uptake leads to increased ROS production through protein kinas C (PKC)-NOX pathway, and activate the expression of scavenger receptors through p38 MAPK pathway. The scavenger receptors increased uptake excessive lipoproteins leading to FCF [52-54]. The excessive lipid uptake and ROS production during hypoxia are augmented by the knock-down of HMG box-transcription protein-1 (HBP1). The downregulation of HBP1, a transcriptional repressor of macrophage migration inhibitory factor (MIF, which is known to facilitate lipid uptake in macrophages), during inflammation could enhance FCF. Indeed, HBP1 has been found suppressed in monocytes derived from $ApoE^{(-/-)}$ mice and in patients suffering from coronary heart disease [55, 56].

TLR signalling pathway plays a vital role in the innate immune response by recognizing specific pathogen associated molecular patterns, and also implicated in FCF. TLR-4/MyD88-mediated pathway activates NF-κB and induces FCF by establishing a cross-talk with SCAP-SREBP pathway as discussed earlier. NF-κB activation by TLR pathway has been shown to increase the translocation of SCAP-SREBP from cytoplasm to nucleus where it upregulates biosynthesis and uptake of lipid through increased transcription of lipid synthetic enzymes and scavenger receptors genes [57, 58] (Fig. **2**).

Proprotein convertase subtilisin/kexin 9 (PCSK9), a member of the protein-converting enzyme family, regulates cholesterol homeostasis by controlling NF-κB activity. Silencing of PCSK9 has been shown to inhibit secretion of IL-1α, IL-6, TNF-α and NF-κB-mediated inflammatory response in THP-1 cells [59]. These evidences suggest that PCSK9 could be an inducer of NF-κB activation possibly through inflammatory cytokines, ILs and/or TNF-α. These inflammatory outbursts could be responsible for the metabolic changes that promote FCF.

Recently, inflammasome activation has been implicated in atherosclerosis, and is commonly associated with the presence of endogenous molecules such as cholesterol crystals, saturated fatty acids, oxLDL and adenosine triphosphate (ATP) [60, 61]. ATP triggers the purinergic receptors such as P2X and P2Y which through K^+ efflux, Ca^{2+} release and ROS generation cause NALP3 inflammasome activation in the macrophages [41, 62, 63]. Inflammasome activation by these molecules during MS releases inflammatory cytokines such as IL-1β and IL-18 and thus increases the plaque burden [41]. For example, the inflammatory cytokines IL-18 binds to IL-18 receptor and Na-Cl co-transporter, an ion transporter protein of 12-transmembrane domain with newly identified IL-18 binding site [64]. The IL-18 binding to these receptors leads to Erk1/2 and p38 MAPK phosphorylation which results in cytokine and chemokine release, and induces inflammatory signals in monocytes and MDM. Palmitate, one of the most common saturated fatty acids, induces IL-1β secretion and decreases IL-1 receptor antagonist (IL-1Ra) secretion in monocytes and MDM [65]. The conditioned media of monocytes and MDM cultured with palmitate induced the expression of adhesion markers ICAM-1 and E-selectin in human aortic and vein ECs, and enhanced the adhesion of monocytes to ECs indicating their contribution to FCF and ED [65].

Taken together, metabolic stress-induced chronic inflammatory conditions may lead to altered oxidized/modified lipids uptake and/or *de-novo* synthesis, which in turn lays the molecular basis for transformation of MDMs into foam cells. In this process scavenger receptors, TLR-4 signalling, SREBP, NALP3 inflammasome and various other inflammatory mediators play contributory roles. These advanced understandings could be helpful in the identification of potent molecular targets for prevention and therapy of atherosclerosis.

