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Cellular Heterogeneity in Atherosclerosis: Unraveling Complexity and Guiding Therapeutic Strategies

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Abstract

Atherosclerosis, a chronic inflammatory disease of the arterial walls, is characterized by intricate cellular heterogeneity within the plaques. The identification of highly plastic and heterogeneous cell populations has added a layer of complexity to atherosclerosis research, challenging traditional cell markers used for plaque analysis. To overcome this, advanced techniques such as lineage tracing and single-cell omics have emerged, enabling a deeper understanding of different cell subsets and their functional roles. Moreover, technological advancements in lipidomics and metabolomics have provided insights into the molecular landscape of atherosclerosis, shedding light on metabolic adaptations and cellular plasticity in diseased arteries. These approaches hold potential for developing antigen-specific therapies by elucidating the adaptive immune response and identifying specific targets for intervention. The need for innovative therapeutic strategies is evident, as investment in cardiovascular drug development has lagged behind other areas of research. Targeting inflammatory responses driven by impaired immune cell activation is a promising avenue, either by focusing on specific immune cell subsets or their effectors. Additionally, site-specific therapies and timing-optimized strategies may enhance drug efficacy while minimizing side effects. Identifying key regulatory pathways controlling the phenotypic modulation of endothelial cells and vascular smooth muscle cells could pave the way for converting them into atheroprotective phenotypes. Furthermore, the integration of spatial omics techniques, protein profiling, and Mendelian randomization can provide valuable insights into the adaptive immune response, antigen-specific targets, and the potential effectiveness of pharmacological modifications. These multidimensional approaches offer the potential for personalized and targeted therapies against atherosclerosis. In conclusion, a comprehensive understanding of cellular heterogeneity, immune mechanisms, and metabolic adaptations in atherosclerosis is essential for the development of innovative therapeutic interventions. By unraveling the complexities of this disease, we can pave the way for precision medicine and improved management of atherosclerosis, thus alleviating the global burden of cardiovascular disease.

Keywords: Cellular heterogeneity; Atherosclerosis; Plaque composition; Lineage tracing; Single-cell omics, Lipidomics; Metabolomics; Antigen-specific therapy; Immune cell; Inflammatory responses; Site-specific therapies; Endothelial cells; Vascular smooth muscle cells

Introduction

The introduction of advanced technologies enabling the identification of multiple cellular parameters and spatial organization has significantly contributed to our understanding of atherosclerosis. These techniques have revealed a remarkable heterogeneity of cellular subsets involved both locally within the plaque and systemically throughout the body (Figure 1). This heterogeneity encompasses not only different cell populations but also varying stages of cell activation, further complicating the intricate network of factors contributing to the development, progression, and exacerbation of atherosclerotic plaques and their clinical manifestations [1,2]. By integrating these single-cell techniques with cell-specific lineage tracing, known as fate mapping, we have obtained deeper insights into the underlying mechanisms driving plaque formation. A comprehensive depiction of the heterogeneous cellular and molecular architecture within the atherosclerotic plaque highlights the complexity of the disease and underscores the challenge of identifying effective therapies. Remarkable levels of heterogeneity and plasticity are observed in major cell types within the plaque, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and immune cells. This heterogeneity presents an opportunity for the discovery of new therapeutic targets beyond traditional lipid species. Unraveling the underlying mechanisms that drive the acquisition of a "plaque stabilizing" phenotype in cells such as VSMCs holds significant potential for the development of novel therapeutic strategies. While there is considerable interest in targeting systemic inflammatory mediators, the identification and targeting of atherosclerosis-specific antigens, such as oxidation-specific epitopes, may offer promising prospects for treating atherosclerosis in a disease- and site-specific manner. The exploration of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in this context holds significant relevance [3].

