CXCL12 INHIBITION PREVENTS TELOMERE SHORTENING AND REPRESSION OF TELOMerase ACTIVITY IN BOTH EARLY AND LATE POST-MENOPAUSAL ATHEROSCLEROSIS VIA ABCA1 UPREGULATION

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Abstract

Vascular senescence is a key component in the initiation of atherosclerosis and its related cardiovascular disorders. C-X-C Motif Chemokine Ligand 12 (CXCL12), the CXC chemokine receptor 4 (CXCR4) ligand is a chemokine known to promote atherosclerosis. However, the role of telomeres in CXCL12-mediated senescence-induced atherosclerosis remains obscure. This study aimed to unravel the role of CXCL12 and its association with telomeres in the context of menopause-induced arterial senescence and atherosclerosis. Apoe\(^{-/-}\) mice underwent bilateral ovariectomy (OVX) to simulate early and late post-menopausal (EPM) conditions (1 and 5 weeks post-OVX, respectively). POL5551, a selective CXCR4 antagonist, was administered as a continuous infusion (30 mg/kg/day in PBS) using a subcutaneously implanted osmotic minipump for two weeks. ATP binding cassette transporter A1 (ABCA1), a cholesterol efflux regulator was significantly downmodulated (\(p < 0.05\)), while NOD-, LRR- and pyrin domain-containing protein 3 (NLR) family pyrin domain containing 3 (NLRP3), inducible nitric oxide synthase (iNOS) and the inflammatory mediators were considerably enhanced (\(p < 0.05\)) in both EPM and LPM mice groups. Notably, atherosclerosis was more prominent in the

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EPM than in the LPM mice. However, POL5551 treatment effectively ameliorated the reduced ABCA1 expression and the increased inflammatory response. Hence, we propose that inhibition of CXCL12 proffers robust anti-senescent and anti-atherosclerotic effects.

**Key words:** ABCA1, atherosclerosis, CXCL12/CXCR4, telomere length, vascular senescence

**Introduction.** Atherosclerosis is one of the major risk factors for a variety of cardiovascular diseases (CVD), including myocardial infarction, ischemic stroke, and pulmonary diseases. The concept of atherogenesis – from a focal lipid accumulation or an extended cellular-lipid matrix formation to a cascade inflammatory/immunological event – has changed over some time [1]. Although various factors are known to trigger atherogenesis, vascular cell senescence due to ageing is considered to be a predominant risk factor for atherosclerosis [2]. Several epidemiological and clinical studies have highlighted that the incidence of CVD and related fatal events is higher in post-menopausal women than in pre-menopausal women and age-matched men.

Various epidemiological and clinical studies accentuated that incidence of CVD and associated fatal events are more prominent in post-menopausal women than pre-menopausal women and age-matched men [3]. While hormone replacement therapy (HRT) has been proven effective in preventing the onset and progression of atherosclerosis in younger post-menopausal women, the “timing hypothesis” sounds like a note of caution for the use of HRT for atherosclerosis in older women. Hence, there is a dire need to understand the intertwined molecular mechanisms underlying menopause-induced arterial senescence and atherosclerosis.

Arterial senescence is manifested by an irrevocable cell cycle arrest in the arterial vasculature which is associated with a plethora of vascular pathological events including increased inflammation, telomere shortening, abnormal endothelial infiltration, disruption of cell-cell junctions, structural, and functional abnormalities in mitochondrial, lysosomal and other sub-cellular components, resistance to apoptosis, and defective tissue remodelling [4]. Further, a recent study by Munoz-Cordova et al. [5] depicted that postmenopausal atherosclerosis can be prevented by the augmentation of autophagic flux and repression of oxidative stress, inflammation and apoptosis. Chemokines have been associated with atherosclerosis and other age-related disorders [6].

CXCL12, the solitary ligand of CXCR4, is a chemokine and a homeostatic regulator expressed by a wide array of cells. CXCL12 exhibits a dual physiological-pathological role in various conditions including angiogenesis, tissue repair, hypoxia and growth arrest [6]. Hence, CXCR4 and its receptor CXCL12 are known to work in an intricate cell- and context-specific manner in the atherosclerotic milieu. A recent study based on the Mendelian randomization analysis revealed that CXCL12 is one of the prime causal mediators of coronary artery disease (CAD) in humans [7]. In addition, a meta-analysis of genome-wide association studies...
GWAS indicated that endothelial cell-derived CXCL12 accelerates the progression of atherosclerosis in CAD [8]. In addition to the atherogenic role, CXCL12 acts as a topological central node in the differentially expressed genes’ network related to senescence [9]. GROOTAERT et al. [10] showed that autophagic dysfunction in the vascular smooth muscle cells (VSMCs) upregulates CXCL12 and other factors, hastens senescence in VSMCs, and triggers neointimal thickening and atherogenesis.