3.2. Stress-mediated Deregulation of miRNAs in FCF

Several miRs have been identified to play a pivotal role in FCF either through regulating the lipoprotein uptake machinery or the lipid biosynthetic gene expression. As mentioned earlier, ANGPTL-4 inhibits LPL activity and regulates lipid uptake by scavenger receptors. It has been shown that miR-134a inhibits ANGTPL-4 expression in atherosclerotic macrophages by directly binding to 3'UTR of its target mRNA. As a result, suppressed ANGPTL4 leads to increased LPL expression which helps in enhanced lipid accumulation and inflammation in macrophages. The release of pro-inflammatory molecules including TNF-α, IL-1α and IL-6 are increased in the presence of miR-134a in atherosclerotic macrophage [47]. Thus, miR-134a could be an important target in preventing foam cell phenotype [47]. Further, increased expression of miR-155 in the presence of oxLDL has been shown to downregulate HBP1, which subsequently leads to MIF overexpression and facilitates excessive lipid uptake and ROS production in macrophages and contributes to FCF (Fig. **2**) [55]. Thus, miR-155 could be another important target to regulate the FCF and atherosclerosis during MS. The decreased level of miR-33 in the atherosclerotic macrophage has been shown to augment SREBPs level, which could lead to increased lipid accumulation through biosynthesis and uptake [66]. MiR-33 also caused mitochondrial dysfunction in atherosclerotic macrophages by inhibiting PGC-1α, thereby hampering mitochondrial biogenesis and ATP production [67]. The reduced function of mitochondria could cause apoptosis and formation of cell debris, which may accumulate and contribute to plaque development.

Several other miRs also play active roles in establishment of anti-inflammatory environment, *e.g.* miR-223 inhibits inflammatory cytokine production by attenuating TLR-4/NF-κB signalling pathway (Fig. **2**), and activating pro-survival PI3K/Akt pathway. It has also been shown to abrogate lipid accumulation and thereby reduces FCF [68].

Thus, FCF stands out as one of the crucial components in arterial wall that initiates inflammatory breakout through the release of large number of cytokines and chemokines involving several miR regulators, *e.g*. miR-134a, miR-155 and miR-33, which could be important targets in controlling lipid accumulation and inflammation in macrophages.

4. MODULATION OF VSMC DYNAMICS AND ATHEROSCLEROSIS

VSMCs migration and proliferation is one of the important events in the atherosclerosis, after ED and FCF. VSMCs, occupying the medial layer in the blood vessels, form a critical and dynamic component in the vasculature providing trophic and structural support [69]. The presence of inflammatory cytokines, secreted by different cellular players, contributes towards the proliferation and migration of VSMCs in the vascular lesion. Excessive proliferation, migration and accumulation of VSMCs in the tunica intima, also known as neointimal hyperplasia, contribute to the development and progression of atherosclerotic plaque [70]. During oxidative and metabolic stresses, VSMCs undergo phenotypical changes and extracellular matrix remodelling. Following EC activation and FCF, inflammatory mediators such as growth factors and cytokines in the arterial lesion induce phenotypical changes in VSMCs. As a result of these changes, quiescent contractile VSMCs convert into actively dividing state followed by proliferation and migration from the media into the intimal layer. Their normal function of maintaining blood flow by vasodilation and vasoconstriction also gets altered [71]. Accordingly, the VSMCs transform into non-contractile form and acquire the ability to produce high amounts of extracellular matrix [72]. Under these conditions, VSMCs also excessively uptake modified lipids. The overexpression of CD36 scavenger receptors and inhibition of efflux proteins ABCA1, in the presence of acetylated LDLs lead to excessive lipid uptake and accumulation in VSMCs which become hypertrophied [73]. These evidences indicate that inflammation and oxidative stress not only affect the normal functioning of VSMCs but also alter the lipid metabolism in atherogenic environment.

4.1. Dysregulated Inflammatory Pathways in VSMCs Dysfunction

Chronic inflammation during MS contributes towards proliferation and migration of VSMCs through the activation of several inflammatory pathways. OxLDL, a marker of oxidative and metabolic stress, triggers an atherogenic response and proinflammatory phenotype in VSMCs by binding and triggering urokinase pathogen-associated receptor (uPAR) [74]. In addition to CD36 and TLR4, uPAR also induces NF-κB-mediated expression of granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. **3**) by VSMCs. These factors activate monocytes to transform into macrophages