Vascular smooth muscle cells (VSMCs) play a crucial role in maintaining vascular tone through their contractile properties. However, during the formation of atherosclerotic plaques, VSMCs undergo migration and proliferation from the media into the lesion, leading to the formation of the fibrous cap. In this process, VSMCs undergo a phenotypic switch from a contractile to a synthetic, de-differentiated state, losing their contractile markers [4]. Recent clonal lineage tracing studies have revealed that VSMCs in the plaque originate from a small number of pre-existing cells in the media [5]. This highlights a greater degree of plasticity in VSMCs than previously recognized, as they can trans differentiate into various alternative phenotypes, including "macrophage-like," "foam cell-like,"

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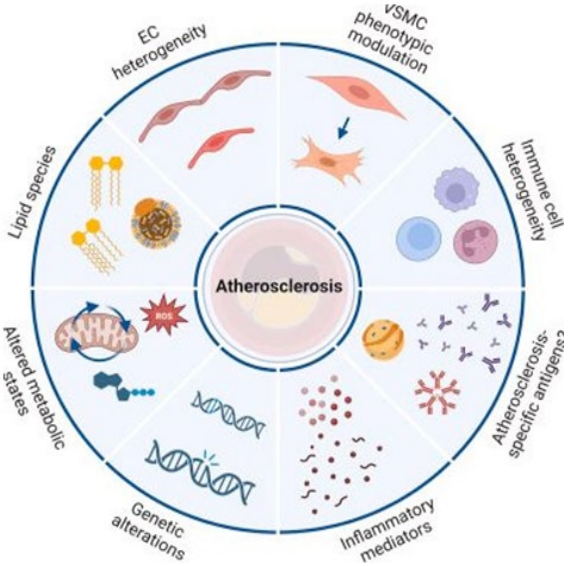

Figure 1: Intraplaque heterogeneity driven by multiple factors.

"myofibroblast-like," "osteoblast-like" cells, or even endothelial cell (EC)-like cells [6,7]. Single-cell transcriptomic studies have uncovered significant transcriptional heterogeneity underlying these diverse VSMC phenotypes. However, the relative proportion of these VSMC subtypes may vary depending on the disease context, vascular bed type, and micro environmental cues within the plaque. The question of whether contractile VSMCs can directly transdifferentiate into alternative phenotypes or first adopt a de-differentiated state, from which other VSMC-derived cell types arise, remains unclear. Emerging evidence suggests the existence of a de-differentiated multipotent population called "SEM cells" (stem cell, EC, and monocyte) as an intermediate VSMC state, revealed through the integration of VSMC fate mapping and single-cell RNA sequencing (scRNA-seq) with trajectory analysis [8,9]. The high level of VSMC plasticity adds complexity to the cellular architecture of the plaque and poses both technical and conceptual challenges. For instance, immunostaining for the contractile marker ACTA2 underestimates the VSMC content in the plaque since over 80% of VSMC-derived cells in mouse plaques are ACTA2 negative. Moreover, misidentification of VSMCs as macrophages has occurred, as more than 40% of CD68+ cells in human and mouse plaques were found to be of VSMC origin. Although VSMCs can adopt alternative phenotypes, their full functionality in these states remains uncertain. Notably, macrophage-like VSMCs exhibit distinct transcriptional and functional characteristics compared to myeloid-derived macrophages, raising questions about the extent of their contribution to plaque stability [10].

Therefore, it is challenging to determine whether macrophage-like VSMCs have the same detrimental impact on plaque stability as bone marrow-derived macrophages. The success of single-cell technologies lies in the comprehensive analysis of the VSMC-specific transcriptomic signature within the plaque, focusing on the co-clustering of cells based on VSMC contractile or de-differentiation genes. However, it is crucial to move beyond associative studies and advance current technologies to elucidate the precise functions of these phenotypically modulated VSMCs and, more importantly, their causal role in plaque vulnerability. One approach to achieve this is by integrating single-cell transcriptomics with epigenomic profiling and human genomics. This integration can help identify novel candidate targets that regulate VSMC phenotypic modulation by altering chromatin accessibility and identifying VSMC-associated risk genes for coronary artery disease (CAD) [11,12]. Spatial transcriptomics techniques offer valuable insights by mapping the distribution of these phenotypically modulated VSMCs to specific regions of the plaque, providing a better understanding of their relationship to vulnerability. Currently, the field emphasizes the need to develop future therapeutic interventions that convert undesirable VSMCs into a beneficial atheroprotective and plaque-stabilizing phenotype. However, it remains unclear which cells precisely fit this profile.