Telomeres, the chromosomal ends comprising DNA-protein complexes, offer protection against genome instability in various pathological conditions including senescence and atherosclerosis [11]. Reduced telomere length, increased telomere attrition rate and repressed telomerase reverse transcriptase (TERT) activity are known to increase the risk for atherosclerosis [11]. SHEN et al. [12] have demonstrated that CXCL12 controls endothelial progenitor cell senescence by modulating telomerase. Recently, GAO et al. [13] have demonstrated that CXCL12 causes atherosclerosis by suppressing ATP-binding cassette transporter A1 (ABCA1). Therefore, the current investigation was carried out to clarify the functioning mechanism of CXCL12 and its association with telomeres in the context of atherosclerosis and arterial senescence brought on by menopause.

Material and methods. Study area. The present study was carried out in the Department of Cardiovascular Disease, Jinling Hospital, Medical School, Nanjing University, from January to May 2022.

Animals and experimental design. In this investigation, female C57BL/6 mice (8–9 weeks old) were obtained from the animal house facility of the Centre at Jinling Hospital, Nanjing University. During the animal assay, test animals were isolated in wide, clean cages with a constant temperature of 23±1°C, 40–60% humidity and exposed to a 12-hour dark-light sequence. Animals were subjected to either sham surgery or bilateral ovariectomy (OVX). Two OVX groups were made: early postmenopausal (EPM) and late postmenopausal (LPM) groups (1 and 5 weeks post-OVX, respectively) as described earlier by CAMPOS et al. [14]. Both EPM and LPM groups were further treated with the selective CXCR4 antagonist, POL5551 (Polyphor Ltd., Switzerland) as a continuous infusion (30 mg/kg/day in PBS) using a subcutaneously implanted osmotic minipump (model 1007D, Alzet, USA) for two weeks [14]. Mice in both sham-operated groups corresponding to EPM/LPM groups received only PBS through a subcutaneously implanted osmotic minipump. This investigation was conducted in harmony with the guidelines for animal handling and care in the laboratory prescribed by the institutional ethical committee and also, by the international guidelines as per the National Institutes of Health.

Measurement of lipid profile. All the animals in all the cohorts were euthanized and the blood was drawn and the serum was collected by centrifugation at 3000 rpm for 10 min. Collected serum was used to analyse the TG, TC, HDL, and LDL levels, as per the manufacturer’s instructions (Nanjing JianCheng
**ELISA assay.** TNF-α, IL-6 and IL-1β levels in plasma from blood samples were measured using an ELISA kit (R & D Systems, USA) following manufacturer’s instructions. Tissue protein was assessed by using a Bradford protein test kit (Beyotime Institute of Biotechnology, Jiangsu, China).

**Measurement of telomere length using reverse transcription quantitative PCR (qPCR) analysis.** The vascular smooth muscle cells (VSMCs) from all the animal groups were separated and the telomere length was quantified by using qPCR on a Rotor-Gene (Corbett Research, UK) [14]. Briefly, the master mix reaction for a single-copy control gene (36B4) and telomere length was conducted at 95°C for 15 min and then subjected to elongation steps. Then, serial dilution of Apoe−/− mouse DNA was done and a reaction standard curve was drawn. Finally, the amount of telomeric DNA to the amount of 36B4 product was divided to obtain the average telomere length (T/S ratio).

**Western blot analysis.** The specimens were homogenised in a chilled RIPA buffer using an electric homogenizer and then centrifuged at 18 000 × g for 18 min at 4°C. The proteins were separated using an SDS-polyacrylamide gel and Tris blocking buffer system, then incubated overnight with the primary antibodies CXCL12, CXCR4, ABCA1, NLRP3, iNOS, and TERT (Abcam, USA). After rinsing the membranes, secondary HRP (horseradish peroxidase)-conjugated antibodies (Santa Cruz Biotechnology, Inc., USA) were added. Finally, antibodies were analysed quantitatively by using enhanced chemiluminescence (ECL) assay kit (GE Healthcare, USA).

**Histopathological examination.** All of the animals’ aorta tissues were dissected and placed in paraffin for staining with hematoxylin and eosin (H & E) solution. A qualified pathologist evaluated the abnormal alterations utilizing microscope imaging cell software linked to an Olympus BX40 light microscope (Olympus Optical Co., Japan). The whole aorta was subjected to Oil red O staining (0.5% w/v stain in isopropyl alcohol) to measure the atherosclerotic lesion area using iSolution imaging analysis software.