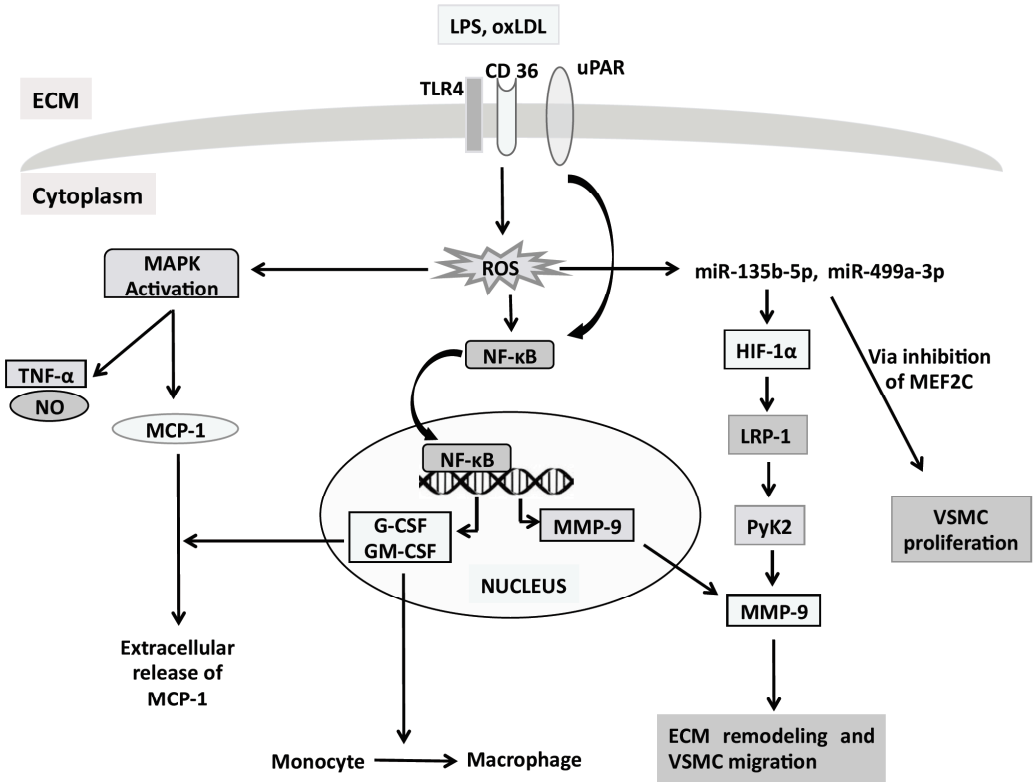

Fig. (3). Schematics depicting various mechanisms involved in VSMCs proliferation and migration during atherosclerosis. OxLDL accumulation and LPS stimulation cause ROS generation that activates MAPK and NF-κB pathway leading to increased expression of TNF-α, NO and MCP1. NF-κB also gets activated by uPAR in combination with CD36 and TLR4 in presence of oxLDL. Activated NF-κB increases the release of polarizing factors which facilitates the conversion of monocytes to macrophages. Expression of MMP-9 through NF-κB and ROS-mediated overexpression of miR135b-5p results in VSMCs remodelling and migration. Further, increased expression of miR-135b-5p and miR-499a-3p cause VSMC proliferation *via* downregulation of MEF2C.

leading to increased inflammation that promotes plaque development [74]. The activated VSMCs also overexpress iNOS, MCP-1 and TNF-α that accelerate plaque formation [75]. Further, upregulation of MMP-9 degrades ECM and facilitates the migration of VSMCs [76]. In addition, another metalloproteinase, ADAM17, in presence of angiotensin II, trans-activates epidermal growth factor receptor (EGFR) which mediates hypertrophy in VSMCs [77].

Platelet-derived growth factor (PDGF), another player identified in promotion of atherosclerosis, is known to be upregulated during oxidative and metabolic stress [78]. PDGF acts as a mitogen and chemoattractant for VSMCs and induces transition of the mature contractile VSMCs into a proliferative and secretory phenotype. Further, cigarette smoke induced PDGF- protein kinase C signalling has been shown to induce VSMC proliferation [79]. PDGF, *via* PDGF receptor beta (PDGFRβ), also induces STAT-1 signalling pathway that leads to disease progression. The cascade of inflammatory response also initiates an increased secretion of chemokines which lead to accumulation of leukocyte in the media of aorta.