Immune cells

Extensive research has significantly contributed to the characterization of diverse immune cell populations involved in atherosclerosis, encompassing both the innate and adaptive immune response. These studies have shed light on their distinct contributions to the pathophysiology of this disease. The chronic inflammatory nature of atherosclerosis, where acute and reparative phases coexist, has become a fundamental concept in understanding its underlying mechanisms and potential therapeutic approaches. However, two key questions continue to stimulate active research: i) whether chronic inflammation arises from excessive effector function rather than impaired immunosuppression, and ii) the specific triggers responsible for immune cell activation and the pathways involved in their recruitment within the atherosclerotic plaque [13,14]. Resolving these questions holds significant promise for advancing our understanding of atherosclerosis.

Macrophages

Macrophages, initially recognized as one of the primary and abundant cell populations within the atherosclerotic plaque, predominantly originate from the infiltration of circulating monocytes and local proliferation. A study employing scRNA-seq and genetic fate mapping of CX3C motif chemokine receptor 1 (CX3CR1) derived cells during atherosclerosis revealed the presence of an unexpected cluster of proliferating monocytes with a stem cell-like signature. This finding suggests that monocytes may persist in a self-renewal state rather than undergoing immediate differentiation into macrophages upon entry into the plaque [15-18]. However, a recent study proposes a different perspective, suggesting that specialized aortic intimal resident macrophages with limited proliferation capacity represent the earliest foam cells in murine plaques. These resident macrophages are subsequently replaced by recruited proliferating monocytes from the circulation [19]. Upon entering the atherosclerotic plaque, monocytes undergo differentiation into macrophages, whose phenotypic diversity is now recognized to encompass a wide spectrum, exhibiting a dynamic continuum of phenotypes [20]. This expanded perspective on macrophage polarization states reveals a greater complexity beyond the classical classifications. Through the utilization of scRNA-seq and CyTOF data obtained from lineage-traced atherogenic mouse models, researchers have identified five distinct macrophage subtypes to date: Classical foam cell macrophages, characterized by high expression of Trem2, Mmp12, Mmp14, CD11c, cathepsins, and markers of steatosis [21]. These macrophages exhibit low expression of inflammatory genes and a significant portion is considered to originate from VSMCs. CCR2 macrophages, believed to derive from circulating monocytes with a pro-inflammatory potential. Residential vascular macrophages, expressing markers such as Lyve1 and Mrc1, and displaying a transcriptomic signature similar to macrophages found in healthy arteries [22]. Cavity macrophages, sharing similarities with small peritoneal macrophages.

A group of macrophages characterized by the expression of IFN-inducible genes (such as Ifit3, Irf7, and Isg15), whose origin is yet unclear.

The proportions of these macrophage subtypes undergo changes during the progression and regression of the disease, ultimately influenced by various factors within their microenvironment, such as cytokines, chemokines, and lipids [23]. A recent study conducted by Goossens et al. employed an advanced platform that combines multiplex immunofluorescent and mass spectrometry imaging techniques to elucidate the connections between these micro environmental niches and myeloid cell phenotypes. This study represents a significant advancement by linking the phenotypic heterogeneity of myeloid cells to their spatial cellular and molecular context, including metabolic and lipidomic profiles. The findings provide unprecedented insights into how the plaque microenvironment influences the diversity of myeloid cells in atherosclerosis [24]. By integrating these techniques with the characterization of cellular functions such as migration, efferocytosis, lipid accumulation, and lysosomal hydrolysis, a more comprehensive understanding of the contribution of macrophage dysfunction to inflammation and the progression of atherosclerosis can be achieved.