**Statistical analysis.** The data was analysed using the SPSS tool (V13.0; SPSS, Inc., USA). The statistical assessment was conducted using a one-way analysis of variances (ANOVA) with Tukey’s post hoc analysis for comparison among various animal groups. A p value of less than 0.05 was considered significant.

**Results.** CXCR4/CXCL12 signalling is a nexus signalling axis of two pathological processes: senescence and atherosclerosis [9,10]. Hence, we investigated the menopause-induced arterial senescence and atherosclerosis by using an Apoe−/− mouse model. To imitate the EPM and LPM statuses, long-term (five weeks post-ovariectomy) and short-term (one-week post-ovariectomy) investigations were done by Campos et al. [14] with or without the selective CXCR4 antagonist, POL5551. The percentage of atherosclerotic lesions was substantially higher (p < 0.05) in the aorta of both EPM and LPM mice, when compared to their
corresponding sham-operated controls (Fig. 1A). We noticed that pharmacological inhibition of CXCR4 significantly ($p < 0.05$) attenuated atherosclerotic lesion formation in the aorta of both EPM and LPM mice. An interesting piece of observation is that atherogenesis was more prominent in the EPM than in the LPM mice, perhaps due to the early induction of senescence-induced atherosclerosis (Fig. 1B).

In this study it has been demonstrated that CXCR4 expression was substantially upregulated ($p < 0.05$) in the OVX mice compared to the sham-operated controls (both EPM and LPM animals) (Fig. 2A). The CXCR4 and CXCL12 were more profoundly ($p < 0.05$) increased in the EPM than in the LPM mice as shown in Fig. 2B, C. Besides, treatment with POL5551 effectively ($p < 0.05$) downmodulated the protein expression levels of both CXCR4 and CXCL12, which in turn reflected the decrease in atherosclerosis in both OVX mice.

ABCA1, a prime cholesterol efflux regulator, is a downstream target of CXCL12. In our study, we observed that ABCA1 was downmodulated in both EPM and LPM mice, although the ABCA1 protein level was relatively more affected in the EPM mice (Fig. 2D). As expected CXCR4 inhibition remarkably ($p < 0.05$) upmodulated the ABCA1 protein levels in both EPM and LPM mice when compared to the untreated OVX mice. No significant difference in the sham-operated controls corresponding to the EPM/LPM mice was observed (Fig. 2E).
Inflammation is a major pathological event in the development of atherosclerotic plaque formation. The inflammatory mediators (IL-1β, IL-6 and TNF-α), NLRP3 and iNOS were remarkably ($p < 0.05$) elevated in both EPM and LPM mice groups when compared to the sham-operated controls as shown in Fig. 3A. However, the plasma levels of inflammatory mediators and also the protein expression levels of NLRP3 and iNOS were significantly ($p < 0.05$) reduced when treated with POL5551 (Fig. 3B, C). This underscores that inhibition of the CXCR4/CXCL12 signalling axis plays a critical role in the anti-inflammatory reaction against the development of atheroma.

Telomere length is considered a key index of cellular senescence. We ob-
erved that menopause-induced arterial senescence was remarkably ($p < 0.05$) high manifested by the significant ($p < 0.05$) reduction in telomere length and TERT activity in the EPM mice than that of the LPM mice when compared to their sham-operated counterparts (Fig. 4A, B). Noticeably, telomere dysregulation is more pronounced in the EPM mice than in the LPM mice (Fig. 4C). However, CXCR4 inhibition effectively ameliorated the telomere dysregulation by attenuating telomere shortening and reduction in the TERT activity.

**Discussion.** CXCL12, a pro-inflammatory and pro-fibrotic chemokine, is a prime regulator of vascular senescence and atherosclerosis $^{[6,14]}$. In this investigation, it was observed that the pharmacological inhibition of CXCL12 prevents menopause-induced arterial senescence and atherogenesis; also, atherogenesis is more pronounced in the early postmenopausal (EPM) state than in the late post-menopausal (LPM) state, which was in line with an earlier report of Wang et al. $^{[15]}$. Luo et al. $^{[16]}$ have shown that the CXCL12/CXCR4 axis is implicated in the formation of ovary injury in an animal model of early ovarian dysfunction, a subset of early menopause.