PDGF further decreases the expression of contractile proteins such as smooth muscle myosin heavy chain (SMMHC), smooth muscle 22 alpha (SM22α) and alpha smooth muscle actin (αSMA) that lead to stiffness and hardening of VSMCs [78]. Stiffness may render VSMCs in a constant contractile state curbing its dilatory properties, narrowing the arteries lumen and increasing the shear stress caused due to the blood flow. The vasorelaxation property of VSMCs is normally maintained by the AMPK pathway, however, atherogenic agent such as angiotensin II downregulates this pathway. A deficiency of AMPK leads to ER stress-mediated vascular contractility in smooth muscle cells thereby leading to hypertension [80]. These mechanisms indicate the explicit role of oxidative stress-induced inflammation in VSMCs contractibility, proliferation and migration that could contribute to plaque development.

4.2. Stress-mediated Alteration of miRNAs in VSMCs Dysfunction

The proliferation and migration of VSMCs have been shown to be regulated by several miRs during MS [81]. Presence of metabolic stress-related endogenous biomolecules regulate the expression and function of various miRs responsible for the dysregulation in VSMCs physiology. For example, excessive levels of PDGF-BB in blood vessels supresses the expression of miR-599 which negatively regulates its target gene, transforming growth factor beta-2 (TGF-β2). It means that reduced level of miR-599 during chronic inflammation results in increased production of TGF-β2 which promotes the proliferation of VSMCs and also increases expression of matrix protein genes such as type I collagen, type V collagen and proteoglycan [82]. Apart from regulating miR-599, PDGF-BB also suppresses the expression of miR-34a. MiR-34a is an inhibitor of Notch-1 expression, thus its own inhibition by PDGF leads to activation of Notch-1 signalling, which induces VSMC proliferation and migration [83].

Establishment of hypoxia during excessive lipid accumulation increases the expression of HIF-1α. Increased HIF-1α has been shown to downregulate the expression of miR-34a and thereby promotes VSMCs proliferation through Notch-1 activation, analogous to the PDGF-BB-miR-34a-mediated Notch-1 regulation as mentioned earlier [84]. Further, miR-135b-5p upregulates the expression of HIF-1α in the presence of hypoxia [85]. Upregulated HIF-1α increases low-density lipoprotein receptor-related protein 1 (LRP1) - mediated proline-rich tyrosine kinase 2 (Pyk2) phosphorylation which activates MMP-9 leading to ECM remodelling and VSMCs migration [86] (Fig. **3**). Also, the expression of miR-499a-3p has been found increased in atherosclerosis patients. It inhibits the expression of MEF2C, a protein responsible for cellular development of cardiovascular system, which causes excessive cell proliferation and migration of VSMCs [87]. Therefore, a multi-pronged regulation of a single phenomenon regulates a single target through various interconnected pathways during oxidative stress in an inflammatory milieu.

These studies clearly implicate role of metabolic stress in modulation of several miRs, which in turn induce proliferation and migration of VSMCs leading to progression of atherosclerostic plaque. The downregulation of miR-34a is a preeminent biomarker of VSMCs dysfunction in these conditions. The studies discussed above have further revealed the novel roles of miRs and advanced the understanding of VSMCs pathophysiology during atherosclerosis, suggesting their potential as molecular targets.

5. POTENTIAL THERAPEUTIC APPROACHES

The global status of atherosclerosis has been predicted to be of epidemic proportion leading to increased burden on the health-care services around the world. Currently, the well-known drugs clinically available to treat atherosclerosis, such as aspirin, statins, PCSK9 inhibitors, are known to reduce plaque build-up and inflammation by reducing excessive lipid synthesis and the effects caused by LDL-cholesterol [88, 89]. Additionally, beta-blockers and angiotensin-converting enzyme (ACE) inhibitors are administered to control the blood pressure and irregular heart rhythms in the patients [90, 91]. Long-term usage of these drugs are however accompanied with a diverse set of side effects [92, 93]. Therefore, search for novel regimen of alternative therapeutics is pertinent. In this context naturally available phytochemicals have been investigated and shown to reduce the incidence rate of atherosclerosis [94]. Phytonutrients when given alone or in combination could impart their beneficial effects by regulating the metabolic homeostasis and molecular derangement caused by oxidative stress. Phytochemicals such as resveratrol, epigallocatechin gallate, phytosterol and other flavonoids impart athero-protective effects. They regulate signalling pathways that contribute to inflammation, oxidative stress and dyslipidemia which are independent as well as convergent risk factors for atherosclerosis [95]. Obesity and MS are major risk factors involved in atherosclerosis. These metabolic alterations could be targeted to decrease the incidence of atherosclerosis. As discussed in this article, many novel mechanisms of cellular pathophysiology of atherosclerosis have been identified that may provide new avenues for potential therapeutic interventions.