Neutrophils

ScRNA-seq studies have revealed the plasticity of neutrophils at the chromatin, transcriptome, and proteome levels, contributing to their phenotypic and functional heterogeneity [25]. However, the investigation of neutrophil heterogeneity in atherosclerosis is limited due to technical challenges. These include the low number of neutrophils detected in atherosclerotic plaques, low RNA content per cell, and high ribonuclease content. Nevertheless, recent studies have demonstrated that modified methodologies for scRNA-seq data analysis can enhance the identification and characterization of neutrophil subtypes in atherosclerosis. Additionally, antibody-based detection methods such as CyTOF offer a reliable approach for detecting neutrophils in the vascular wall and studying their heterogeneity. Currently, there is more available data on the heterogeneity of circulating neutrophils than on those residing within the atherosclerotic lesion [26]. Within the plaque, neutrophils can be categorized into two subtypes based on the expression level of Sialic acid-binding Ig-like lectin F (SiglecF), which mirrors neutrophils found in the myocardial infarction and tumor microenvironments. SiglecFhi-neutrophils are characterized as aged, long-lived cells expressing higher levels of inflammatory and profibrotic cytokines. The concept of neutrophil aging has been extensively reviewed elsewhere, and it is hypothesized that SiglecFhi-neutrophils share similarities with aged CD62Llo and CXCR4hi circulating neutrophils, such as an increased ability to form neutrophil extracellular traps and produce reactive oxygen species. However, they also possess unique traits influenced by the local microenvironment during their aging within the tissue. Integration of CyTOF and scRNA-seq holds promise for further research into the involvement of different neutrophil subtypes in atherogenesis, providing new insights into the phenotypic, transcriptional, and functional characteristics of purified neutrophil subpopulations in atherosclerosis [27].

T and B cells

T and B lymphocytes have a significant impact on atherosclerosis, with CD4+ T cells being extensively studied in experimental models. CD4+ T cells encompass both "inflammatory cell subsets," such as Th1 and Th17 cells that produce inflammatory cytokines (e.g., IFNγ and IL-17), and "tolerogenic cell subsets," including Treg cells that produce pro-resolutive cytokines (IL-10 and TGFβ). However, plasticity within these subsets has been observed, adding complexity to our understanding of their role in the disease. Recent scRNA-seq analyses conducted in human and murine atherosclerotic plaques have identified multiple lymphocyte subsets expressing varying levels of cytokines, chemokine receptors, and markers of cell exhaustion [28]. Yet, it remains unclear whether these subsets are associated with the severity of atherosclerosis and whether they represent potential targets for therapeutic intervention. The presence of lymphocytes activated against atherosclerosis-specific antigens (ASA), which are antigens selectively generated or involved due to the atherosclerotic environment, remains poorly understood. This is particularly relevant for B lymphocytes, considered key players in the initiation and progression of atherosclerosis. B cells primarily influence plaque development through the secretion of antibodies. Antibodies are classified into four distinct classes: IgM, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4 in humans, and IgG1, IgG2a/c, IgG2b, and IgG3 in mice), IgE, and IgA [29]. Almost all antibody classes are implicated in atherosclerotic disease. IgM antibodies are generally recognized as atheroprotective due to their ability to recognize oxidation-specific epitopes (OSEs), products of lipid peroxidation. OSEs are modifications that alter the immunogenicity of host molecules, such as proteins and lipids, making them targets for the immune system. These OSEs are abundant on oxidized LDL and cellular debris within atherosclerotic plaques. IgG antibodies exhibit both proatherogenic and atheroprotective properties. This duality can be attributed to the different IgG subclasses and the wide range of antigens recognized by IgG antibodies. On the other hand, IgE antibodies appear to have a detrimental role in plaque formation. However, it is currently unknown whether antibodies impact atherosclerosis by recognizing ASA. This unanswered question holds significant importance as it could determine the most effective immunomodulatory therapeutic strategy against atherosclerosis, whether it involves targeting specific antigens or inflammatory mediators. Studies have demonstrated antigen-mediated activation of naïve cells within the murine aorta, occurring in structures known as tertiary lymph nodes associated with arterial adventitia, which act as reservoirs of activated and naïve lymphocytes. Simultaneously, circulating lymphocytes infiltrate atherosclerotic plaques through chemokine receptors, contributing to disease severity. The presence of circulating antigen-experienced lymphocytes, called effector memory T cells, has been linked to atherosclerosis severity in both mice and humans. However, it remains unclear whether this reflects local hyper-reactivity against specific antigens, a response to altered local and systemic metabolic pathways, or the result of impaired immunosuppressive mechanisms. Recent research by Depuydt et al., combining scRNA-seq with T cell receptor (TCR) sequencing of human carotid plaques and matched peripheral blood mononuclear cell (PBMC) samples, revealed autoimmune-like features of effector T cells in plaques. Interestingly, clonally expanded CD4 T cells exhibited a signature indicative of antigen-specific activation, suggesting potential interactions with TREM2 foam cells [30]. Therefore, understanding the dynamics of lymphocyte biology, including their phenotype, localization, activation, and recruitment, would accelerate the translation of basic research into the identification of novel targets for limiting cardiovascular inflammation.