Elucidation of the molecular mechanism underlying CXCL12/CXCR4-mediated anti-atherosclerotic activity revealed that pharmacological inhibition of CXCL12 prevents menopause-induced arterial senescence and atherogenesis via up-modulation of ABCA1-mediated cholesterol efflux and attenuation of NOD-, LRR- and pyrin domain-containing protein 3 (NLR) family pyrin domain containing 3 (NLRP3)-mediated inflammatory response. Several studies showed that augmentation of ABCA1-mediated cholesterol efflux and attenuation of inflammatory response abrogate atherosclerotic plaque formation in Apoe$^{-/-}$ mice via

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Fig. 3. Protein expression levels of NLRP3 and iNOS in POL5551 treated mice; A) NLRP3 and iNOS expression Western blotting analysis; B) NLRP3 levels in POL5551 treated mice; C) iNOS levels in POL5551 treated mice.
various signalling pathways including Tie2/TFE3/LXRα, miR-33 and TLR-4/NF-κB, and GSK3/β-catenin/Tcf21 [13]. Results show that the inhibition of CXCL12 in the LPM mice displayed better ABCA1-mediated cholesterol efflux and lesser NLRP3-mediated inflammation than that of the EPM mice.

EPM mice had a higher inflammatory response in terms of elevated levels of inflammatory mediators such as IL-1, IL-6, and TNF-α than LPM mice in terms of plasma levels of inflammatory mediators. In harmony with this increased inflammatory pathology observed in the EPM mice, altered lipid levels in terms of increased lipid parameters including TC, TG, and LDL-C, as well as decreased HDL-C were more pronounced in the EPM mice than that in the LPM mice. One plausible explanation is that reduced inducible NO synthase in the LPM mice might have contributed to the relatively decreased dyslipidemia and atherosclerosis progression in the LPM group. This finding was consistent with the findings of Miyoshi et al. [17], who discovered enhanced iNOS-mediated oxidation of LDL by
activation of smooth muscle cells in advanced atherosclerosis in the EPM mice. Indeed, inhibition of CXCL12 more effectively ameliorated the altered levels of iNOS, inflammatory mediators and lipid parameters in the LPM mice than in EPM mice.

An interesting study by Herrmann and Herrmann \cite{18} revealed that for an increase of 1 kb in leukocyte telomere length (LTL), there was about 10 months increase in the average age. This indicates that telomere shortening is more robustly associated with the EPM state than the LPM state. Willeit et al. \cite{19} proposed that accelerated vascular ageing and advanced atherosclerosis are associated with decreased LTL. Besides, the shortening of telomere length, telomerase inhibition in the VSMCs is known to accelerate vascular senescence and atherosclerosis \cite{20}. In this line, we observed that TL and TERT in the VSMCs were drastically decreased in EPM mice, which implies increased atherosclerosis in EPM mice compared to LPM mice. However, inhibition of CXCL12 prevented the reduction in TL and telomerase in both EPM and LPM mice, with a notably better ameliorative effect in the LPM mice. Indeed, remarkable attenuation of senescence and atherosclerosis might be achieved through the restoration of telomerase activity and TL.

This study has a few shortcomings, including restricted measurement of telomere length and telomerase in only VSMCs rather than measurement of TL in endothelial cells, leukocytes and other relevant cells. Further, studying the role of various biomarkers in the scope during premature, stable and advanced atherosclerosis in both EPM and LPM statuses might have unravelled several valuable insights into senescence-associated atherosclerosis. However, further progress in this research is under design and we warrant detailed prospective research in this area using clinical models.

**Conclusions.** In conclusion, this study proposed that the inhibition of CXCL12 offers robust anti-senescent and anti-atherosclerotic effects. Furthermore, we hypothesise that CXCL12 inhibition might be a useful technique in the treatment of atherosclerosis in postmenopausal women and other senescence-associated clinical problems.

**Significance of the study.** Vascular senescence is a critical component in the growth of atherosclerosis and related cardiovascular diseases. CXCL12, a chemokine known to induce atherosclerosis, is the CXC chemokine receptor 4 (CXCR4) ligand. But the mechanism of XCL12-mediated senescence-induced atherosclerosis remains unclear. The present research aimed to explore the role of CXCL12 and its interaction with telomeres in the setting of menopause-induced arterial senescence and atherosclerosis. This research demonstrates that inhibiting CXCL12 has strong anti-senescent and anti-atherosclerotic properties. Moreover, we believe that CXCL12 suppression might be a beneficial approach in the therapy of postmenopausal atherosclerosis and other senescence-related clinical issues.
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Contribution of each author: WL conducted research, while YL assisted with article writing. SL performed animal experiments. FL then conducted ELISA and Western blot analyses. JBG coordinated the research activities and contributed to the correction and modification of the research paper.

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