Ursolic acid, a pentacyclic triterpenoid exclusively found in herbs and fruits, executes its biological effect by curbing an obesity associated adipokine, resistin-induced ROS production and NF-κB activation in ED [96]. It also inhibits the transvasation of monocytes into the sub-endothelial space [96]. Further, inflammatory molecules such as IL-6 and MCP-1 are major contributors of atherosclerotic pathophysiology. Dietary supplements of cocoa polyphenols attenuate the levels of these inflammatory cytokines [97]. However, the application of antioxidant therapy for atherosclerosis in humans has shown meagre success rates. The reason could be inappropriate selection of antioxidant molecule, doses and issues with their bioavailability. Further, the solubility and tissue storage of the naturally derived carotenoids and flavonoids could be additional concerns. For example, high solubility of the lycopene supplements in lipids leads to it accumulation in those organs and tissues rich in fat such as liver. Further, vitamin E, an antioxidant, has shown prooxidative nature at higher doses in studies and may not be suitable for therapeutic intervention. Thus, choosing phytochemicals as antioxidant should not be based on its easy availability, rather it should be based on proper experimental evidences regarding their solubility, doses, bioavailability and tissue storage. The efficacy of phytochemicals must be tested through well designed experiments and clinical studies before being used for therapeutic intervention in humans.

Importantly, novel molecular approaches using miR inhibitors or mimics could also be utilized. Various studies as discussed in this article indicate strong potential for their future application in clinical studies. In this approach use of CRISPR technology holds greater promise to intervene for prevention or treatment of atherosclerosis in predisposed individuals [98, 99]. Gene mutation in miRs sequence induced through this method may result in loss of function of atherogenic miRs and may help in preventing the disease progression. Also, the delivery of mimics of atheroprotective miRs can work as endogenous miRs and may help in silencing the expression of target genes. As one miR can behave pleiotropically *i.e*. affecting multiple target mRNAs, or conversely multiple miRs may control a single mRNA, therefore application of miRs in therapeutics must be designed and targeted for affected cells and not the normal cells in the tissue.

CONCLUSION

Atherosclerosis is a progressive pathology and reversal of this condition remains critical for its management. Presence of several atherogenic molecules in the circulation stimulates ECs, macrophages and VSMCs during metabolic disruption and activates a plethora of receptors on these cells which in turn lead to activation of various signalling pathways. Activation of these pathways especially NALP3 activation, p90RSK/Erk-5 pathway, TRAF3IP2/JNK pathway, TLR4/MyD88 pathway, uPAR/CD36/NF-κB pathway and Na^+/K^+-ATPase/Lyn /JNK pathway leads to transcription of different inflammatory cytokines, chemokines and growth factors. These mediators play crucial role in the initiation and progression of atherosclerotic plaque by creating suitable milieu such as excessive ROS production and inflammation. In recent years, several new and potential molecules of interest including phytochemicals such as ursolic acid, cocoa polyphenols, resveratrol, and also endogenously produced miRs such as miR-33, miR-146b-5p, miR-155 and miR-135b-5p have been identified that play vital roles at different stages of atherosclerotic plaque development. The recent advancement in the understanding of atherosclerosis pathophysiology has elaborated various aspects of vascular dysfunction such as ED, FCF and VSMCs dysfunction. Thus, based on these findings new studies should be designed for accurate and early diagnosis as well as for identifying novel molecular medicine for therapeutic interventions in atherosclerosis.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The funds from GSBTM (GSBTM/MD/PROJECTS/SSA /5050/2016-17) and SERB (ECR/2015/000266), awards of Ramanujan Fellowship to UCSY from Department of Science and Technology (DST), Government of India, Junior Research Fellowship (JRF) to JFV from GSBTM, Government of Gujarat, and to RP from SERB, Government of India is thankfully acknowledged.

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