Significance of cellular heterogeneity in atherosclerosis

The discovery of highly plastic and heterogeneous cells within the atherosclerotic plaque introduces a new level of complexity to future research on atherosclerosis. Traditional methods of identifying cell subsets based on specific markers are no longer sufficient for assessing plaque composition or determining plaque stability, as vascular wall

cells and macrophages can express overlapping markers. Instead, lineage tracing combined with analysis of transcriptional or proteomic signatures are necessary to accurately identify and understand these cells and their progeny. Furthermore, the diverse and complex nature of the plaque necessitates innovative approaches in basic research to identify potential targets for therapeutic intervention in atherosclerosis. While single cell transcriptomics has provided valuable insights into cellular networks within the plaque, additional information on cell activation and intercellular interactions is crucial for identifying key drivers of the disease. Advancing single cell technologies and bioinformatic tools is essential to comprehensively unravel the functional complexity of cells in the plaque microenvironment. Integrating cell-specific fate mapping, single cell omics, and human genetics will further enhance our understanding of the causal relationship between cellular heterogeneity and atherosclerosis. Moreover, advancements in lipidomics and metabolomics have the potential to provide molecular insights into the key players involved in atherosclerosis. For instance, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) enables the profiling of lipid compositions in specific regions of the plaque. Application of this technique to symptomatic plaques has revealed that regions rich in macrophages exhibit an abundance of sphingomyelins, while intimal vascular smooth muscle cells are enriched in cholesterol and cholesteryl esters. By incorporating lipidomics and metabolomics, we can gain a deeper understanding of how metabolic adaptations within diseased arteries contribute to the cellular plasticity and heterogeneity observed in atherosclerotic plaques. This integration of phenotype and cellular metabolism would provide insights into the localized immune-metabolic responses occurring within specific regions of the plaque, potentially facilitating the design of targeted delivery systems for metabolic interventions. Additionally, this technology would greatly enhance the investigation of spatial immune cell reactivity at the single-cell level. Combining MALDI MSI with high-throughput techniques such as single-cell-based B and T cell receptor sequencing and transcriptomics would allow for the characterization of lymphocytes' antigen specificity against atherosclerosis-specific antigens (ASA) while considering the unique composition of lipids, peptides, or metabolites in different areas of the arterial lesion. By merging the phenotypic profiles and antigen specificity of lymphocytes with the distinct milieu of various plaque regions, we can gain valuable insights into the spatial immune cell dynamics within the atherosclerotic lesion. This approach would provide valuable insights into the mechanisms underlying the adaptive immune response in atherosclerosis. It would help determine the extent to which immunoglobulins and T cell activation are mediated through the recognition of ASA, potentially opening doors for the development of antigen-specific therapeutic approaches for managing atherosclerosis. Investment in cardiovascular drug development has shown limited growth over the past twenty years, despite the persistent global burden of cardiovascular disease, and has not received the same level of attention as other research fields such as oncology. Within the field of atherosclerosis, apart from the lipid component, there is a perceived lack of innovative drugs. However, there is a growing interest in targeting inflammation to reduce major coronary events. While there is clear evidence pointing to the role of specific immune subsets in atherogenesis, further investigation is needed to effectively target the inflammatory responses driven by impaired immune cell activation. In the future, we envision therapies that aim to target either specific immune cell subsets (though this may carry certain health risks such as increased susceptibility to infections) or their effectors (e.g., secreted antibodies and ASA). However, the current knowledge derived from single-cell studies is insufficient to implement the latter approach. As discussed earlier, a deeper understanding of immune mechanisms at the protein level through advanced techniques is necessary to develop therapeutic strategies against ASA. In addition to targeting circulating mediators such as cholesterol and cytokines, it is crucial for the field to explore the potential of developing site-specific therapies for atherosclerosis.

The emergence of spatial omics techniques in combination with protein profiling holds promise for enabling more site-specific treatment of atherosclerosis in the near future. By identifying ASA, it becomes possible to design drugs that can be precisely targeted to the affected area. Although currently employed primarily in cancer therapy, the identification of ASA may facilitate the application of chimeric antigen receptor (CAR) T cell therapy in atherosclerosis. This approach involves isolating a patient's T cells and modifying them to specifically recognize and attack cells expressing the antigen. Healthy cells would remain unharmed, resulting in targeted treatment. Another intriguing approach to dampen immune cell activation involves targeting the interaction between chemokine receptors and ligands using therapies based on miRNA or siRNA, as well as employing peptide and non-peptide antagonists for heterodimer inhibition. Considering the rhythmic release of cytokines and the dynamic recruitment of myeloid cells, chrono-pharmacology-based therapy may offer improved drug effectiveness and reduced risk of side effects by optimizing therapeutic strategies based on timing.

Therefore, considering the distinct distribution and dynamic nature of immune cells and vascular wall cells, a different strategy may be necessary to target endothelial cells (ECs) or vascular smooth muscle cells (VSMCs). Instead of aiming at the entire cell population, it is worth considering the selective targeting of subsets of cells that contribute to plaque instability. Alternatively, efforts can be directed towards converting cells into a "plaque stabilizing" phenotype. However, determining which phenotypes of ECs and VSMCs are truly detrimental for plaque stability and which ones are not still requires experimental evidence. By identifying the regulatory pathways that govern phenotypic modulation, we can make progress towards discovering therapeutic options that can convert ECs or VSMCs into an atheroprotective phenotype. To mitigate the high costs associated with clinical trials, which are often attributed to the high failure rate of newly tested drugs, and to minimize potential risks of adverse side effects, Mendelian randomization provides an alternative approach for identifying future therapeutic targets for atherosclerosis. This approach has been successfully employed in lipid research and the investigation of inflammatory mediators like IL-6. Mendelian randomization utilizes genetic variants in a gene that encodes a specific drug target to assess the effects of pharmacological modification of this target and its potential effectiveness in treating or preventing coronary artery disease (CAD).

Discussion

'In conclusion, the study of cellular heterogeneity in atherosclerosis has revealed a complex and dynamic landscape within the plaques. The identification of highly plastic and heterogeneous cell populations has added an extra layer of complexity to future research in atherosclerosis. Traditional cell markers are no longer sufficient to assess cellular plaque composition and stability, highlighting the need for advanced techniques such as lineage tracing and single-cell omics to accurately characterize and understand the different cell subsets and their functional roles. Technological advancements in lipidomics and metabolomics offer promising avenues for exploring the molecular mechanisms underlying atherosclerosis. Techniques like MALDI mass spectrometry imaging enable the profiling of lipid compositions

in specific regions of the plaque, providing insights into metabolic adaptations and their contributions to cellular plasticity. Combining this information with immune cell reactivity and antigen-specificity could pave the way for the development of antigen-specific therapies for atherosclerosis, targeting immune subsets or their effectors.

Conclusion

Furthermore, the field of atherosclerosis research would benefit from increased investment and innovation in drug development. With a focus on reducing inflammation and targeting specific immune cell subsets or their effectors, novel therapeutic strategies can be explored. Site-specific therapies and timing-optimized therapeutic approaches may enhance drug efficacy and minimize side effects. Identifying key regulatory pathways controlling phenotypic modulation of endothelial cells and vascular smooth muscle cells could open up new possibilities for converting these cells into atheroprotective phenotypes. To guide future therapeutic interventions, the integration of spatial omics techniques, protein profiling, and Mendelian randomization can provide valuable insights into the adaptive immune response, identify antigen-specific targets, and evaluate the potential effectiveness of pharmacological modifications. Overall, a comprehensive understanding of cellular heterogeneity, immune mechanisms, and metabolic adaptations in atherosclerosis will fuel the development of innovative and targeted therapies, offering hope for improved management and prevention of this global burden of cardiovascular disease.

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Conflict of Interest

Author declares no conflict of interest.

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